Modular Biogenesis of Mitochondrial Respiratory Complexes

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Abstract

Mitochondrial function relies on the function of oxidative phosphorylation to synthesise ATP and generate an electrochemical gradient across the inner mitochondrial membrane. These coupled processes are mediated by five multi-subunit complexes that reside in this inner membrane. These complexes are the product of both nuclear and mitochondrial gene products. Defects in the function or assembly of these complexes can lead to mitochondrial diseases due to deficits in energy production and mitochondrial functions. Appropriate biogenesis and function are mediated by a complex number of assembly factors that promote maturation of specific complex subunits to form the active oxidative phosphorylation complex. The understanding of the biogenesis of each complex has been informed by studies in both simple eukaryotes such as *Saccharomyces cerevisiae* and human patients with mitochondrial diseases. These studies reveal each complex assembles through a pathway using specific subunits and assembly factors to form kinetically distinct but related assembly modules. The current understanding of these complexes has embraced the revolutions in genomics and proteomics to further our knowledge on the impact of mitochondrial biology in genetics, medicine, and evolution.

Introduction

Mitochondrial electron transport chain complexes exist in the inner mitochondrial membrane (IMM) and are composed of multiple subunits that either span the IMM or associate with the IMM exposed to either the matrix or inter-membrane space (IMS). The structure of these complexes is further complicated by associations between complexes, referred to as supercomplexes. The architecture of these supercomplexes is proposed to mediate efficient electron transfer from reducing equivalents generated during oxidative metabolism such as NADH and $FADH_2$ via electron transport complexes and mobile electron carriers ultimately reducing molecular oxygen to water.

Each electron transport complex harbours co-factors that enable electron transfer in a specific and rapid manner. These include flavin mononucleotide (FMN), iron-sulphur (Fe-S) clusters, haem/cytochromes, and metal ions such as copper. Electrons are passed from co-factors with higher redox states to those with lower redox states terminating in the reduction of molecular oxygen. The location of each co-factor is determined by the association with the specific subunit where it is bound. This allows a predictable transfer of electrons from entry points in complex I (NADH:ubiquinone oxidoreductase) and complex II (succinate dehydrogenase – SDH) that converge at the mobile electron carrier co-enzyme Q (ubiquinone/ubiquinol) prior to transfer to complex III (co-enzyme Q:cytochrome *c* oxidoreductase or bc_1 complex). The association of each co-factor is mediated at a specific stage in the biogenesis of the complex by a specific assembly factor. This

ensures the potentially redox active co-factor has minimal time as a free and potentially highly reactive molecular entity.

Most eukaryotic electron transport complexes are the product of two genomes. The majority of mitochondrial proteins are encoded by the nuclear genome and undergo cytosolic translation and incorporation into the mitochondria through outer mitochondrial membrane (OMM) and IMM specific protein translocation complexes (Calvo et al., 2016). A small, but essential, number of electron transport complex genes are encoded by the mitochondrial genome. These undergo mitochondrial transcription and translation and are incorporated into the IMM via specific translocation complexes (Ott and Herrmann, 2010). Once in the IMM these proteins associate with cognate assembly factors and structural subunits in a series of kinetically related assembly complexes.

Owing to their complexity, the assembly of each of these electron transport complexes needs to be a tightly regulated and efficient process that minimises generation of non-functional assembly intermediates and/or toxic by products such as reactive oxygen species (ROS). Biogenesis is also controlled by availability of required gene products and co-factors. An imbalance in electron transport complexes can lead to a decrease in ATP production and also a back flow of electrons that could result in excessive generation of ROS. These are thought to be the causes of a number of pathologies associated with impaired electron transport chain biogenesis.

Characterisation of the assembly of electron transport chain complexes originally stemmed from the genetic investigations of budding yeast, *Saccharomyces cerevisiae*, due to the inability of respiratory incompetent cells to grow on substrates such as ethanol, glycerol and lactate. This allowed for rapid identification of many genes whose absence impaired electron transport chain biogenesis (Tzagoloff and Dieckmann, 1990). The elucidation of the function of each of the genes has identified functions related to mitochondrial translation, messenger RNA stability, tRNA, structural subunits of the electron transport chain complexes and multiple assembly factors for each electron transport complex. The functions of these assembly factors are wide-ranging and are responsible for translation control, co-factor incorporation and potentially chaperone-like activity.

In this review article, we will discuss the assembly complexes associated with each electron transport chain complex highlighting the composition of the complexes and their modular nature as well as unanswered questions related to each of these electron transport chain complex assembly pathways.

Complex I – NADH:ubiquinone oxidoreductase

1 - Structure and function

Complex I (NADH:ubiquinone oxidoreductase) is an IMM complex comprised of 45 subunits, derived from both the nuclear and mitochondrial genomes in humans (Figure 1). Fourteen central subunits are structurally conserved from bacteria to mammals and seven of these subunits are encoded by the mitochondrial genome and synthesised using the mitochondrial transcription and translation machinery, while the remaining subunits are encoded in the nucleus and synthesised in the cytosol and require import into the IMM. In consequence of such large multimeric structure, isolated complex I deficiency is the major cause of mitochondrial disease in infancy and childhood with a wide range of clinical phenotypes such as Leigh syndrome, mitochondrial encephalomyopathy, lactic acidosis with stroke-like episodes (MELAS), neonatal cardiomyopathy and

hepatopathy with tubulopathy (Rodenburg, 2016). Mitochondrial encoded complex I subunits are found in most eukaryotes and therefore assembly can be determined using in fungal models such as *Yarrowia lipolytica* and *Neurospora crassa*. Of the 45 different proteins of mammalian complex I, eight have no orthologue in *Y. lipolytica*, while five of the 42 subunits of *Y. lipolytica* enzyme are not found in mammals (Wirth et al., 2016). However, in *Saccharomyces cerevisiae* complex I exists as one of three single polypeptide NADH dehydrogenase enzymes, Ndi1p, Nde1p and Nde2p, that accept electrons from extra-mitochondrial or intra-mitochondrial sources and pass onto ubiquinone, much like higher eukaryotic complex I. As these are single subunit enzymes and do not possess proton translocation activity, *S. cerevisiae* can not be used as a model for Complex I assembly or function.

The assembled multi-subunit complex I is a proton pump, translocating protons from the matrix to the IMM to participate in the generation of the mitochondrial membrane potential, in fact complex I is the major contributor of the proton motive force that drives aerobic ATP synthesis. An electron transfer complex (the N module) harvests electrons from NADH on the matrix side through Flavin Mononucleotide (FMN) reduction and passes them on to a series of eight canonical iron-sulphur clusters to ubiquinone, an IMM lipid soluble electron carrier, at the Q module. Complex I also contain a P module that is responsible for proton translocation that is coupled to electron transfer. With a molecular mass of about 1 MDa, complex I is the largest component of the respiratory chain, with many subunits, that need to be assembled appropriately to maintain cellular vitality. In many organisms, Complex I presents as an L-shaped architecture with a membrane arm and a peripheral arm. The initial redox reactions occur in the N module peripheral arm that is exposed to the matrix containing the flavoprotein and the eight iron-sulphur clusters that ultimately transfer the electrons. Electron leakage in these transfer paths account for significant formation of mitochondrial ROS, which has a direct impact in cell physiology (Barrientos and Moraes, 1998). Rotenone inhibits complex I activity by blocking ubiquinone that can promote apoptotic cell death by increasing complex I ROS release (Chance et al., 1963; Li et al., 2003). In many organisms, Complex I presents a regulatory transition in active (A-form) and deactivated (D-form) forms (Kotlyar and Vinogradov, 1990). The reversible switch between A-form and D-form is important, for instance, to minimize electron leakage, as well it was shown to prevent cardiac ischemia/reperfusion damage (Chouchani et al., 2013). The complexity of Complex I enzyme has made the study of assembly more technically challenging, because of the size of the complex and the number of subunits, but recent publications using high content proteomics and cryo-electron microscopy have revealed complex organisation and assembly (Fiedorczuk et al., 2016; Guerrero-Castillo et al., 2017). More recently, Complex I structure described in cryo-EM studies allowing mechanistic proposal for the Active/Deactivated transitions (Blaza et al., 2018; Parey et al., 2018; Zickermann et al., 2015).

2 – Protein Assistance during Assembly

There are approximately 15 proteins that act as assembly factors for complex I (Figure 1). Patients with mutations in complex I subunits and assembly factors have provided important information regarding the function of assembly factors and the assembly pathway along with bioinformatic and proteomic studies. The control of ND subunits mitochondrial gene expression is not well understood, only C20orf7 has been described as necessary for the maintenance of healthy steady state levels of ND1, but its specific function remains elusive (Sugiana et al., 2008). The assembly factor C8orf38/NDUFAF6 was identified by subtractive proteomics of purified mitochondria from a number of mouse tissues (Pagliarini et al., 2008). The function of this gene as an assembly factor was confirmed by the identification of a patient suffering from lethal infantile

complex I deficiency due to a mutation in the gene. NDUFAF6 codes for a 333 amino-acid protein that is not present in all metazoans (Lemire, 2017). As multi-subunit Complex I is not conserved in all eukaryotic organisms phylogenetic profiling and bioinformatic analysis has been used to identify unique genes. This approach identified the human orthologue of *Neurospora crassa* CIA30/NDUFAF1 (Dunning et al., 2007). NDUFAF1 together with ECSIT helps the assembly of a sub-assembly intermediate containing the membrane arm subunits ND2, ND3, ND6 and NDUFB6 (Vogel et al., 2007). The B17.2L/NDUFAF2 gene was identified using a similar approach using whole genome subtraction of yeast strains lacking or containing complex I (Ogilvie et al., 2005). Identification of this complex I assembly factor coincided with the identification of a patient harbouring a mutation in this gene. B17.2L is a paralogue of the B17/NDUFB6 structural subunit of complex I that exists in the beta subcomplex that resides in the IMM (Vinothkumar et al., 2014). NDUFAF2 is present in assembly intermediates with a proposed role as an important check-point in the assembly pathway (Mckenzie and Ryan, 2010). Another two factors NDUFAF3 and NDUFAF4 are proposed to have a role in early stages of the formation of the peripheral arm with the possible anchoring of the nuclearly encoded subunits NDUFS2, 3, 7, 8 and NDUFAF9 (Mckenzie and Ryan, 2010; Saada et al., 2009). Mutations in NDUFAF3 cause infantile lactic acidemia (Saada et al., 2009). Complex I activity requires the presence of iron-sulphur clusters in the N module in the subunits NDUFS1, NDUFS7, NDUFS8, NDUFV1, NDUFV2. Incorporation of Fe-S clusters are mediated by the assembly factor NUBPL/IND1. Mutations in NUBPL are associated with a number of Complex I diseases. The NDUFS7 subunit is hydroxylated on an arginine residue. This post-translational modification is mediated by NDUFAF5 early in the assembly of complex I and this step is crucial for early steps on biogenesis of early assembly intermediates. The FAD-dependent oxidoreductase domain containing protein (FOXRED1) is involved in steps part-way through Complex I assembly and is associated with certain Complex I deficiencies (Calvo et al., 2010; Formosa et al., 2015).

3 – Modular Assembly

Synthesis of mitochondrially encoded subunits can be followed by metabolic labelling using radioactive amino acids. Newly synthesised mtDNA-encoded Complex I subunits assemble into a number of distinct membrane arm intermediates and require significant chase times for their integration into the holoenzyme. In contrast, nuclearly encoded subunits integrate with pre-existing Complex I subunits to form intermediates to assembly holoenzyme (Lazarou et al., 2007). Original studies into the assembly of Complex I made use of rho^0 (Ugalde et al., 2004). BN-page analyses of complex I sub-assembly intermediates in patient cell lines containing different Complex I mutations indicated that both matrix and membrane arm subunits are found together in early-stage intermediates of Complex I assembly process (Antonicka et al., 2003). With the more recent advances in mass spectrometry analysis new insights into the assembly of this complex multi-subunit enzyme have been revealed. A complexome approach proposed a Complex I assembly pathway. Cells depleted of Complex I by treatment with chloramphenicol, a mitochondrial specific translation inhibitor, revealed a step-wise appearance of complexes that could be recognised using the composition of subunits as well as assembly factors. Inhibition of mitochondrial translation depleted mitochondrial gene products, but could not deplete all nuclear encoded subunits, and these remained in complexes associated with the Q (ubiquinone electron transfer) and P (proton pumping) modules of the enzyme (Guerrero-Castillo et al., 2017). Using systematic gene deletion of Complex I accessory subunits, 6 distinct modules of the enzyme were identified. Subunits displaying destabilisation after specific accessory subunit deletion were grouped together as this indicated a defect in assembly of that particular assembly module (Stroud et al., 2016) (Figure 1).

The study of patients suffering from a variety of mitochondrial diseases caused by mutations in Complex I subunits has identified a number of sub-assemblies. A ND1 disease causing inversion mutation results in an altered sequence without any decrease in complex I expression, but results in decreased activity (Blakely et al., 2006). Other mutations in ND1 cause MELAS and hearing loss that result in decreased complex I activity and assembled enzyme without evidence of any sub-assemblies (Gutiérrez Cortés et al., 2012; Kirby et al., 2004). ND3 mutations causing infantile mitochondrial encephalopathy also displayed reduced expression of complex I but with a much larger impact on complex I activity implying mutations in this subunit affect activity much more than assembly of the complex (McFarland et al., 2004). ND6 mutations causing Leigh syndrome result in decreased amounts of complex I activity that also displayed two sub-assemblies detected by immunoblotting for subunits NDUFS5 and NDUFA9 (Ugalde et al., 2003). Mutations in ND4 are one of the most common causes of LHON, causing decreased activity of Complex I but little demonstration of sub-assemblies of Complex I biogenesis. Mutations of the other mitochondrial encoded complex I subunits, ND2, ND4L, and ND5, have been demonstrated to be causative for a variety of diseases, but analysis of sub-assemblies has not been performed.

Several nuclear encoded Complex I core conserved subunits are responsible for disease when mutated and mitochondria from these patients express different sub-assemblies. Mutations in NDUFS7 cause Leigh syndrome which result in decreased Complex I activity and almost complete absence of assembled Complex I. This mutation in NDUFS7 also results in lack of expression of complex I subunits NDUFA13/GRIM19, NDUFA9 and NDUFS3, however there was no evidence of sub-assemblies (Lebon et al., 2007). Mitochondria derived from a separate patient with NDUFS7 mutations displayed sub-assemblies containing NDUFA9, NDUFS3 and NDUFS5 (Ugalde et al., 2004). Differences in the presentation of disease in these two patients results from different mutations which has very different effects on complex I activity, perhaps indicating a difference in the association or function of the Fe-S cluster in this subunit. A mutation in NDUFS8 was found to cause late-onset Leigh syndrome and this resulted in decreased NDUFS8 expression along with other Complex I subunits NDUFS2, NDUFV1 and NDUFA9 indicating a general instability of the complex possible due to defects in assembly (Procaccio and Wallace, 2004). Mutations in NDUFB3 causing mitochondrial Complex I deficiency result in complete absence of Complex I containing supercomplexes and severe loss of Complex I activity (Haack et al., 2012). Mitochondria from fibroblasts derived patients with mutations in NDUFS2 that cause inherited Complex I deficiency display a number of sub-assemblies that contain NDUFS3 and NDUFS5 (Ugalde et al., 2004). Mutations in NDUFS4 associated with a Leigh-like syndrome also displayed sub-assemblies containing NDUFA9, NDUFS3, and NDUFS5 (Ugalde et al., 2004). Mutations in NDUFV1 characterised in the Complex I containing yeast, *Yarrowia lypolytica*, demonstrated the decreased expression and activity of fully assembled complex I along with increased amounts of ROS production (Varghese et al., 2015). NDUFS1 mutations result in isolated Complex I deficiency which result in decreased expression of fully assembled Complex I and also the presence of a sub-assembly containing NDUFA9 that has a lower molecular size than fully assembled Complex I but is not catalytically active. Sub-assemblies much lower in molecular size than assembled Complex I also exist when detected using NDUSF3 (Hoefs et al., 2010). NDUFS1 mutations that cause cavitative leukoencephalopathy result in decreased expression of several other subunits, including NDUFA9, NDUFS3, NDUFB8 and NDUFB4, resulting in decreased expression of fully assembled complex I (Ferreira et al., 2011). A destabilising mutation of NDUFA9 found in Leigh syndrome also causes decreased expression of NDUFA13/GRIM19 and NDUFB8, resulting in decreased complex I activity and expression, again indicating that certain subunits, such as NDUFA9, when absent exert effects on other subunits resulting in their decreased expression (van den Bosch et al., 2012). NDUFA2 mutations leading to

destabilisation of NDUFA2 and NDUFA9 result in sub-assemblies containing NDUFA9 and NDUFS3 (Hoefs et al., 2008). NDUFA1 mutations cause widespread decreased expression of a number of subunits (Potluri et al., 2009). A central nervous system specific deletion NDUFA5 in mice causes decreased expression of other Complex I subunits (Peralta et al., 2014). Patients with NDUFA6 mutations display defects in complex I assembly and the presence of a late stage assembly intermediate lacking N-module subunits (Alston et al., 2018). Short interfering RNA inhibition of NDUFA11 expression leads to the decrease in assembled complex I and the accumulation of sub-assemblies of the hydrophobic arm containing NDUFB8 (Andrews et al., 2013). Short hairpin mediated depletion of NDUFB11 in HeLa cells results in decreased expression of fully assembled complex I, decreased expression of other subunits and the presence of sub-assemblies containing NDUFS3 (van Rahden et al., 2015). NDUFA13/GRIM19 mutations are found in patients with early onset hypotonia, dyskinesia and sensorial deficiencies which is associated with decreased complex I assembly and destabilisation of subunits (Angebault et al., 2015). NDUFA10 mutations are associated with Leigh syndrome and cause decreased complex I assembly with a series of assembly intermediates containing NDUFS3 (Hoefs et al., 2011). Mutations in the structural subunit NDUFB8 result in decreased activity and expression of the assembled Complex I enzyme, with no reported sub-assemblies (Piekutowska-Abramczuk et al., 2018). The accessory subunit NDUFAB1 (also known as the acyl carrier protein - ACP) is a vital protein and cells lacking this protein do not survive (Stroud et al., 2016). Mutations in NDUFV2, NDUFS6 are associated with disease but no evidence of effects on Complex I expression have been presented.

Mutations in Complex I assembly factors are also responsible for a number of mitochondrial disorders and this has also given further insight into the assembly process. NDUFAF2 mutations lead to the accumulation of Complex I assembly intermediates and decreased expression of assembled Complex I (Hoefs et al., 2009). This assembly factor associates with several other assembly factors and structural subunits (Dunning et al., 2007; Fassone et al., 2011). A mutation in the assembly factor NDUFAF3 resulted in early childhood death due to a much reduced expression and activity of complex I. NDUFAF4 mutations associated with infantile mitochondrial encephalopathy (Saada et al., 2009) and Leigh syndrome result in accumulation of sub-assemblies containing NDUSF3 (Baertling et al., 2017). Mutations in C20orf7/NDUFAF5 cause a lethal form of neonatal Complex I deficiency or Leigh syndrome (Gerards et al., 2010; Saada et al., 2012). When expression of NDUFAF5 is inhibited by RNA interference, Complex I activity is severely decreased, which is due to a decrease of fully assembled Complex I. This decrease is due to an inability to assemble Complex I in the absence of NDUFAF5 as newly synthesised subunits ND1 and ND2 are unable to assemble into Complex I (Sugiana et al., 2008). Depletion of NDUFAF5 also causes the disappearance of a Complex I sub-assembly containing NDUFS2 and the appearance of a normally low abundance sub-assembly containing NDUFB8 (Rhein et al., 2016).

Complex II – Succinate Dehydrogenase

Complex II (succinate dehydrogenase/succinate – co-enzyme Q reductase) is a highly conserved ETC complex that oxidises succinate as part of the citric acid cycle to pass electrons to co-factors to flavin adenine nucleotide that are coupled to Fe-S clusters and co-enzyme Q reduction. Complex II is a 4 subunit complex that in eukaryotes is encoded completely by nuclear genes – the only OXPHOS complex to be expressed in this manner. Subunits SDHA/SDH1 and SDHB/SDH2 are catalytic subunits and reside in the complex facing the matrix which are anchored to the IMM through SDHC/SDH3 and SDHD/SDH4. Complex II harbours a haem prosthetic group between subunits SDHC and SDHD. The functional importance of this haem group is not known, but is

required for assembly of the complex (Lemarie and Grimm, 2009). The assembly of complex II is mediated by a number of assembly factors similar to the other OXPHOS complexes (Figure 2).

Diseases associated with mutations in SDH structural subunits and assembly factors result in paragangliomas and phaechromocytomas, and also Leigh syndrome. The first Complex II assembly factor, SDHAF1, was identified in patients with infantile leukoencephalopathy. Patients with mutations in SDHAF1 have decreased amounts of assembled Complex II and also RNA interference reduces assembled Complex II in cell lines (Ghezzi et al., 2009). SDHAF1 is homologous to the yeast gene, SDH6, and can complement the corresponding deletion. SDHAF1 contains a LYR tripeptide motif that is associated with Fe-S homeostasis and is likely involved in Fe-S incorporation into Complex II. SDHAF1 function was confirmed to be required for Fe-S incorporation into SDHB through studies in yeast using the homologs SDH2 and SDH6. A second assembly SDHAF3 is also involved in Fe-S incorporation into SDHB (Na et al., 2014). The flavination of SDHA is mediated by SDHAF2 (SDH5 in yeast) and mutation in this assembly factor is associated with paragangliomas (Hao et al., 2009).

Complex III - Ubiquinol-cytochrome *c* oxidoreductase.

1 - Structure

Ubiquinol-cytochrome *c* oxidoreductase, or respiratory complex III, herein referred to as the bc_1 complex is an integral multisubunit membrane enzyme that catalyses the transfer of reducing equivalents from ubiquinol to cytochrome *c*, coupling electron transfer to proton translocation across the IMM contributing for the formation of the electrochemical gradient, used for the synthesis of ATP by the mitochondrial F_1-F_O ATP synthase complex (Trumpower and Gennis, 1994).

In *Saccharomyces cerevisiae* functional bc_1 complex exists only in a dimeric form, with each monomer containing ten different subunits associated to the IMM (Hunte et al., 2000). Only the cytochrome *b* is encoded by the mitochondrial DNA, whereas the other nine proteins are nuclearly encoded. Cytochrome *b* (Cobp) is the central core of the bc_1 complex and together with cytochrome c_1 (Cyt1p) and the Rieske Fe-S protein (Rip1p/UQCRFS1) are the redox centre, or catalytic subunits of the enzyme. Cobp is embedded via eight transmembrane spans in the inner membrane and harbours two redox centers: haem b_{562} (b_H) and haem b_{565} (b_L), which are located on distant positions in the membrane. Haem b_L is positioned near the IMS, and haem b_H is located in the interior of the enzyme, oriented towards the matrix space (Zara et al., 2009a). The two haems groups are coordinated by histidine residues in a four-helix bundle (Hunte et al., 2000). Cyt1p contains haem c_1 as its redox center exposed in the IMS. Rip1p presents a topology similar to Cyt1p, with a mobile catalytic domain in the IMS and a hydrophobic α helix bound to the inner membrane (Hunte et al., 2000).

The bc_1 complex couples electron transfer to proton shuttling by a mechanism known as the Q-cycle (Mitchell, 1975). In a bifurcated reaction (Trumpower, 2002), one electron is delivered from ubiquinone at the quinone oxidation (Q_O) site to Rip1p and Cyt1p, which reduces soluble cytochrome *c* in the IMS. Indeed, Rip1p cycles between two conformational states to transfer electrons from the ubiquinone bound in the Q_O site to Cyt1p (Brugna et al., 2000) a very important feature for this mechanism to work is the large scale domain movement of Rip1p (Rathore et al., 2019; Zhang et al., 1998). The second electron passes through haem b_H and haem b_L in Cobp, reducing ubiquinone to semi-ubiquinone at the quinone reduction (Q_i) site at the matrix side of the membrane (Trumpower, 1990). As a result, two protons are released at the IMS per electron transferred to cytochrome *c*. Interestingly, cytochrome *c* binds to the homodimeric bc_1 complex in a monovalent mode, the binding correlates with a conformational change of the Rip1p head domain and subunit Qcr6p in one monomer turning the dimer into an asymmetric form (Solmaz and Hunte, 2008). Indeed, Rip1p physically cross-links the two functional units of the intertwined dimer and probably act as a

regulatory element in the mechanistic alternating site model, with only one monomer of the bc_1 complex active at a time (Solmaz and Hunte, 2008). The understanding of the structure and function of this complex has furthered by high resolution cryo-EM of the bc_1 complex dimer revealing 12 phospholipid molecules including four phosphatidylcholines, four phosphatidylethanolamines, two cardiolipins, and two 1,2-diacyl-sn-glycero-3-phoshocholines (Rathore et al., 2019). There are two classes of specific bc_1 complex inhibitors: the first class inhibits the electron transfer at the Q_O site and includes stigmatellin and myxothiazol (von Jagow et al., 1984), the second class exemplified by antimycin A (Wikström and Berden, 1972), block the Q_i site by interrupting the electron transfer from haem b_H to quinone or semiquinone.

In addition to the three catalytic subunits, the other seven structural subunits are referred to as supernumerary subunits, which include Cor1p, Cor2p, Qcr6p, Qcr7p, Qcr8p, Qcr9p and Qcr10p. All subunits, except for Qcr6p and Qcr10p, they are essential for yeast respiration.

Cor1p and Cor2p are the two largest subunits of the complex and were named core proteins on the assumption that they would form the core of the complex. *COR1* codes for the 44-kDa core protein, which is the largest subunit of the bc_1 complex (Tzagoloff et al., 1986). The protein is bound to the IMM but mostly is exposed to the mitochondrial matrix, where it interacts with Cor2p and Qcr7p (Hartley et al., 2019; Rathore et al., 2019).

Qcr6p is an acidic subunit that assists Cyt1p association into the complex (Yang and Trumpower, 1994) and cytochrome *c* binding to the homodimeric bc_1 complex (Solmaz and Hunte, 2008). The protein is loosely associated with the complex since it is easily released into the intermembrane space. Indeed, *qcr6* yeast mutants grow normally on a non-fermentable substrate (Crivellone et al., 1988).

Qcr8p, Qcr9p and Qcr10p are small proteins that cross the IMM with a hydrophobic α-helix (Brandt et al., 1994; Maarse and Grivell, 1987; Schmitt et al., 1990). The structure and peripheral binding of Qcr10p to the bc_1 complex could only be recently resolved in cryo-EM structure studies (Hartley et al., 2019; Rathore et al., 2019). In the matrix, the extended N terminus of Qcr10p interacts with Cor2p and Qcr7p of the other monomer, while in the IMS Qcr10p interacts with Cyt1p (Hartley et al., 2019). An eleventh subunit present in the human complex turned out to be the leader sequence of Rip1, which is retained within the mature complex (Brandt et al., 1993).

2 - Mitochondrial Gene expression

The *COB* gene of yeast mitochondria contains two, or three and in some cases as many as six introns (Lazowska et al., 1980; Nobrega and Tzagoloff, 1980). Some introns code for splicing factors that promotes the excision of their own coding sequences whereas other depends on nuclear encoded products. For instance, the terminal intron (bI2) is a group I intron, capable of self-splicing *in vitro* but dependent of the Cbp2 protein *in vivo* (Gampel et al., 1989). Mrs1p and Mss116p are also nuclear encoded proteins required for *COB* intron removal, however they are also necessary for intron processing in other mitochondrial genes, indeed, Mss116p has a RNA chaperone function that belongs to the DEAD-box protein family, and it is required for efficient splicing of all mitochondrial group I and group II introns present in *COX1*, *COB* and 21S rRNA, as well as for mitoribosome biogenesis and *COX1* translation (De Silva et al., 2017; Huang et al., 2005; Kreike et al., 1986). The bicistronic *COB* primary transcript also contains the tRNA E coding sequence, which is processed by RNase P along with auxiliary proteins (Guedes-Monteiro et al., 2019). The 5' end of *COB* RNA is further processed by Pet127p and finally stabilized by Cbp1p within the AU-rich *COB* mRNA 5'-UTR that contains a unique CCG trinucleotide (Chen and Dieckmann, 1997; Dieckmann et al., 1984; Fekete et al., 2008; Mittelmeier and Dieckmann, 1993).

The dependence on two separated genomes for proper assembly of the respiratory complexes required the development of a balanced output of gene products destined to be subunits of the same enzyme. Mitochondria translational activators are the primary candidates to exert such control (Costanzo and Fox, 1990) in three proposed ways: through the recognition of a specific 5' UTR end, which is the case of Cbp1p, Cbs1p and Cbs2p; by a direct interaction with the mitoribosome (Cbs1p,

Cbs2p, Cbp3p and Cbp6p); and finally interacting with the nascent polypeptide chain at the mitoribosome tunnel exit (Cbp3p and Cbp6p) (Derbikova et al., 2018; Ndi et al., 2018).

Besides its function on mRNA stabilization Cbp1p might also play a role in co-translational insertion of Cobp into the inner membrane (Krause et al., 2004). Cbs1p and Cbs2p were shown to interact with the 5' UTR of *COB* mRNA as well as with the mitoribosome itself (Krause-Buchholz et al., 2005, 2004; Rödel and Fox, 1987; Rödel, 1986). Cbp3p and Cbp6p are involved in a feedback loop mechanism required for the coordination of Cobp synthesis and assembly (Dieckmann and Tzagoloff, 1985; Gruschke et al., 2012, 2011). It has been proposed that the Cbp3p-Cbp6p complex associates with newly synthesized protein preventing apocytochrome b from degradation and assist the early steps of bc_1 complex assembly (Gruschke et al., 2012). Upon early failure in bc_1 biogenesis, Cbp3p-Cbp6p remains associated with the intermediate and is not available to stimulate a further round of *COB* mRNA translation (Hildenbeutel et al., 2014).

3 – Protein Assistance in the Assembly process

Yeast mutants of bc_1 complex were isolated in terms of the activity of the electron transfer complexes, ATP synthase activity and cytochrome spectra. Hallmarks of these mutants include absence of the characteristic spectra of mitochondrial cytochrome *b*, and specific reduced activity of NADH or CoQH2 – dependent reduction of cytochrome *c* in the presence of cyanide (Tzagoloff and Dieckmann, 1990; Tzagoloff, 1995).

Numerous assembly factors are required for proper bc_1 assembly. They assist the modular assembly of the complex as well as maturation and topogenesis of the prothestic groups present in Cobp, Cyt1p and Rip1p proteins (Ndi et al., 2018; Smith et al., 2012).

Besides their role in Cobp translation, Cbp3p and Cbp6p are chaperones of the newly synthesized Cobp, which is a crucial step for Cobp maturation. Indeed, null mutants of *cbp3* or *cbp6* are still able to translate Cobp at a reduced rate but the protein is rapidly degraded (Gruschke et al., 2011). Cbp4p is also a chaperone essential in the process of bc_1 biogenesis; the protein is tightly associated with the mitochondrial membrane (Crivellone, 1994) and it is required for Cobp stabilization during the haemylation process of apocytochrome b (Hildenbeutel et al., 2014).

The haem *c* addition to Cyt1p is also a protein-assisted process. Cyt1p has a bipartite signal sequence. The first part is removed by the mitochondrial processing peptidase (MPP) in the matrix; secondly, Imp2p only performed the removal of the remaining presequence after the covalent addition of haem *c* (Arnold et al., 1998a). The covalent attachment of haem c_1 to apocytochrome *c*1 is assisted by Cyt2p a haem lyase (Zollner et al., 1992). In certain *cyt1* mutants and in the absence of Cyt2p the haem lyase function can be performed by Cyc3p, the haem lyase of soluble cytochrome *c* (Bernard et al., 2003). HCCS is the human Cyt2p homolog and covalently links a haem group to the apoprotein of cytochrome *c*.

Bcs1p and Mzm1p are both necessary for the maturation and insertion of Rip1p into the bc_1 complex. Mzm1p stabilizes Rip1p Fe-S cluster in the mitochondrial matrix, protecting it from aggregation and degradation prior to integration and association with Bcs1p into the IMM (Atkinson et al., 2011; Cui et al., 2012). The AAA ATPase Bcs1p mediates Rip1p translocation with its bound Fe-S cluster across the IMM from the mitochondrial matrix (Cruciat et al., 1999; Nobrega et al., 1992; Wagener et al., 2011). Bcs1p has a single transmembrane domain near the N-terminus spanning the IMM, which is crucial for the assembly of Rip1p into the bc_1 complex (Sawamura et al., 2014). Downstream of the N-terminal transmembrane region the protein is divided into three regions: a non-canonical mitochondrial-targeting signal just following the transmembrane region, a AAA ATPase and a highly conserved middle domain of unknown function (Fölsch et al., 1996; Ndi et al., 2018).

Finally, Bca1p is present only in fungi; it is bound to the IMM and protrudes into the IMS. Its specific function is still unknown, but it seems necessary in early assembly steps of bc_1 assembly process (Mathieu et al., 2011).

4 - Modular Assembly

Biogenesis of the bc_1 complex was recently scrutinised in five different stable intermediates that are observed from Cobp mitochondrial translation and IMM insertion to Rip1p addition to the dimeric complex (Ndi et al., 2018) (Figure 3).

The newly synthesized Cobp protein is rapidly associated to the Cbp3p-Cbp6p complex, which is bound to the tunnel exit of the mitochondrial ribosome, the association of apocytochrome b with Cpb3p-Cbp6p release the complex from the ribosome (Gruschke et al., 2011).

The first proposed intermediate is formed after hemylation of apocytochrome b, which occurs in a two-step process. The first haem *b* is inserted at the b_L site favouring the binding of Cbp4p, which allow the addition of the second haem *b* at the b_H site and subsequent release of Cbp3p-Cbp6p (Hildenbeutel et al., 2014). The mature Cobp associates with Qcr7p and Qcr8p subunits forming the second intermediate (Gruschke et al., 2012; Ndi et al., 2018; Zara et al., 2009b).

The subsequent association of Cobp-Qcr7p-Qcr8p-Cbp4p with Cor1p and Cor2p lead to Cbp4p dissociation, and the association with matured Cyt1p and Qcr6p (Gruschke et al., 2012), forming the third intermediate of about 500kDa (Zara et al., 2007). The addition of Qcr9p and the recruitment of Bcs1p generate the fourth intermediate. As described before, Bcs1p assists the insertion of the Fe-S protein Rip1p into the precomplex and, consequently, the formation of the fifth intermediate. Qcr9p binds to the transmembrane segment of Rip1p, integrating Rip1p firmly into the precomplex (Rathore et al., 2019). Binding of Bcs1p to and release from the cytochrome bc_1 precomplex appears to be a dynamic process regulated by ATP hydrolysis (Cruciat et al., 1999), therefore, Bcs1p dissociation and perhaps Qcr10p binding at this step ends the process with the formation of mature bc_1 complex (Ndi et al., 2018).

The dimerization process is an early event in the bc_1 biogenesis. Indeed, it was demonstrated that the bc_1 late core intermediate lacking Rip1p possesses a dimeric structure excluding a potential role of this catalytic protein in the dimerization process (Conte et al., 2015). It is plausible that dimerization occurs just after the addition of haem *b* into Cobp protein, or Cbp4p dissociation (Ndi et al., 2018).

In a recent study it was shown a spatial compartmentalization of mitochondrial translation and the assembly of the respiratory complexes through the identification of different sites of the IMM sites: the inner boundary membrane (IBM), which parallels the outer membrane, and the *cristae* membrane (CM) (Stoldt et al., 2018). The modular assembly of the bc_1 complex begins with mitochondrial Cobp translation at IBM, the association with Cbp3p-Cbp6p also occurs mainly at this site, but the association of Cbp4p, an intermediate phase event, is less enriched at IBM; on the other hand factors involved in late assembly events, such as Bcs1p, are enriched at CM (Stoldt et al., 2018), which shows a distinct spatial distribution of the different assembly steps of the bc_1 complex.

Deficiencies in bc_1 complex are the least common oxidative phosphorylation defects associated with mitochondrial disease. Mutations in the mitochondrial CYB gene have been so far described in 45 different positions (MITOMAP). Patients with mutations in nine nuclear genes required for bc_1 assembly have also been reported presenting a wide range of tissue-specific defects including the structural components UQCRQB, UQCRQ and UQCRC2 and the accessory factors BCS1L, TTC19, LYRM7, UQCC1, UQCC2 and UQCC3 (Ghezzi and Zeviani, 2018; Gorman et al., 2016). Defective BCS1L is the most frequent cause of CIII-defective mitochondrial disease, as more than 25 different pathological mutations associated to very variable clinical presentations ranging from multivisceral GRACILE to congenital metabolic acidosis, neonatal proximal tubulopathy and/or liver failure with or without encephalopathy (Fernández-Vizarra and Zeviani, 2015) CYC1 mutations present ketoacidotic and lactic acidotic encephalopathy and insulin-responsive hyperglycemia (Gaignard et al., 2013).

TTC19 is only found in metazoans and probably function as a scaffold for bc_1 complex biogenesis, in its absence a subcomplex containing Cor1/Cor2 is accumulated (Smith et al., 2012). LYRM7 is the human Mzm1p counterpart required for the stabilization of UQCRFS1 (Sánchez et al., 2013). UQCC1, UQCC2 and UQCC3 are the human othologs for yeast Cbp3p, Cbp6p and Cbp4p

respectively. UQCC1 binds to newly synthesized Cyt b and is required for UQCC2 function (Tucker et al., 2013; Wanschers et al., 2014).

5 - Supercomplex with cytochrome *c* oxidase

The use of mild detergents for mitochondrial protein solubilization revealed that respiratory chain complexes form supramolecular assemblies known as supercomplexes, which composition and abundance may vary among organisms and tissues depending on the metabolic and physiological conditions (Lobo-Jarne and Ugalde, 2018). In yeast the bc_1 complex dimers associate with either one or two copies of mature cytochrome *c* oxidase to form supercomplexes of the respiratory chain (Cruciat et al., 2000; Schägger and Pfeiffer, 2000). Although, the physiological importance of supercomplex formation has not yet been determined, their presence and higher distribution in the inner membrane *cristae* (Stoldt et al., 2018) can maximize the proton gradient and therefore the efficiency of the oxidative phosphorylation process. In mammalian mitochondria the respiratory supercomplexes also harbour complex I and is termed the respirasome since they contain all the components required to transfer electrons from NADH to molecular oxygen. The most abundant form of respirasome in mammalian cells consists of one unit of complex I, the dimeric complex III and one unit of complex IV (Lobo-Jarne and Ugalde, 2018). In yeast the supercomplexes are stabilized by cardiolipin and phosphatidylethanolamine (Pfeiffer et al., 2003) and at least three proteins: Rcf1p, Rcf2p and Coi1p (Singhal et al., 2017; Strogolova et al., 2019, 2012; Vukotic et al., 2012).

Cryo-EM studies revealed protein–protein interactions in the interface of bc_1–COX on either side of the IMM and interactions via bridging lipids in the membrane region itself (Hartley et al., 2019; Rathore et al., 2019). The majority of electrostatic interactions occur on the matrix side between Cor1p and Q residues at the N terminus of Cox5ap in a combination of hydrogen bonds, for example between K240--Q46, D192--Q35, via Cor1p K239 ε amino group with the side-chain carbonyl of Q47, and a pi-pi stacking interaction between the aromatic rings of Cor1p Y65 with Cox5ap W38 (Hartley et al., 2019; Rathore et al., 2019). In the IMS, the C-terminal domain of Cox5ap is in position to interact with both the C terminus of Qcr6p and a loop region between helices 6 and 7 of cytochrome c_1. Within the membrane, Cox5ap contacts the N-terminal helix of Rip1p and Qcr8p via a cardiolipin molecule and another lipid modelled as phosphocholine (Hartley et al., 2019).

Complex IV – Cytochrome *c* oxidase

1 – Structure and function

Cytochrome *c* oxidase (COX) is the terminal electron acceptor in the ETC of many organisms. In the case of organisms that use molecular oxygen as the terminal electron acceptor COX accepts electrons from cytochrome *c* and uses this to reduce molecular oxygen to water. We will only consider eukaryotic terminal electron acceptors of this nature. Other terminal electron acceptors and alternative oxidases exists and are mainly found in bacteria and plants. COX is composed of three catalytic core subunits, and in most cases, these subunits are encoded by mitochondrial DNA. The remaining subunits and assembly factors are encoded by genes present in the nucleus, but the number of these varies between organisms which will be highlighted here.

Many of the structural subunits and assembly factors of COX were identified in *Saccharomyces cerevisiae* and functions have been elucidated since. More recently, large-scale genomic analysis of patients and proteomic approaches have revealed new proteins associated with COX assembly and function. Structural studies initially carried out using bovine heart COX determined the 3-dimensional organisation of structural subunits and co-factors that has informed the catalytic mechanism of the enzyme as well as the proton channelling required to contribute to the generation of the mitochondrial membrane potential (Tsukihara et al., 1996). The conservation of COX structural subunits from many organisms is high and this allows for interpretation of all sources of

COX. The subunit contacts within COX are predicted to be conserved based on the conserved function of the enzyme (Hartley et al., 2019; Rathore et al., 2019). Knowledge of the subunit contacts allows for predictions of which subunits should interact as the enzyme is undergoing assembly. In higher eukaryotes, COX has a conserved structure with homologous subunits found in yeast. Complexity is increased due to the presence of more structural subunits, subunit isoforms and differential regulation of expression and assembly using different mechanisms. Studies related to the assembly of COX in higher eukaryotes initially came about after human genetic studies of patients suffering from mitochondrial diseases. The absence of any vital structural subunit in humans would be expected to result in a non-viable embryo/foetus or a severe disorder. Many mutations in COX subunits and assembly factors have been identified over the years, that result in lower activity or expression of the functional enzyme, indicating an inefficiency in biogenesis or function. Mutations in the mitochondrial-encoded COX subunits are associated with mitochondrial disease, such as Leber's Optic Atrophy and Complex IV deficiency syndromes (COX6B1, COX7, COX8A, MTCO1, MTCO2, MTCO3). Mutations in structural subunits leading to COX deficiency syndromes have more than one isoform (COX6A, COX6B, COX7) which may be compensated by inappropriate expression of the isoform in the defective tissue or are derived from mitochondrial DNA where heterogeneity of mitochondrial mutations can be variable (Rak et al., 2016).

2 - Mitochondrial Gene Expression

The three COX mitochondrial-encoded genes have specific protein factors involved in regulating their expression and translation. In yeast, *COX1* is transcribed as a polycistronic transcript containing *ATP6/ATP8* of ATP synthase (Manon and Guerin, 1989). Transcript processing requires splicing to generate a *COX1* transcript without introns, a process assisted by maturases encoded by open reading frames located within some of the introns (Lazowska et al., 1980) and nuclear genes such as *MSS116* and *COX24* (Barros et al., 2006; Séraphin et al., 1989), which are curiously also involved in mitoribosome biogenesis (De Silva et al., 2017; Mays et al., 2019). Indeed, some of these factors have several functions in mitochondrial RNA metabolism. For instance, the *COX1* specific translational activator, Pet309p, binds to COX1 messenger RNA prior to processing of the polycistronic mRNA - an interaction that depends on the pentatricopeptide repeat (PPR) motifs (Zamudio-Ochoa et al., 2014) and - is also required for translation (Tavares-Carreón et al., 2008). Translation of the mature *COX1* transcript is controlled by a post-transcriptional mechanism reliant on the function of Mss51p, a translational activator. Mss51p, when free, associates with *COX1* mRNA and promotes translation (Barrientos et al., 2004). Mss51p function is regulated by the presence of haem and Mss51p mutants that cannot bind to haem or when cells are depleted of haem, Mss51p cannot act as a translational activator of COX1 mRNA (Soto et al., 2012). Mss51p is also part of Cox1p assembly complexes (see later) that sequester Mss51p and prevent it from acting as a translational activator. *COX2* mRNA associates with Pet111p which binds to a site in the 5' untranslated region (UTR) and a site in the coding sequence. Pet111p acts as a translational activator potentially by preventing the formation of secondary structure in the translation initiation region (Jones et al., 2019). *COX3* mRNA interacts with Pet54p, Pet122p and Pet494p, which are all translational activators of the COX3 mRNA transcript and associate in the 5' UTR (Costanzo and Fox, 1988, 1986; Costanzo et al., 1986). These are present in an IMM associated complex that interacts with the ribosome to enable translation of COX3 mRNA (Brown et al., 1994). Interestingly, Pet54p also interacts with *COX1* mRNA to allow for intron splicing but also efficient translation (Mayorga et al., 2016; Valencik et al., 1989). As a mechanism to co-ordinate translation of these three COX mitochondrial mRNAs, a complex of translational activators has been described (Naithani et al.,

2003) and translation has been visualised occurring at specific intra-mitochondrial sites (Stoldt et al., 2018). Translation of mitochondrial COX transcripts is dependent on the function of mitochondrial ribosome subunits, such as COX24/mS38 and mL38 (Box et al., 2017; Mays et al., 2019). These point to a highly co-ordinated system to regulate COX subunit translation within mitochondria to enable efficient biogenesis.

3 – Protein Assistance in the Assembly Process

Protein assistance is required at all stages of the assembly process including the insertion of highly hydrophobic mitochondrially encoded components, as well as the maturation of their catalytic subunits through addition of two haem group and three copper prosthetic groups (Barrientos et al., 2002). COX subunit I (Cox1p) is a 12 trans-membrane domain spanning integral IMM protein. This catalytic core subunit contains the haem groups from cytochrome *a* and a_3 and a copper ion in the CuB centre required for COX electron transfer as the catalytic site for reduction of molecular oxygen to water. The low spin haem *a* and the high spin *a3* are non-covalently bound to Cox1p and are synthesized from protoheme by the addition of a farnesyl group by Cox10 a farnesyl transferase (Tzagoloff et al., 1993), followed by a monoxygenase reaction catalysed by Yah1p-Cox15p (Barros et al., 2002, 2001). The final hemylation of Cox1p is still elusive, however it depends on Cox10p and Cox15p oligomerization (Bestwick et al., 2010; Khalimonchuk et al., 2012) and additional factors such as Coa2p, Shy1p and Pet117p (Kim et al., 2012; Taylor et al., 2017). Cox1p is translated and inserted in to the IMM by Oxa1p, Mba1p and Mrx15p IMM insertases (Hell et al., 2001; Möller-Hergt et al., 2018; Preuss et al., 2001) most likely through specific juxta-membrane localisation of mitochondrial ribosomes. When in the IMM, Cox1p is then part of a complex containing Coa1p, Coa3p/Cox25p and Cox14p (Fontanesi et al., 2011; McStay et al., 2013a, 2013b; Mick et al., 2010; Pierrel et al., 2007). These three assembly factors are single trans-membrane domain spanning integral IMM proteins whose functions are unknown but essential for COX biogenesis. This complex then recruits Mss51p and the first structural subunit Cox5ap/bp. In this complex, Mss51p is sequestered away from *COX1* mRNA and is unavailable for further activation of translation. This epistatic mechanism of regulation ensures that further biogenesis of Cox1p cannot occur in the absence of required assembly factors, structural subunits or prosthetic groups, such as haem and copper. There are two homologous genes for COX subunit 5, *COX5A* and *COX5B*, which are 66-67% homologous at both the nucleotide and amino acid sequence (Cumsky et al., 1985). The difference between these two genes is how gene expression is regulated. COX5A is the major transcription product under conditions of normoxia, while *COX5B* is the major transcription product under conditions of hypoxia (Hodge et al., 1989). Incorporation of Cox5bp into COX provides an increased affinity for molecular oxygen to allow OXPHOS to continue under hypoxic conditions. Cox5a/bp are trans-membrane proteins that display associations only with Cox1p in the fully assembled COX enzyme based on structural studies in mammalian and yeast COX (Tsukihara et al., 1996). In yeast, COX5 is the only COX subunit to have alternative isoforms and potentially incorporation of this subunit allows for altered biogenesis or overall assembled structure that enables increased affinity for oxygen. In higher eukaryotes, other structural subunits have multiple isoforms giving rise to greater structural and functional diversity. This discrete assembly complex was identified using affinity purification of these aforementioned assembly factors and structural subunits to identify associated mitochondrial gene products that were metabolically labelled. These complexes only contained newly synthesised Cox1p and there is no evidence of newly synthesised Cox2p or Cox3p indicating a discrete assembly pathway of Cox1p prior to association of other catalytic core subunits of COX. The maturing Cox1p sub-complex then recruits Cox6p and Cox8p, both structural subunits associated with the final assembled COX enzyme

(McStay et al., 2013a). Cox8p is another single trans-membrane spanning subunit that is encoded by the nuclear genome. Cox6p is the only peripheral membrane structural subunit of COX which resides on the matrix facing side of the IMM. The next sub-complex is formed by the addition of Shy1p, an assembly factor that has been implicated in haem addition to assembling COX (McStay et al., 2013b). Shy1p is a multi-trans-membrane spanning protein. Interestingly, Shy1p is a homolog to all related forms of the COX enzymes from bacteria to humans, indicating its importance in COX biogenesis, and is most likely one of the most fundamental proteins required for COX assembly (Greiner et al., 2008). Copper is the other required co-factor for Cox1p. Incorporation of copper into Cox1p is mediated by the copper chaperones Cox17p (Glerum et al., 1996a) and Cox11p (Hiser et al., 2000; Khalimonchuk et al., 2005; Tzagoloff et al., 1990). Like Cox17p, other soluble factors such as Cmc1p, Cox19p, and Cox23p also contain a CX9C motif and are required for the maturation of Cox1p copper centre (Barros et al., 2004; Bode et al., 2015; Bourens and Barrientos, 2017; Dela Cruz et al., 2016; Horn et al., 2008; Khalimonchuk et al., 2010; Nobrega et al., 2002).

The other two catalytic core subunits of COX, Cox2p and Cox3p are both trans-membrane spanning domain proteins. Cox2p has two trans-membrane spanning domains and has an associated copper co-factor in the CuA binuclear centre that accepts electrons delivered by cytochrome *c*. Cox3p has a single trans-membrane domain with no co-factors or prosthetic groups but is thought to participate in proton translocation. Cox2p is the only mitochondrial genome encoded structural subunit that requires proteolytic processing before incorporation into COX. Amino-terminal processing removes the first 15 amino acids from the polypeptide and this occurs before association with Cox2p assembly factors. The inner membrane peptide complex catalytic subunit Imp1p is responsible for the removal of the propeptide (Behrens et al., 1991; Pratje et al., 1983). Cox20p associates with the Cox2p pre-cursor prior to cleavage by Imp1p (Hell et al., 2000). Cox18p acts to assist with the entry of the Cox2p C-terminus into the IMM in association with Pnt1p and Mss2p (Lode et al., 2000; Pacheu-Grau et al., 2015; Saracco and Fox, 2002; Schulze and Rödel, 1988). Copper does not reside freely in yeast and is in complex with the copper chaperone Cox17p that delivers copper to Cox11p for to incorporate into Cox1p (Cobine et al., 2006; Hiser et al., 2000; Jett and Leary, 2017). The formation of the binuclear CuA center depends on Sco1p, which contains bound copper (Beers et al., 2002) and probably transfer its copper to the CuA site (Glerum et al., 1996b), as well as alter the oxidation state of the cysteine ligands in Cox2p (Chinenov, 2000). The yeast paralog Sco2p is probably the human SCO1 counterpart and is not required for COX assembly. In contrast, human patients with mutations in either SCO1 or SCO2 present a COX deficiency that cannot be rescued by the respective homolog, indicating non-overlapping activities of the two proteins (Leary et al., 2007). Accordingly, while one Sco protein promotes maturation of the CuA site, the second regulates copper efflux under conditions of excessive cellular copper (Leary et al., 2007). Coa6 is an additional factor that together with human SCO2 promote the maturation of the CuA center (Pacheu-Grau et al., 2015). Interestingly, newly synthesised Cox2p does not make associations with any structural subunits until the Cox1p or Cox2p assembly modules associate. Indeed, Cox2p assembly intermediates were mainly found associated to Cox18p and Cox20p, and a small fraction with Sco1p and Coa6p (Franco et al., 2018). Cox16p has been detected in both Cox1p and Sco2p-Cox2p assembly complexes and has been proposed to be assembly factor involved in the association of Cox1p and Cox2p assembly modules (Aich et al., 2018; Cerqua et al., 2018; Su and Tzagoloff, 2017). The structural subunits, Cox9p and Cox12p, would be hypothesised to associate with Cox2p based on the structure of the final assembled COX enzyme, but the association of Cox9p and Cox12p with any COX catalytic subunit assembly modules has not been observed (Franco et al., 2018; Su et al., 2014a). Newly synthesised Cox3p associates with the structural subunits found in the final assembled enzyme (Cox4p, Cox7p, Cox13p) and Rcf1p, a protein involved in supercomplex

formation with complex III (Chen et al., 2012; Strogolova et al., 2012; Su et al., 2014a; Vukotic et al., 2012).

There are still unanswered questions that need to be addressed in the assembly of yeast COX. There are still several subunits and assembly factors that have not been observed to associate with newly synthesised catalytic core subunits, e.g., Cox9p, Cox12p. The steps where haem and copper are incorporated into the assembling COX enzyme has not been directly demonstrated. Proteins associated with incorporation these co-factors are known, for the case of Shy1p with Cox1p and Sco1p with Cox2p. However, these complexes have not been directly analysed for content of the appropriate co-factor. The final association of the three distinct assembly modules is also not known. Some of these unanswered questions may be due to limitations of the experimental technique where assembly is being followed *in vitro* where limiting factors may be absent or sensitivity to detergents may result in disruption of associations.

Some very interesting observations from the analysis of the assembly process require follow up. OXPHOS complexes are known to exist in supercomplexes, thought to promote efficient transfer of electrons from one complex to another which also decreases the opportunity for electron loss resulting in dangerous radicals in the cell. Cox6p associated with newly synthesised Atp9p, which forms the rotor component assembly module of ATP synthase. The association of Cox6p with Atp9p is independent of the requirement to for COX or ATP synthase, as mutants unable to form either of these complexes are able to form these complexes. This demonstrates a cross-talk between these OXPHOS complexes during biogenesis. The reason for the existence of this interaction has not yet been elucidated (Su et al., 2014b). The associations of assembly factors with COX structural subunits is thought to be transient to enable a required step in biogenesis to occur. However, prolonged associations with Shy1p and Cox16p in assembling and fully assembled COX have been observed (McStay et al., 2013b; Su and Tzagoloff, 2017). The purpose of these associations with fully assembled COX has not been investigated. Potential functions could be to mark newly synthesised COX or in maintenance of assembled COX. The formation of a fully assembled COX *in organello* is observed by BN-PAGE and 2D SDS-PAGE. However, the stoichiometry of catalytic core subunits is not representative of an equal amount of newly synthesised complex being present in the complex. Assembled COX after *in organello* labelling was composed of only newly synthesised Cox2p and Cox3p, with very minimal amounts of newly synthesised Cox1p (McStay et al., 2013b). This indicates there is a source of Cox1p within mitochondria that is primed and ready to be incorporated into COX when there are sufficient amounts of Cox2p and Cox3p. This perhaps indicates that Cox1p exists in a complex where haem and copper are already associated and awaits the synthesis of Cox2p and Cox3p. Cox1p containing complexes not part of fully assembled COX have been detected in yeast mitochondria and these could potentially represent a supply of pre-formed Cox1p modules awaiting incorporation into the fully formed enzyme. The complexity of the Cox1p subunit structure and requirements for associated co-factors and structural subunits supports this hypothesis. The number of steps required for Cox1p biogenesis may be rate-limiting and result in slow assembly of COX or present reactive intermediates that could result in electron leakage. However, this needs to be investigated in much more detail using *in vivo* conditions that may be more representative of normal physiological conditions.

The COX assembly process has been studied extensively in model organisms as well as using mitochondrial disease patient samples providing insights into the process in humans. This work has revealed many similarities but also many differences between the yeast and higher eukaryotic systems of COX assembly. Mutations in human COX assembly factor genes give rise to disease (COA3/MITRAC12, COA7, COA8/APOPT1, COX20, SCO1, COX10, TACO1, PET100). Fatal infantile

encephalocardiomyopathy is caused by mutations in SCO2, COX15, COA5, and COA6; and Leigh Syndrome by mutations in SURF1, COX10, COX15, TACO1, PET100, and LRPPRC.

Regulation of mitochondrial gene expression relies heavily on post-transcriptional steps. Mutations in the mammalian MTCO1 translational activator TACO1 are responsible for certain forms of Leigh Syndrome. Fibroblasts derived from these patients express novel complexes containing COX subunits. TACO1 mutation results in decreased translation of MTCO1, implying the novel complex is lacking MTCO1. Expression of MTCO2 and MTCO3 is also decreased, however translation occurs but theses subunits have decreased stability indicating the assembly process associated with these two subunits is also impaired (Weraarpachai et al., 2009). Mutations in LRPPRC are associated with French-Canadian type Leigh Syndrome (Lee et al., 2001; Mootha et al., 2003). Fibroblasts derived from these patients display reduced COX activity and assembled enzyme which is due to a decreased expression of COX mitochondrial transcripts, as well as those of some of Complex I (Sasarman et al., 2010). This is most likely due to its role as a mRNA chaperone in mitochondria (Siira et al., 2017).

4 – Modular Assembly

In addition to the studies in budding yeast, investigations into COX assembly complexes were carried out in assembly factor and structural subunit deficient human cell lines that revealed a number of complexes not previously detected in wild-type cells. These complexes were ordered based on antibody detection and metabolic radiolabelling of mitochondrial polypeptides into each complex. This investigation identified COXIV (homolog to yeast COX5) as one of the first structural subunits to associate with COXI (Nijtmans et al., 1998), in agreement with yeast studies. These subunits retain alternative isoforms that are regulated by oxygen concentration, perhaps indicating this is one of the first steps of COX assembly in eukaryotes where alternative subunits can be incorporated. This study also proposed that haem was incorporated into COXI early in the assembly process, followed by associations with the majority of other structural subunits. However, the resolution of each structural subunit association was not accomplished in this work but paved the way for the study of the step-by-step assembly pathway. A patient suffering from Leigh Syndrome-like symptoms was found to harbour a mutation in COX8A, a ubiquitously expressed isoform of COX8. This resulted in decreased COX activity in muscle biopsies and decreased expression of assembled COX without any evidence of accumulation of sub-assemblies (Hallmann et al., 2016). The other human isoform of COX8, COX8C, has a more restricted tissue distribution than COX8A, may be able to fulfil the role of COX8A when absent (Figure 4).

Cells derived from Leber's optic atrophy cells lacking SURF1 characterised using 2D gel electrophoresis identified four distinct COXI containing complexes similar to those identified in wild-type cells. Despite the absence of SURF1 protein, there was still some fully assembled COX present in these mutants (Tiranti et al., 1999). This is consistent with yeast, where the absence of SHY1 does not completely inhibit COX assembly. This indicates the function of SURF1/SHY1 can be by-passed, either by the natural kinetics of assembly or by another protein. Gene deletion approaches in cultured human cells have revealed the composition of some of these complexes. These complexes include an early CMC1-COA3-COX14 complex that assembles with newly synthesised COXI. A subsequent complex, known as MITRAC, contains COA3 (MITRAC12), COA1 (MITRAC15), COX1, COX4, COX5, COX6 with SURF1 and TIM21, a component of the IMM translocation machinery (Dennerlein et al., 2015; Mick et al., 2012). Deletion of CMC1 results in a decreased expression of functional COX, but synthesis is not inhibited, like in budding yeast, indicating an alternative mechanism of regulation in human cells (Bourens and Barrientos, 2017). RNA interference of

MITRAC12 results in the complete loss of the MITRAC complex, reduced COXI synthesis and reduced COX activity (Mick et al., 2012).

Several assembly factors have been identified as causative of disease for several groups of patients. This is the case for COA7, COA8, COX20, SCO1, SCO2, COA6 where deficiency of function or expression leads to decreased COX activity due to a decrease in the expression of fully assembled COX (Bourens et al., 2014; Leary et al., 2004; Martinez Lyons et al., 2016; Melchionda et al., 2014; Signes et al., 2018). Using RNA interference, an assembly pathway in humans was proposed where COX1 assembles with COX4 and COX5a initially (sub-assembly S2), which then recruits mature COX2. The remaining structural subunits then associate including COX3 (sub-assembly S4* and S4). However, the order where subunits associated is not clear (Fornuskova et al., 2010). Deficiency in COX20 results in an accumulation of S2 prior to the association of COX2 and other structural subunits. This accumulation supports the role of COX20 in the maturation of COX2, most likely through the copper centre formation (Szklarczyk et al., 2013). Disease causing mutations associated with SCO2 deficiency also display an accumulation of sub-assemblies. SCO2 mutations result in decreased COX2 synthesis and distinct sub-assemblies (Leary et al., 2009). These sub-assemblies are different from the sub-assemblies associated with SURF1 deficiency – pointing to the roles these two assembly factors have for different catalytic core subunits (Stiburek et al., 2009). SCO1 is also required for COX2 maturation and mutations found in patients results in similar sub-assemblies as SCO2 mutations, indicating they are in the same pathway. Mutations in COA6 also reveal sub-assemblies containing COXI and COXIV. COA6 is a copper binding chaperone required for the biogenesis of COX2 (Ghosh et al., 2016, 2014; Pacheu-Grau et al., 2015; Stroud et al., 2015). Similarly, to budding yeast studies the assembly process of COXII and COXIII is less well understood. This is again likely to be due to the lower number of assembly factors required for COXII and COXIII assembly. Assembly intermediates containing COXII and COXIII have not been described.

The Assembly process of F_1-F_O ATP synthase.

1 - Structure

The mitochondrial proton-translocating ATP synthase , the F_1-F_O complex, or complex V, utilizes a proton motive force to produce ATP from ADP and inorganic phosphate. The complex consists of the membrane-embedded F_O sector, responsible for the proton translocating activity, and the F_1 sector, which catalyses the ATP synthesis. F_O and F_1 sectors are connected by the F_1 central stalk and the peripheral stalk (Boyer, 1997). Human complex V is an assembly of 29 subunits, the F_1 sector consists of a catalytic globular hexamer and the central stalk. The globular part is arranged in dimers of α (Atp1p) and β (Atp2p) subunits: $\alpha 3\beta 3$. Each dimer α/β possesses a nucleotide binding pocket. Of the six adenine nucleotide binding sites, three catalytic are located in the β subunits, and alternate position with the three non catalytic in the α (Ackerman and Tzagoloff, 2005). The core of the spherical hexamer is occupied by the γ (Atp3p) subunit, which protrudes from the central part where interacts with δ (Atp16p) and ε (Atp15p) subunits to form the central stalk (Rak et al., 2009).

Differently of F_1, F_O protein composition of is not fairly constant across evolution. The core subunits of F_O found in both mitochondria and bacteria, are subunits *a* (Atp6p), *b* (Atp4p), and *c* (Atp9p). In eukaryotes the F_O sector embedded in the inner membrane contains the Atp9p/*c* ring with eight identical subunits in human or ten in yeast. Atp6p*/a*, which is associated to the Atp9p/*c* ring and forms the proton conduction pathway contains five transmembrane α-helices and an amphipathic α-helix that lies along the matrix surface (Guo et al., 2017). Atp4p*/b* has two N-terminal transmembrane α-helices, which form a domain with subunits *e* (Atp21p) and *g* (Atp20p); the soluble C-terminal portion of Atp4p*/b* enters the mitochondrial matrix as part of the peripheral stalk

(Guo et al., 2017). Subunit *f* (Atp17p) consists of a soluble N-terminal sequence of ~50 residues that binds to the base of the peripheral stalk, followed by a single transmembrane α-helix. The subunit 8 (Atp8p) N-terminus is intimately associated with Atp6p*/a* subunit and the C-terminal contributes to the base of the peripheral stalk (Guo et al., 2017; Srivastava et al., 2018). Subunit *i/j* (Atp18p in yeast or 6.8PL in human) is important for the stability of the F_1-F_O ATP synthase dimer.

The protonation and deprotonation events at the interface between Atp6p*/a* and Atp9p*/c* ring couple the translocation of protons to the rotation of the Atp9p/*c*-ring (Stock et al., 2000; Symersky et al., 2012). The C-terminal of the Atp9p*/c* protein contains negatively charged Glutamate residues exposed on the outside of the ring, which are neutralized by protons from the IMS. The protonation pathway during ATP synthesis is a path formed by subunits Atp6p*/a*, Atp4p*/b*, and Atp17p*/f* with the final course formed by helices 5 and 6 of Atp6p*/a* (Srivastava et al., 2018). As the protons passed by the channel and neutralize these residues, they change their conformation to move to a more hydrophobic environment, making the ring movement of the rotor, which induces conformational changes to the central stalk Atp3p (γ) subunit. Therefore, Atp3/γ transmits these rotational movements to the F_1 catalytically head altering the nucleotide binding pockets conformation that leads to ATP production, while the peripheral stalk keeps the catalytic head static for the energy transducing process preventing from rotating along with the central rotor (Ackerman and Tzagoloff, 2005). Based on high resolution structural information, the peripheral stalk is composed of subunits OSCP (Atp5p), *b* (Atp4p), *d* (Atp7p), *h* (Atp14p or F_6 in human), *f* (Atp17p), *i/*j (Atp18p/6.8 PL) and Atp8p. Subunits Atp5p, Atp4p, Atp7p, Atp14p/F_6, and Atp17p primarily serve as structural components of the stator (Guo et al., 2017) while, subunits Atp4p, Atp7p, Atp17p, Atp18p/6.8 PL and Atp8p are also part of the complex purified as F_o, and may have a role in proton movement in addition to a structural role in forming the stator (Srivastava et al., 2018).

Non-essential structural components of F_1-F_O ATP synthase include subunits *e* (Atp21p), *g* (Atp20p) and *k* (Atp19p in yeast or DAPIT in human), which are associated with the dimeric form of the enzyme (Arnold et al., 1998b). Subunits *e, f, g ,* DAPIT and 6.8PL are also known as supernumerary with no direct role in the synthesis of ATP. They are all localized in the region of interaction between monomers in the dimeric complex. Indeed, individual deletion of human subunits *e, f, g*, and 6.8PL disrupts dimerization, while deletion of DAPIT influences oligomerization of dimers (He et al., 2018).

The fully assembled F_1F_O-ATP synthase dimerizes via Atp4p transmembrane helixes by the incorporation of Atp20p and Atp21p and by the assistance of Atp18p and Atp19p/DAPIT (Davies et al., 2014, 2012; Wagner et al., 2010). The dimer is held together by subunits Atp6p, Atp18p/6.8PL, Atp19p/DAPIT, and Atp21p/*e* (Guo et al., 2017; He et al., 2018). The formation of F_1-F_O ATP synthase dimers plays a major role in determining the structure of mitochondrial morphology and consequently the *cristae* formation and local increase in membrane potential (Paumard et al., 2002). The curved structure of the domain formed by Atp20p, Atp21p and the N-terminal of Atp4p, with further support from Atp19p/DAPIT explain how these subunits bend the lipid bilayer (Guo et al., 2017). Indeed, ATP synthase dimers are self-assembled into rows, which are able to bend the membrane (Blum et al., 2019). It was recently demonstrated that assembly of the F_1-F_O ATP synthase complex occurs in the *cristae* bends favouring the complex V intermediate accumulation and consequently a continuous of cristae formation (Stoldt et al., 2018).

Regulatory auxiliary proteins are also associated to F_1-F_O ATP synthase. Inhibitory proteins Inh1p, Sft1pand Stf2p modulates the hydrolytic activity of the complex in yeast. In human mitochondria IF1 inhibits the complex by binding to one of the catalytic α-β interfaces (Ackerman and Tzagoloff, 2005; Hashimoto et al., 1990; Robinson et al., 2013). Dephosphorylated IF1 inhibits the synthase and hydrolytic activity of the ATP synthase (García-Bermúdez et al., 2015) the protein has a short half-life, its expression is tissue dependent and controlled at posttranscriptional levels by S39 phosphorylation (García-Aguilar and Cuezva, 2018).

2 - Mitochondrial Gene expression

The monomeric form of F_1-F_O ATP synthase contains 18 subunits in humans, including the regulatory IF1 (Srivastava et al., 2018), whose expression relies on information from two compartmentally separated genomes. An extraordinary situation, which raise the question of how two compartmentally separated genome communicate to express balanced output of gene products destined to be subunits of the same enzyme. In the yeast S*. cerevisiae* three subunits: Atp6p, Atp8p and Atp9p are mitochondrially encoded while in animals and in filamentous fungi only Atp6p and Atp8p remained encoded by the organelle. Curiously, *Neurospora crassa* and *Aspergillus nidulans* have both the nuclear and the mitochondrial version of Atp9p (Rak et al., 2009). At any rate, synthesis of Atp9p and Atp6p must be strictly regulated to prevent accumulation of unassembled proton channels that may lead to dissipation of the membrane potential.

Biogenesis of F_1-F_O ATP synthase is assisted by some dozen nuclear gene products that intercede at all stages of the assembly pathway (Ackerman and Tzagoloff, 2005). Most of these factors in yeast target the mitochondrial *ATP6*, *ATP8*, *ATP9* mRNAs and their products since regulatory mechanisms can occur in the different steps of the mitochondrial gene expression, for instance, every single mRNA needs to be activated by a specific protein in order to be translate in the mitoribosomes (Costanzo and Fox, 1990). *ATP8* and *ATP6* are transcribed on a polycistronic RNA together with *COX1,* while *ATP9* is co-transcribed with $tRNA^{ser}$ and *VAR1* (Rak et al., 2009). *ATP9* mRNA requires Aep1p/Nca1p, Aep2p/Atp13p and Atp25p (Ackerman et al., 1991; Zeng et al., 2008) for its stabilization. Aep1p/Nca1p and Aep2p/Atp13p are also considered potential translational activators of the *ATP9* mRNA (Godard et al., 2011). In human, *ATP8* and *ATP6* are also transcribed in a bi-cistronic mRNA unit with a 46-nucleotide overlap (Fearnley and Walker, 1986). Differently of other mitochondrial mRNAs, addition of the poly-A tail to ATP8/6 mRNA decreases its stability, while its removal depends on the PDE12 factor (Rorbach et al., 2011). According to the MITOMAP database 48 different mutations in these two genes have already been identified. As typically found in mitochondrial genetic disorders mutations that cluster in specific regions of *ATP6* or *ATP8* also give rise to a wide variety of clinical symptoms (Dautant et al., 2018).

In contrast to the human *ATP6-ATP8* transcript, various proteins have been described in yeast affecting *ATP6-ATP8* expression. Nca2p, Nca3p, Aep3p and Atp22p were previously shown to affect expression of Atp6 and Atp8p by either stabilizing (Camougrand et al., 1995; Ellis et al., 2004; Pélissier et al., 1995) or activating translation of the bicistronic *ATP8-ATP6* mRNAs (Barros and Tzagoloff, 2017; Zeng et al., 2007a). Although together in the same mature mRNA *ATP6* and *ATP8* have different translation activators; translation of *ATP6* is strictly dependent on Atp22 (Zeng et al., 2007a) and translation of *ATP8* depends on Aep3p, which is also necessary for mRNA stabilization as mentioned above (Barros and Tzagoloff, 2017; Ellis et al., 2004).

The Aep1p/Nca1p, Aep2p/Atp13p, Aep3p and Atp22p proteins required for *ATP9* and *ATP6/8* mRNA stabilization and translational activation contains (PPR) motifs (Herbert et al., 2013), which consists of repeated in tandem in two antiparallel α-helices leading to a helix-turn helix domain, which form an RNA-binding groove (Manna, 2015).

Translational control of the bicistronic *ATP8/ATP6* mRNA by assembled F_1 ATPase ensures a balanced output of the nuclear F_1F_O-ATPase complex and mitochondrial subunits (Rak and Tzagoloff, 2009). In a very interesting mechanism, translation of *ATP6* mRNA is repressed by Smt1p and maintains the *ATP8/ATP6* mRNA in a translation mute form when F_1 is in limiting conditions (Rak et al., 2016), with fully assembled F_1, Smt1p is displaced allowing the recognition of the mRNA by Atp22p, the Atp6p translational activator. Indeed, in the absence of Aep3p, the putative Atp8p translational activator, low expression of a nuclear version of *ATP8* can complement the *aep3* mutant if *smt1* is also mutated (Barros and Tzagoloff, 2017).

To date it is still elusive the existence of mitochondrial translational activators in human mitochondria, however many unstudied factors have been identified in mitochondrial RNA granules, which are centers for posttranscriptional RNA processing (Antonicka and Shoubridge, 2015).

Mitochondrial translation has been demonstrated to occur at different sites of the IMM: the inner boundary membrane (IBM), which parallels the outer membrane, and the *cristae* membrane

(CM), which projects into the mitochondrial matrix (Stoldt et al., 2018). Proteins involved in the biogenesis of of F_1-F_O ATP synthase such as Atp22p, Atp25p (C-terminus) and Atp10p are particularly enriched in the CM, which as mentioned earlier favoured the formation of the *cristae* structures in the mitochondrial inner membrane (Stoldt et al., 2018).

3 – Protein Assistance in the Assembly process

3.1 - F_O assembly

Formation of F_O is a protein assisted process, dependent of Atp10p, Atp23p, Ina17p and Ina22p proteins. Following synthesis on mitoribosomes Atp6p is bound to Atp10p, whose association protects Atp6p from proteolysis and assists the assembly of Atp6p into F_O (Ackerman and Tzagoloff, 1990a; Tzagoloff et al., 2004). In fact, Atp6p is subjected to proteolysis processes in mitochondria with reduced levels of the stator component Atp14p (Goyon et al., 2008). Interestingly the *atp10* null mutant can be suppressed by mutations in the Atp6p C-terminus that increase the protein stability (Paul et al., 2000). In fact, it was demonstrated that in the process of F_O assembly Atp10p associates with the Atp6p/Atp8p intermediate (Naumenko et al., 2017; Rak et al., 2011) in a complex that also contains components of the peripheral stalk and Atp23p (Naumenko et al., 2017). Atp10p is a 30 kDa IMM protein; homologues of Atp10p can be found in many eukaryotes groups but not in metazoa and green algae (Pícková et al., 2005). Atp23p is a metalloprotease with the catalytic center formed by the HEXXH motif that processes the 10 amino acid N-terminal signal sequence of Atp6p (Osman et al., 2007; Zeng et al., 2007c). ATP23 is amplified in human glioblastomas, and several transcript variants encoding different isoforms have been found for this gene. The construction of an *atp23* mutant with a point mutation in the catalytic center revealed that the removal of the Atp6p N-terminus is not essential for biogenesis of the F_1-F_O ATP synthase (Zeng et al., 2007b). However, the physical participation of Atp23p in F_O assembly process can be considered the essential role of Atp23p for F_1F_O-ATPase assembly. A genetic association between *ATP23* and *ATP10* was also observed, *ATP23* overexpression rescues the respiratory growth of *atp10* mutants (Zeng et al., 2007c), suggesting that Atp23p excess can overcome the lack of Atp10p. Finally, the importance of Atp6p N-terminus was assessed in *atp6* variants lacking the N-terminus, these mutants although respiratory competent presented decreased efficiency in the assembly of the F_1-F_O ATP synthase (Zeng et al., 2007c).

It was demonstrated that Atp23p interacts with Atp10p in multiple protein complexes that also contains Atp6p/Atp8p intermediates in *ina22* null mutants (Naumenko et al., 2017). According to a steady state study performed in yeast cells there is no free pool of Atp23p in mitochondria, and the protein seems to exist only associated to F_1F_O-ATPase assembly intermediates (Naumenko et al., 2017). Ina22p together with Ina17p constitute the INA complex (INAC). Both proteins interact with each other in coiled-coil domains, Ina22p becomes unstable in *ina17* mutants (Lytovchenko et al., 2014). Ina22p is integrated in the IMM with a single transmembrane anchor, interacting with subunits of the peripheral stalk specially Atp4p, which has a central role in the peripheral stalk structure. Mitochondria lacking Ina22p show defects in the assembly of the stator proteins Atp4p, Atp5p, and Atp14p resulting in the accumulation of free F_1 module in the matrix (Lytovchenko et al., 2014). Importantly, INAC is not essential for F_1F_O-ATPase biogenesis, as the mature complex is still present in mutant mitochondria (Lytovchenko et al., 2014). ATPase mitochondrially encoded genes have their expression altered in *ina22* mutants, i.e., the rate of Atp6p and Atp8p translation is augmented (Naumenko et al., 2017), probably due to an augment of free F_1 , which as mentioned before control the translation of the bicistronic *ATP8/ATP6* mRNA (Rak and Tzagoloff, 2009). Oppositely, the level of newly synthesized Atp9 and consequently ring formation is reduced in these mutants (Naumenko et al., 2017).

3.2 - Atp9p/c ring formation

The human genome contains three genes *ATP5G1*, *ATP5G2*, and *ATP5G3* encoding for the *c* subunit, they are identical with slight differences in the amino-terminus mitochondrial targeting sequence. The disruption of the three genes altogether leads to formation of an incomplete F_1F_O-ATPase not only devoid of the *c* ring but also without subunits a, 8, 6.8PL and DAPIT (He et al., 2017b).

Newly synthesized Atp9p subunits are not immediately incorporated into the Atp9p ring. Even though, the oligomerization of Atp9p has been assumed to occur spontaneously, different proteins have been described to be involved in this process. In *E. coli c*-ring oligomerization depends on the *uncI* gene product (Ozaki et al., 2008), and in yeast this process depends on the two-domain protein Atp25p (Zeng et al., 2008). *ATP25* codes for a protein that consists of two isolated and independent domains: an N-terminal domain related to the Rsf (ribosome silencing factor) of bacteria and the C-terminal necessary to stabilize the *ATP9* mRNA (Woellhaf et al., 2016; Zeng et al., 2008). The Atp25p N-terminus was shown to be associated to the mitoribosomes (Woellhaf et al., 2016) as well as a role in the *c*-ring oligomerization has been proposed (Rak et al., 2011; Zeng et al., 2008). In fact, the oligomerization of Atp9p is considered a slow process, in comparison to Atp6p/Atp8p association (Zeng et al., 2008), but some factors have also been described as facilitators of this process, such as Oxa1p the mitochondrial translocase protein (Jia et al., 2007). The insertion of Atp9p into the IMM does not depend on the translocase activity of Oxa1p, but the formation of the Atp9p ring is facilitated by Oxa1p chaperone ability (Jia et al., 2007). Also interestingly, the Atp9p ring formation is specifically reduced in the *cox6* mutant, a cytochrome *c* oxidase deficient mutant (Su et al., 2014b). Indeed, newly synthesized Atp9p can be pulled down with a tagged version of Cox6 (McStay et al., 2013a). Therefore, the presence of unassembled Atp9p in large molecular complexes can be part of a regulatory process necessary for the proper assembly of the Atp9p ring into the mature F_1F_O-ATPase.

3.3 - The F_1 catalytic head

The F_1 sector assembles independently of the F_O subunits (Schatz, 1968; Tzagoloff, 1969), its biogenesis requires the general chaperones Hsp60, Hsp70, Hsp90 and at least three specific factors Atp11p/ATPAF1, Atp12p/ATPAF2 and Fmc1p (Ackerman and Tzagoloff, 2005; Wang et al., 2001).

Therefore, the formation of the $\alpha 3\beta 3$ hexamer sub-structure is a protein-assisted process. Atp11p and Atp12p interact with the β and α subunits, respectively, and function as molecular chaperones that stabilize unassembled α and β subunits by shielding their hydrophobic surfaces (Ackerman and Tzagoloff, 2005, 1990b). Atp11p binds to the β subunit at the nucleotide binding domains (Wang and Ackerman, 2000), while Atp12p binds to the α subunit at the pre-interface surface that will form the non-catalytic site with a neighbouring β subunit (Wang et al., 2000). Therefore, loss of Atp11p and Atp12p leads to aggregation of subunits α and b, and consequently impairs the assembly of the F_1 sector (Wang et al., 2000). Indeed, efficient production of recombinant bovine mitochondrial F_1 in *Escherichia coli* depends on the expression of human Atp11p (AF1) and Atp12p (AF2) (Suzuki et al., 2016).

It has been proposed that the association of the central stalk to the Atp11p-Atp12p- $\alpha 3\beta 3$ hexamer provoke the release of Atp11p and Atp12p and the formation of the F_1 sector. Structural analyses reveal that the C-terminal region of Atp12p and the coiled coil tail of the γ-subunit are structurally similar, prompting the hypothesis that the binding of Atp3p to the Atp12p-α complex initiates the release of Atp12p and Atp11p chaperones from α and β and their further assembly into the mature sector (Ludlam et al., 2009). Fmc1p is not present in metazoa (Pícková et al., 2005), it is considered an Atp12p specific chaperone and it is required for F_1 assembly under conditions of heat stress (Lefebvre-Legendre et al., 2001). Interestingly, *fmc1* mutants can be suppressed by *ATP12* excess that indicates a function of Fmc1p in Atp12p folding or stabilization. A direct role of Fmc1p in Atp12p was also suggested in a study using the pathogenic human Atp12p W94R mutation in yeast, this *atp12* mutant became insoluble in yeast only in the absence of Fmc1p (Meulemans et al., 2010). Proteins homologous to Atp12p are widely present in eukaryotes; the same is true for Atp11p,

except for some groups of nematodes, on the other hand, Fmc1p is restricted to the fungi kingdom (Pícková et al., 2005).

4 - Modular Assembly

In the process for the assembly of the mature F_1F_O-ATPase complex, different modules have been identified, particularly in the formation of the F_O sector and the peripheral stalk (Figure 5). As mentioned earlier the soluble F_1 sector was shown to assemble as an independent unit. Two distinct modules assemble the F_O sector: the Atp9p ring (*c*-ring) and the Atp6p/Atp8p (*a*/8) complex (Naumenko et al., 2017; Rak et al., 2011). The modular assembly F_1F_O-ATPase depends on the INA complex, which interacts physically with two distinct subassemblies modules: the Atp9p ring and Atp6p/Atp8p intermediate (Naumenko et al., 2017). Human ρ^0 cells devoid of mitochondrial DNA, which cannot make subunits Atp6p/*a* and Atp8p are still able to synthesize the *c*-ring and F_1-peripheral stalk module (He et al., 2017b).

In contrast to the Atp9p ring formation, the Atp6p/Atp8p stator subcomplex intermediate is a very fast process that include the association of the peripheral stalk subunits Atp4p, Atp7p, Atp14p, OSCP/Atp5p and the chaperones Atp10p and Atp23p (Naumenko et al., 2017; Rak et al., 2011). In fact, the observed association between the Atp6p/Atp8p intermediate with OSCP protein could only be detected under low salt conditions (Naumenko et al., 2017). Based on Atp10p pull down assays performed from cells devoid of *ina22*, it has been proposed a mechanism in which the INAC mediate the assembly of the mature F_1F_O-ATPase complex, through the association of Atp6p/Atp8p with the peripheral stalk and the F_1 sector. Only after the formation of this intermediate the Atp9p ring is added, ensuring the coupling of proton movement to ATP production (Naumenko et al., 2017). However, in *atp6* or *atp8* null mutants F_1 pull down assays revealed the association of F_1 with the Atp9p ring independently of the presence of the Atp6p/Atp8p intermediate (Rak et al., 2011). The formation of an F_1-Atp9p ring intermediate was also observed in human cells with *ATP6* expression suppressed (Fujikawa et al., 2015). In another study it was shown that in mitochondrial translocase mutant (*oxa1*) the Atp9p ring is accumulated associated with F_1 but not with Atp6p, therefore, it was proposed that Oxa1p makes the Atp9p ring ready for Atp6p/Atp8p intermediate assembly (Jia et al., 2007).

More recently, the use of gene editing techniques has allowed the removal of specific subunits of the F_1F_O-ATPase complex and therefore allowing the study of assembly intermediates and the formation of vestigial F_1F_O-ATPase in human cells (He et al., 2017a, 2017b). Indeed, the study of vestigial F_1F_O-ATPase complex in human cell lines devoid of supernumerary components or peripheral stalk subunits also indicate a different order of events in the human F_1F_O-ATPase assembly in comparison to yeast (He et al., 2018). In this study, cells lacking subunits *e, f, g*, DAPIT, and 6.8PL can assemble the remaining subunits into an incomplete ATP synthase complex with severe outcomes on cell respiration, except for DAPIT removal, which retained almost normal levels of the wild type respiratory capacity. Moreover, with this strategy the authors found that an F_1*/c* ring intermediate is formed prior to the addition of the peripheral stalk components and the last step for the formation of the F_1F_O-ATPase is the association with the Atp6/Atp8 intermediate (He et al., 2018). In another study, the knock down of Atp7, a peripheral stator subunit, human cells accumulated two subcomplexes, one containing a central rotor shaft plus catalytic subunits (F_1- Atp9 ring) and the other containing Atp4, Atp20 and Atp21 stator stalk components (Fujikawa et al., 2015). It is worthwhile to note that the yeast assembly factors Atp10p, Ina22p and Ina17p are not present in human cells, which can account for these differences in the order of events for the assembly of the mature F_1F_O-ATPase complex.

Conclusion

The complexity of the OXPHOS system can be immediately appreciated by the number of complexes, the number of subunits and co-factors each complex is composed of, the functions of each complex, the assembly factors required for each complex, the location of the complexes and the dual genetic

origin of the complexes. With so many requirements there is a seemingly high likelihood of error that would be detrimental to cellular function and organismal health. Owing to the fundamental use of electrochemical gradients across membranes in all forms of cellular life the evolutionary pressures on maintaining functional complexes must be powerful. As life became more complex and adapted to different environments and developmental stages the control of assembly and function evolved to be more intricate. The conservation of function from bacteria to single celled and multicellular eukaryotes has enabled a deep knowledge of OXPHOS complex assembly that is being used to understand the process further but to also understand the origins of eukaryotic life as well treatments of human disease.

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