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Review

Assembly of mitochondrial complex I and defects in disease

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ABSTRACT

Isolated complex I deficiency is the most common cause of respiratory chain dysfunction. Defects in human complex I result in energy generation disorders and they are also implicated in neurodegenerative disease and altered apoptotic signaling. Complex I dysfunction often occurs as a result of its impaired assembly. The assembly process of complex I is poorly understood, complicated by the fact that in mammals, it is composed of 45 different subunits and is regulated by both nuclear and mitochondrial genomes. However, in recent years we have gained new insights into complex I biogenesis and a number of assembly factors involved in this process have also been identified. In most cases, these factors have been discovered through their gene mutations that lead to specific complex I defects and result in mitochondrial disease. Here we review how complex I is assembled and the factors required to mediate this process.

1. Introduction

In most eukaryotes, the mitochondrial respiratory chain consists of four multi-subunit complexes (complexes I–IV) and two electron carriers; ubiquinone and cytochrome *c*. Oxidative phosphorylation (OXPHOS) begins with the entry of electrons into the respiratory chain through either complex I or II (Fig. 1). Electrons enter from NADH via complex I, or from succinate via complex II and are transferred to ubiquinone. Complex III carries electrons from reduced ubiquinone to cytochrome *c*, and complex IV completes the sequence by transferring electrons from cytochrome *c* to oxygen. The transport of electrons is coupled to translocation of protons across the inner membrane into the intermembrane space, creating a transmembrane proton gradient that provides the driving force of ATP production by complex V (ATP synthase). The dependence on OXPHOS and energy consumption for humans was summarized by Rich [1], where it was reported that the average person turns over 65 kg ATP/day. Given the high dependence on OXPHOS for such energy, it comes as no surprise that defects in the OXPHOS system lead to disease.

Representing an incidence of ~1 in 5000 live births, disorders of the mitochondrial OXPHOS system are the most common of inborn metabolic diseases [2,3]. They result in a wide variety of clinical phenotypes that may initially present in infancy or early adulthood. A number of different organs or tissues may be affected, especially brain, heart and skeletal muscle, which rely heavily on OXPHOS for ATP. Isolated complex I deficiency is the most common cause of respiratory chain dysfunction [3,4], with patients displaying varying clinical manifestations that range from single to multiple tissue involvement (Table 1). For example, mutations in mtDNA-encoded complex I subunits are associated with Leber Hereditary Optic Neuropathy (LHON), a form of blindness where only the optic nerve is affected [5,6]. Conversely, other complex I mtDNA mutations can result in Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-like episodes (MELAS), a clinically and genetically heterogeneous disorder comprised of myopathy, encephalopathy, and central nervous system involvement that results in seizures, paralysis, blindness, and episodic vomiting [7]. Complex I defects are also associated with fatal childhood disorders such as Leigh Syndrome, an early-onset progressive neurodegenerative disorder with a characteristic neuropathology consisting of focal, bilateral lesions in one or more areas of the central nervous system [8]. This disease can present with a variety or combination of other clinical features, including; hypertrophic cardiomyopathy, hypotonia, ataxia and deafness. The etiology underlying the variety of clinical phenotypes in complex I deficiency is not well understood, but may be due to different gene mutations that exert variable effects on the stability, assembly and activity of the enzyme complex.

Biochemically, defects in complex I appear to often occur as a result of impaired assembly of the enzyme. However the biogenesis of complex I is not well understood and this is complicated by its large size, regulation by two genomes and lack of a detailed crystal structure. Furthermore, the model yeast *Saccharomyces cerevisiae* lacks a true complex I and this has prevented such detailed analyses into assembly seen for other complexes of the respiratory chain (see other reviews in this issue for details).

2. Mammalian complex I

Complex I is the largest and least understood component of the respiratory chain. It consists of 45 different subunits that assemble together into a structure of ~1 MDa [9]. Mitochondrial DNA (mtDNA) encodes 7

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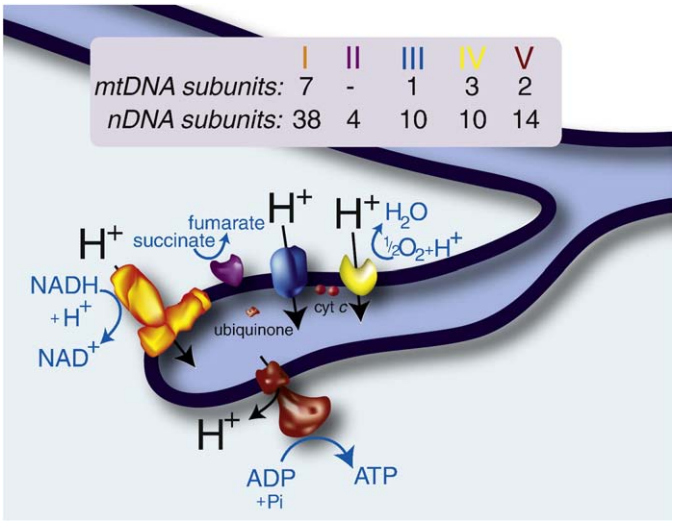


Fig. 1. The mitochondrial OXPHOS machinery. Residing in the inner mitochondrial membrane, the mitochondrial OXPHOS machinery is comprised of 5 complexes. Electrons enter complex I from the oxidation of NADH to NAD^+, or complex II from the oxidation of succinate, and exit at complex IV resulting in the reduction of O_2 to $2H_2O$. Ubiquinone and cytochrome *c* act as electron carriers. Coupled to the flow of electrons between complexes I–IV is the pumping of protons across the inner membrane, creating an electrochemical gradient that is utilized by complex V to generate ATP. Inset: the number of nuclear and mtDNA-encoded subunits within each complex is shown.

complex I subunits. The remaining 38 subunits are encoded by nuclear genes, and following their translation are imported into the organelle through the import machineries [10,11]. Electrons from the oxidation of NADH are transferred to a non-covalently bound flavin mononucleotide (FMN) and are subsequently passed through a series of iron–sulfur clusters (Fe–S) to the final acceptor ubiquinone, which is then reduced to ubiquinol. The transfer of every pair of electrons is energetically coupled to the pumping of 4 protons into the intermembrane space.

3. Modular architecture of complex I

Homologs of mammalian complex I can be found in bacteria, archaea and plants. *E. coli* complex I consists of 14 subunits, all of which have orthologous subunits in the eukaryotic counterpart [12]. The bacterial complex is ~550 kDa and can be broken down into three functional modules – the electron input module (N Module), electron output module (Q Module), and the proton translocation module (P Module) – which have been evolutionarily conserved [13,14].

The functional modules of complex I are depicted in Fig. 2. The N module binds and oxidizes NADH resulting in the liberation of electrons that are transferred via flavin mononucleotide (FMN) onto a series of Fe–S clusters. This functional module has been traced back to two separate origins. The first is from the soluble NAD^+-reducing hydrogenases as found in the chemolithotrophic purple bacterium *Alcaligenes eutrophus* [15]. The other is from the bacterial formate dehydrogenase complex, an enzyme that couples the oxidation of formate with proton reduction [16]. The Q module receives electrons that have been transferred through the Fe–S clusters of the N module and transfers them to ubiquinone. This module shows homology to the water soluble Ni–Fe hydrogenases [17]. While the N and Q modules lie in the mitochondrial matrix (or bacterial cytoplasm), the P module is membrane embedded. The P module is believed to be involved in proton pumping and some of its components seem to have evolved from bacterial Na^+/H^+ and K^+/H^+ antiporters [18]. Furthermore, components of this module also show homology to the membrane bound Ni–Fe hydrogenases. It has been suggested that a common ancestor of complex I lost the Ni–Fe active site in its subunit(s) and gained the ability to react with quinones [19].

4. Structure of complex I

While crystal structures for the other OXPHOS complexes have been solved, only low resolution 3D structures of complex I are available [20–22]. The general consensus for the overall shape of complex I from

Table 1
Complex I activity and assembly status in patients with mutations in genes known to cause complex I deficiency

				Complex I defect[a]		
	Human gene	Gene product description	Disease	Assembly/stability[b]	Enzymatic[c]	References
mtDNA	*ND1*	CI subunit, P module	LHON, MELAS	+++	+++	[6,7]
	ND2	CI subunit, P module	Leigh S.	++	+	[133]
	ND3	CI subunit, P module	Leigh S., LIMD	+	++	[134,135]
	ND4	CI subunit, P module	LHON , Leigh S.	++	++	[136–138]
	ND4L	CI subunit, P module	LHON	N.D.	+	[5]
	ND5	CI subunit, P module	Leigh S., MELAS, LHON	+	++	[78,81,83,139]
	ND6	CI subunit, P module	Leigh S., LHON, MELAS	+++	+++	[80,140,141]
Nuclear	*NDUFA1*	CI subunit, Q module	Leigh S.	+	+	[142]
	NDUFA11	CI subunit, Q module	Cardioencephalomyopathy, LIMD	N.D.	+++	[143]
	NDUFS1	CI subunit, Q module	Leigh S., leukodystrophy	++	++	[144,145]
	NDUFS2	CI subunit, Q module	Cardioencephalomyopathy, Leigh S.	+++	++	[146]
	NDUFS3	CI subunit, Q module	Leigh S.	N.D	++	[147]
	NDUFS4	CI subunit, Q module	Leigh S.	++	++	[85,148]
	NDUFS6	CI subunit, Q module	LIMD	++	+++	[86]
	NDUFS7	CI subunit, Q module	Leigh S.	+	++	[149]
	NDUFS8	CI subunit, Q module	Leigh S.	+++	++	[8,75]
	NDUFV1	CI subunit, N module	Leigh S.	++	++	[87,144]
	NDUFV2	CI subunit, N module	Cardioencephalomyopathy	N.D.	++	[150]
	NDUFAF1	CI assembly protein (CIA30)	Cardioencephalomyopathy	++	++	[91]
	NDUFA12L	CI assembly protein (B17.2L)	Encephalomyopathy	++	++	[87]
	C6ORF66	CI assembly protein	Encephalomyopathy	++	+++	[96]

Abbreviations: LHON, Leber's Hereditary Optic Neuropathy; MELAS, Mitochondrial Encephalopathy, Lactic Acidosis, Stroke-like episodes; Leigh S., Leigh Syndrome; LIMD, Lethal Infantile Mitochondrial Disease, N.D., Not determined.
[a]For some genes, certain mutations cause milder biochemical defects and the data shown are for the most severe defects reported for each gene.
[b]Assembly status was scored as + modest decrease in amount of holocomplex I, ++ marked decrease in amount of holocomplex I or accumulation of subcomplexes, and +++ gross decrease in amount of holocomplex I and subcomplexes.
[c]Activity status was scored as + modest decrease, ++ marked decrease, and +++ gross decrease.

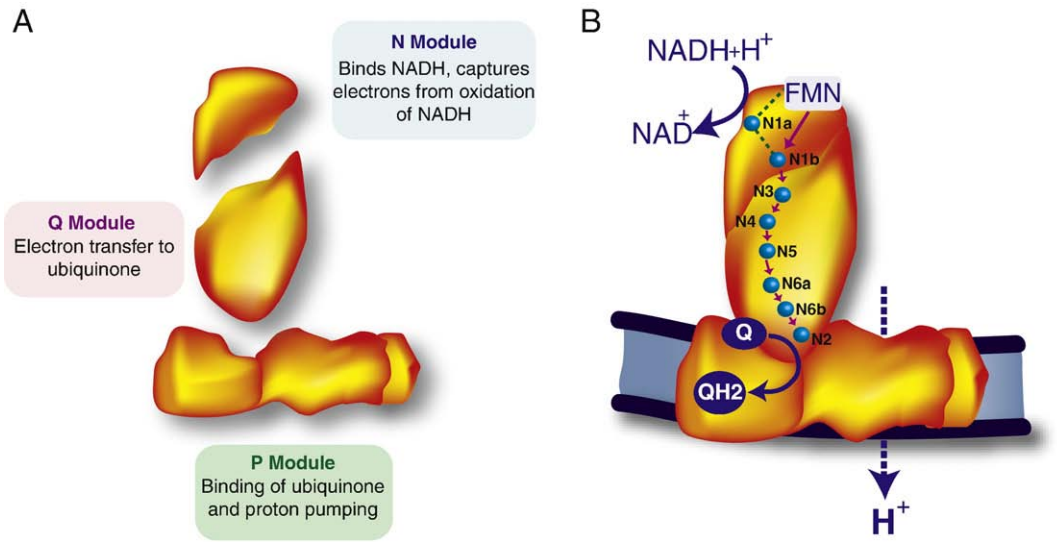


Fig. 2. Complex I modules and activity. A. Complex I is made up of three conserved functional modules; the electron input module (N Module), electron output module (Q Module), and the proton translocation module (P Module). B. Schematic depicting complex I electron-transfer pathway and proton pumping activity. Electrons from the oxidation of NADH are transferred through complex I via flavin mononucleotide (FMN) and a series of iron–sulfur (Fe–S) clusters (blue circles) to ubiquinone (Q), forming ubiquinol (QH_2). The main pathway of electron transfer is indicated by purple arrows and diversions by green dashed lines. For a detailed summary of the electron-transfer pathway see [151]. Cluster N1a is thought to be located in subunit NDUFV2; N3 and FMN in NDUFV1; N1b, N4 and N5 in NDUFS1; N6a and N6b in NDUFS8; and N2 in NDUFS7.

bacteria and mitochondria is of an L-shaped boot [23]. This structure consists of a hydrophilic peripheral arm with a hydrophobic membrane arm lying perpendicular to it (Fig. 3). Work carried out by Sazanov et al. [24] demonstrated that in *E. coli*, this conformation of complex I is maintained in a native lipid bilayer as an active enzyme. This contradicts the observation by Bottcher et al. [25] describing complex I as adopting a 'horseshoe' conformation. It has since been suggested that the 'horseshoe' may represent dimers of complex I with insufficient staining of the peripheral arms [24]. Alternatively, the peripheral arm may have some conformational flexibility and the 'horseshoe' shape represents a conformational extreme [23].

Recently, the atomic structure of the hydrophilic peripheral arm of complex I from the thermophilic bacterium *Thermus thermophilus* was solved using X-ray crystallography [26,27]. The structure has provided insights into the electron transduction pathway of complex I and also the relative positions of the core subunits present in the matrix arm. As the core subunits are evolutionarily conserved, the electron transduction pathway may be applied to mitochondrial complex I. However since mitochondrial complex I contains many additional subunits of unknown function, the bacterial model cannot provide a complete picture.

5. Core subunits of complex I

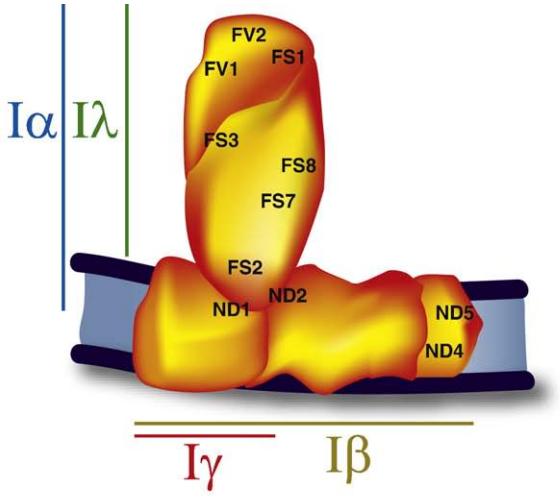
Prokaryotic complex I is made up of 14 subunits that are conserved through evolution. These subunits represent the 'core' of complex I and compose the minimal structural unit required for the enzyme's primary function, i.e. electron transfer coupled to proton translocation. In mammals, half of the core subunits are encoded by nuclear genes and all of these are located in the peripheral matrix arm.

The subunit composition of bovine complex I has been extensively studied [28–33] with the nomenclature of subunits based on subunit size in kDa. With the increasing importance of complex I in human disease being realized, research has shifted its focus on the human complex where the nomenclature differs with subunits adopting the gene name. Nuclear gene-encoded subunits are termed NADH dehydrogenase ubiquinone ("NDU") followed by a description of predicted function/location (FS–iron–sulfur protein region, FV-flavoprotein region, FA-subcomplex α, FB-subcomplex β, FC-undefined subcomplex). mtDNA-encoded subunits are termed NADH dehydrogenase (ND) followed by the subunit number. The core subunits NDUFS1, NDUFS2, NDUFS3 NDUFS7, NDUFS8, NDUFV1 and NDUFV2 are encoded by the nuclear genome and are highly conserved in eukaryotes. These subunits are involved in the oxidation of NADH and subsequent transfer of electrons to ubiquinone. The seven remaining core subunits ND1–6 and ND4L, are encoded by mtDNA. They are all very hydrophobic and are located in the membrane arm of the complex. The mtDNA-encoded subunits, along with ~13 integral membrane proteins encoded by the nuclear genome, contribute more than 60 transmembrane segments to form the membrane arm of complex I [34]. Subunit ND1 harbors a quinone binding site and is therefore predicted to be involved in ubiquinone binding. Subunits ND4 and ND5 may also harbor ubiquinone binding sites [35]. Sequence analysis comparisons of ND2, ND4 and ND5 show that they are related to K^+/H^+ or Na^+/H^+ antiporters, highlighting their likely function in proton pumping [13].

6. Nuclear DNA-encoded supernumerary subunits

Of the 45 subunits that comprise mammalian complex I, 38 are encoded by the nuclear genome. Of these, seven are core subunits with the remainder, termed "supernumerary" subunits, having no bacterial counterparts [36]. Some supernumerary subunits have functions that are unrelated to electron transfer and proton pumping, however most have no known function. They may have general roles including stabilizing the complex, preventing the generation of damaging reactive-oxygen species (ROS), or protecting the complex from oxidative damage [30]. More specific roles for supernumerary subunits in the regulation of activity or for the assembly of other subunits into complex I have also been suggested. For example, subunits of 10 and 18 kDa were found to be phosphorylated in a cAMP-dependent manner. The 10 kDa subunit is NDUFA1 and the 18 kDa subunit was originally thought to be NDUFS4, although it has more recently been suggested to be NDUFB11 [37].

Some complex I subunits also appear to be involved in distinct biochemical functions beyond the scope of complex I enzymatic activity. For example, GRIM-19 was first described as a cell death-regulatory protein induced by interferon-β and retinoic acid [38,39], but was later identified as a subunit of complex I, termed NDUFA13 [38], where it is required for electron-transfer activity [40]. It is not clear how GRIM-19/NDUFA13 might be released from mitochondria as



Iα

Human	Bovine
NDUFS1	75 kDa
NDUFV1	51 kDa
NDUFS2	49 kDa
NDUFS3	30 kDa
NDUFV2	24 kDa
NDUFS8	TYKY
NDUFS7	PSST
NDUFS4	18 kDa
NDUFA2	B8
NDUFS6	13 kDa
NDUFA5	B13
NDUFA7	B14.5a
NDUFV3	10 kDa
NDUFA13	B16.6
NDUFA12	B17.2
NDUFA11	B14.7
NDUFAB1	SDAP
NDUFA9	39 kDa
NDUFA8	PGIV
NDUFS5	15 kDa
NDUFA6	B14
NDUFA1	MWFE
NDUFB4	B15
NDUFA3	B9
NDUFA4	MLRQ
ND6	ND6

Iλ

Human	Bovine
NDUFS1	75 kDa
NDUFV1	51 kDa
NDUFS2	49 kDa
NDUFS3	30 kDa
NDUFV2	24 kDa
NDUFS8	TYKY
NDUFS7	PSST
NDUFS4	18 kDa
NDUFA2	B8
NDUFS6	13 kDa
NDUFA5	B13
NDUFA7	B14.5a
NDUFV3	10 kDa
NDUFA13	B16.6
NDUFA12	B17.2
NDUFA11	B14.7

Iβ

Human	Bovine
NDUFB2	AGGG
NDUFB8	ASHI
NDUFB11	ESSS
NDUFB1	MNLL
NDUFB10	PDSW
NDUFAB1	SDAP
NDUFB5	SGDH
NDUFB9	B22
NDUFB7	B18
NDUFB6	B17
NDUFB4	B15
NDUFC2	B14.5b
NDUFB3	B12
ND4	ND4
ND5	ND5
NDUFA4	MLRQ

Iγ

Human	Bovine
NDUFC1	KYFI
ND1	ND1
ND2	ND2
ND3	ND3
ND4L	ND4L

Fig. 3. Proposed organization of subunits in mammalian complex I. Complex I forms an L-shaped structure in the inner membrane, with a large peripheral arm protruding into the matrix. Through the use of mild chaotropic agents, complex I can be dissociated into subcomplexes Iα, Iβ, Iγ and Iλ. The subunit composition of these subcomplexes is shown (both human and bovine nomenclature) [29]. Subunits in subcomplex Iγ are believed to separate from other subcomplexes during the dissociation process. The predicted location of selected core 'NDU' and 'ND' subunits within complex I is as indicated.

part of apoptotic initiation given that it is a subunit of complex I which, unlike other apoptotic inducing factors, does not reside in the intermembrane space. Nevertheless, reports on GRIM-19 playing a role in cell death continue. This includes the recent finding that induction of mitochondrial-stress and subsequent apoptosis by the complex I inhibitor rotenone can be abrogated by expression of cytomegaloviral-encoded RNA [41]. This RNA (β2.7) was reported to interact with complex I where it prevents apoptosis-mediated re-localization of GRIM19 and maintenance of cellular ATP production required for viral replication. How such a viral RNA might be imported into mitochondria to target complex I remains to be determined.

How do the core and supernumerary complex I subunits fit together into a functional enzyme? The subunit composition and general topology of complex I has been defined through its dissection into four subcomplexes (Iα, Iβ , Iλ and Iγ) using mild chaotropic agents [42–44]. As shown in Fig. 3, subcomplex Iα is made up of subunits from both the peripheral arm and a portion of the hydrophobic membrane arm, while subcomplex Iβ consists of subunits that make up the majority of the membrane arm. Subunits not found in either of these subcomplexes are located in subcomplex Iγ. Altering the conditions can further dissociate subcomplex Iα to produce subcomplex Iλ. This subcomplex represents the hydrophilic peripheral arm and contains the 15 subunits that provide all the redox cofactors [30]. Subunit NDUFA13 represents the only membrane protein of subcomplex Iλ.

7. Interactions between complex I and other respiratory chain complexes

In bacteria, some fungi, plants and mammals, respiratory chain complexes have been found to be organized into supramolecular structures termed "supercomplexes" or "respirasomes" [45–47]. By

employing different detergents respiratory chain complexes can be differentially dissected from these supercomplexes [47,48]. In mammals, respirasomes are present in two major supercomplexes —- the complete respirasome made of complexes I,III,IV, in a 1:2:4 ratio and an additional smaller supercomplex containing complex III_2:complex IV_4,. Complex II is thought to exist as a monomer and complex V as a dimer although a larger form exists in yeast [47,49]. The "respiratory string" model proposed by Schagger [47] posits that the respiratory chain exists as a string of interconnected $I_1III_2IV_4$ and III_2IV_4 in a 2:1 ratio. The functional benefits of supercomplex formation include: (1) channeling of ubiquinol and cytochrome *c*, avoiding competition for these substrates from other enzymes; (2) enhanced catalytic activity from reduced diffusion times of substrates; and (3) the ability to sequester ubisemiquinone thereby preventing the generation of damaging superoxides [50]. Supercomplexes of dimeric complex V have also been suggested to play a role in cristae formation [51,52]. The presence of respirasomes has also been shown to be essential for the assembly/stability of complex I (see below).

8. Supercomplex structure

The projection structure of the bovine CI/$CIII_2$/CIV supercomplex was determined by single particle image analysis [53], and more recently a 3D map was determined using random conical tilt electron microscopy [54]. The crystal structures of dimeric complex III [55,56] and complex IV [57], together with the cryo-EM structure of complex I [20], were fitted into the 3D map. In the model, complex III_2 occupies the central area of the membrane arm of complex I while complex IV is localized at the tip of the membrane arm where it shares a small contact surface with complex III_2. Only one of the complex III monomers in the dimer is attached to complex I while the other faces the lipid. In this configuration, all of the electron carrier binding sites within the complexes are on the same face, thus supporting the idea of reduced diffusion rates for substrates. Of note, the matrix arm of complex I in the supercomplex is bent toward the membrane giving it a different shape from that of isolated complex I [20].

9. Assembly of complex I

The assembly of subunits into complex I has proved to be a very puzzling problem to address, complicated by the size of the enzyme and its dual genomic control. Newly imported subunits, encoded by the nuclear genome must assemble in coordination with the highly hydrophobic subunits encoded by mtDNA. This coordinated process also requires regulation and signaling between the mitochondrion and nucleus [58–60]. Add to this the poorly understood role of assembly proteins and lack of a detailed crystal structure and it becomes clear why the assembly pathway of the so-called 'behemoth' of the respiratory chain has remained largely elusive. This is despite the fact that a number of model systems have been employed to study complex I assembly including fungi such as *Neurospora crassa* and *Yarrowia lipolytica* [61–63], the nematode *Caenornabditis elegans* [64], the protist *Chlamydomonas reinhardtii* [65,66] and cultured mammalian cell lines [67–70].

10. Assembly of complex I in *N. crassa*

The first detailed model of mitochondrial complex I assembly was derived from the aerobic fungus *N. crassa*. This model was developed through pulse-chase labeling of assembly intermediates coupled with the characterization of subcomplexes in mutant strains [61,71,72]. Mutants lacking subunits of the matrix arm could not assemble and accumulated the membrane arm of the complex [73]. Conversely, a mutant lacking a nuclear encoded subunit of the membrane arm accumulated the matrix arm, and two subcomplexes of the membrane arm [71]. From such studies, it was proposed that complex I in *N. crassa* is assembled via evolutionarily conserved modules [16]. In this model, the hydrophilic matrix arm is formed separately while the membrane arm is constructed from ~200 kDa and ~350 kDa intermediates [61]. The ~350 kDa intermediate was found to contain two *c*omplex I *i*ntermediate *a*ssociated proteins, CIA30 and CIA84, which are essential for the assembly of the membrane arm and dissociate prior to the formation of the holoenzyme [72]. Loss of either protein prevents the formation of the ~350 kDa intermediate and results in accumulation of the ~200 kDa intermediate and the hydrophilic matrix arm. While the assembly of complex I in *N. crassa* provides a useful model, its application to mammalian systems is limited due to evolutionary divergence.

11. Piecing together the assembly pathway of mammalian complex I

Most of the current knowledge obtained for the assembly of mammalian complex I has been compiled from observations made in systems where the assembly process is disturbed [34,67,69,74–77]. These studies have allowed for the identification of complex I subunits that are essential for assembly and stability of the holoenzyme. Loss of mtDNA-encoded subunits ND1, ND2, ND4, ND5 and ND6 affect complex I assembly/stability to varying degrees. Mutations in ND1 and ND6 affect the levels of assembled complex I [78], with ND4 and ND6 essential for the integration of other ND subunits into the complex [79,80]. In addition, cells from a patient with a mutation in *ND2* were found to be defective in complex I assembly and accumulated subcomplexes [69]. The absence of ND5 leads to a lower efficiency of assembly/stability of the membrane arm [81], however it is not essential for the assembly of the holoenzyme. Given that ND5 lies at the periphery of the membrane it may be the last of the ND subunits to assemble [42,82].

In the absence of mtDNA-encoded subunits, the levels of some nuclear DNA-encoded subunits of the peripheral arm remain unchanged [83]. Therefore it has been suggested that the presence of mtDNA-encoded subunits is not required for the formation of a peripheral arm subcomplex [34]. Indeed, cells lacking mtDNA (ρ^0), contain a subcomplex of the peripheral arm consisting of at least NDUFS2, NDUFS3 and NDUFS8 [83]. This subcomplex is suggested to link the membrane arm to the peripheral arm, and may therefore represent an early assembly intermediate. While NDUFS7 is thought to reside next to NDUFS2 in the holoenzyme, it was not found in the subcomplex of the peripheral arm in ρ^0 cells [83]. Its absence from this subcomplex suggests that NDUFS7 may be located near the membrane domain and requires the presence of integral membrane proteins (e.g. ND subunits) for its incorporation [83,84]. Subcomplexes have also been identified from patients with complex I defects. Analysis of mitochondria from patients with mutations in subunits NDUFS4, NDUFV1 and NDUFS6, all revealed an accumulation of an ~800 kDa subcomplex [69,85–87].

Another supernumerary subunit, NDUFA1 has also been shown to be important for complex I assembly [88]. Insertion and stabilization of NDUFA1 in the mitochondrial inner membrane was shown to be reliant on the presence of mtDNA-encoded subunits, in particular ND4 and ND6 [74]. Furthermore, using a conditional complex I assembly system developed in Chinese hamster fibroblasts, NDUFA1 was suggested to form part of an assembly intermediate consisting of both mtDNA- and nDNA-encoded subunits [74].

12. Current models of human complex I assembly

A number of recent studies have increased our understanding into the assembly of complex I in humans. Many studies have utilized cell lines from patients with mutations in subunits or assembly factors of complex I (Table 1). One study involved the analysis of a cohort of four patients presenting with isolated complex I deficiency and resulted in the identification of seven putative assembly intermediates [69]. These

intermediates contained a combination of both peripheral and membrane arm subunits. Another study used an inducible complex I assembly system. The approach involved depleting cells of complex I by inhibiting translation of mtDNA with doxycycline, followed by removal of the drug and monitoring assembly of newly synthesized complex I subunits using 2D blue-native polyacrylamide gel electrophoresis (BN-PAGE) and western analysis. It was concluded that complex I assembly involves a semi-sequential pathway involving functional modules, similar to *N. crassa* [67]. The same group recently refined this model by analyzing the assembly of GFP-tagged NDUFS3 within intermediate subcomplexes before maturation into complex I [68]. The final and most recent study monitored the assembly of newly imported nDNA-encoded subunits into isolated mitochondria that contained pre-existing complex I as well as performing chase studies of radiolabeled mtDNA-encoded subunits [70]. While these studies contain some differences with respect to the entry of specific subunits, a general consensus of the subunit assembly pathway in human mitochondria can be drawn. An early subassembly of the Q module is anchored to the membrane by ND1 and other membrane embedded subunits. This is followed by expansion of both the Q and P modules via the addition of subunits/subassemblies and completion of complex I assembly by addition of the N module. While most of the models suggest a progressive pathway of complex I assembly, it was additionally proposed that assembly is a dynamic process in which subunits and/or subassemblies may be exchanged with pre-existing ones [70].

13. Complex I assembly proteins

Given the subunit composition of complex I and its regulation by two genomes, it is expected that many factors are required in its biogenesis. Indeed, as a point of comparison, mammalian complex IV, consisting of only 13 subunits, requires the assistance of at least 14 assembly factors for its correct biogenesis [89]. The role of such factors in complex I biogenesis will include involvement in subunit maturation (e.g. folding/co-factor attachment), chaperoning intermediate assemblies, subunit synthesis and turnover. Only in recent years have a number of assembly factors for complex I been validated while others have been implicated in the process.

13.1. Human CIA30

Functional studies of CIA30 by Vogel et al. [90] found that knockdown of the assembly factor using RNA interference led to reduced levels of both enzymatic activity and assembled complex I. The role of CIA30 in complex I assembly was further demonstrated with the identification of a patient presenting with cardioencephalomyopathy as a result of mutations in the gene encoding CIA30 (*NDUFAF1*) [91]. Low levels of CIA30 in patient mitochondria correlated with decreased levels of assembled complex I and also decreased enzymatic activity. The assembly process of complex I was shown to be disturbed at an early stage. Restoration of wild type CIA30 levels in the patient using a lentiviral-inducible transfection system restored complex I levels [91]. CIA30 is not found with fully assembled complex I, but instead resides within two complexes of ~460 and ~830 kDa [91,92].

CIA30 was originally identified in *N. crassa* within a subassembly of complex I [72]. Another factor termed CIA84 was also identified in this subassembly, but does not associate with CIA30. The putative human homolog of *N. crassa* CIA84, identified recently via comparative genomics [12], awaits further characterization into its potential role in complex I assembly.

13.2. Ecsit

Ecsit, originally identified as a cytosolic adaptor protein essential for the inflammatory response and embryonic development [93,94], has been shown to interact with CIA30 in complex I assembly [95]. Ecsit was identified through co-purification with CIA30 using a tandem affinity purification system on mitochondrial lysates [95]. Although predominantly cytosolic, a small proportion of Ecsit is found in mitochondria where it co-localizes in high molecular weight complexes with CIA30. Knockdown of Ecsit reduces CIA30 levels and leads to impaired complex I assembly. In contrast to CIA30 knockdown, decreased levels of Ecsit lead to the accumulation of complex I intermediates which indicates that although both proteins are present in ~400–830 kDa complexes, their mechanism of action may differ [95].

13.3. B17.2L

Whole genome subtraction studies between fermentative yeasts lacking complex I (e.g. *S. cerevisiae*) and aerobic yeasts that harbor complex I (e.g. *Y. lipolytica*) led to the identification of B17.2L [87]. A mutation in exon 2 resulting in a premature stop codon was identified in the gene encoding B17.2L in a patient with progressive encephalopathy [87]. The patient presented with severe enzymatic deficiency of complex I that also correlated with reduced assembly of the holoenzyme. Re-introduction of B17.2L cDNA in patient fibroblasts rescued complex I assembly and activity. B17.2L was subsequently identified to associate with an ~830 kDa subcomplex in several patients with complex I assembly defects but was not found in the mature holoenzyme [70,87]. This intermediate appears to represent complex I lacking the matrix-located NADH dehydrogenase portion of complex I. Furthermore, given that B17.2L was shown to associate with complex I subunits ND1, NDUFS1, NDUFS2, NDUFS7 and NDUFS4 in normal mitochondria, it has been attributed to play a direct role in complex I assembly [70,87].

13.4. C6ORF66

Homozygosity mapping of patients with isolated complex I deficiency led to the identification of a novel assembly factor encoded by the *C6ORF66* gene [96]. A missense mutation in a conserved residue of C6ORF66 led to a decrease of the protein in muscle and severely reduced levels of complex I activity. BN-PAGE analysis revealed diminished levels of assembled complex I, with the accumulation of two smaller intermediates, one of which resembles the ~830 kDa intermediate associated with B17.2L. Patient cells stably transduced with C6ORF66 displayed a return to normal levels of complex I activity and therefore correction of the defect.

Interestingly, both C6ORF66 and B17.2L were initially identified as proteins involved with cancer. It was reported that C6ORF66 promotes breast cell cancer invasiveness [97], while B17.2L is a transcriptional target of *c-myc*, and reduction of its levels leads to inhibition of tumorigenesis [98]. Given that C6ORF66 and B17.2L, along with Ecsit [93,94], have been implicated with roles outside that of complex I biogenesis, it is possible that mitochondrial function and complex I activity may be linked to these processes. These findings outline the need for further studies on the biochemical functions of these proteins.

13.5. Apoptosis inducing factor (AIF)

The normal function performed by apoptosis-inducing factor (AIF) appears to be in complex I maintenance [99,100]. AIF is a flavoprotein with NADH oxidase activity, which is normally located in the mitochondrial intermembrane space, or loosely associated with the inner membrane [101,102]. Induction of apoptosis results in the translocation of AIF from mitochondria to the cytosol and nucleus where it interacts with cyclophilin A to become an active DNAse [103]. The apoptotic function of AIF is not dependant on its NADH oxidase activity [104,105]. An important step towards elucidating AIF's role in complex I maintenance was made with the discovery of the Harlequin

(Hq) mouse [106]. Expression levels of AIF are reduced to ~20% of wild type levels in Hq mice, and mice exhibit increased degeneration of certain neurons accompanied by oxidative stress [106]. Hq mouse retinal and cerebellar granule neurons show reduced levels of complex I subunits and decreased enzyme activity [99]. Complex I deficiency has also been identified in AIF-deficient cardiac and skeletal muscle from conditional knockout mice [107], in HeLa cells with knocked down AIF [99], and AIF-null embryonic mouse stem cells [99,108]. Loss of AIF activity in cells causes dysfunction of complex I due to failure of proper subunit synthesis or assembly [99]. How AIF, a protein that is normally located in the mitochondrial intermembrane space, functions in complex I maintenance remains unresolved.

14. General assembly proteins and disease

In addition to assembly proteins that appear to have a direct role in complex I assembly, a number of proteins also exist that have general roles in the biogenesis of the OXPHOS machinery. These include Oxa1 [109], prohibitins [110,111]. [83], paraplegin [112,113], and frataxin [114]. Such proteins have broad functions in mitochondrial biogenesis including maturation of protein subunits and protein degradation.

15. Supercomplex involvement in complex I assembly/stability

The formation of respiratory chain supercomplexes is critical for the stability and possibly assembly of complex I. Loss of either complex III or IV in the bacterium *Paracoccus dentitrificans* leads to a decrease in complex I levels [46]. For human mitochondria it has been well established that complex I is stabilized in the mitochondrial membrane by association with dimeric complex III. Genetic defects in humans affecting complex III assembly, such as mutations in the mtDNA cytochrome *b* gene [115] or the nuclear gene encoding the complex III assembly factor BCS1L [116], can lead to secondary defects in complex I. Metabolic labeling of mtDNA products in cells harboring a cytochrome *b* mutation shows that while complex I assembly is unaffected, the stability of complex I is impaired due to failure of supercomplex formation [115]. Mutations in cytochrome *b* that only affect complex III activity, but not assembly, have no effect on complex I [115].

Likewise, observations made in patient cells with mutations in *BCS1L* show that the physical presence of complex III (but not its enzymatic activity), is required for complex I stability [116,117]. Complex IV has also been implicated in the assembly/stability of complex I although its role is not as clear [118,119]. A knockout mouse cell line of the complex IV assembly factor COX10, resulting in no detectable levels of complex IV, also presented with decreased levels of assembled complex I [118]. It has been suggested that complex IV may have a role in complex I assembly since pulse-chase experiments in COX10 knockout cells could not detect any newly assembled complex I [118]. However it appears that unlike complex III, even residual complex IV levels are adequate for complex I assembly/stability as patients with missense mutations in *COX10* have normal levels of complex I [120]. Furthermore, complex I is unaffected in patient fibroblasts with almost undetectable levels of complex IV [48]. The exact role of complex IV in the assembly/stability of complex I awaits further clarification.

It is now apparent that supercomplexes aid in the stability of complex I. However, it is not known whether the stability provided is a result of assistance during assembly of complex I or stability after assembly. As discussed earlier, complexes III and IV share a large contact surface with the membrane portion of complex I while only sharing minimal contact with each other. Given that the major contact site contributed by complex I is through the membrane arm, it can be argued that only once a significant portion of this arm has formed will it be able to interact with complexes III and IV to form supercomplexes. Indeed, a late stage ~830 kDa intermediate of complex I that is associated with the assembly protein B17.2L was identified in a supercomplex with dimeric complex III [70,87]. Furthermore, a membrane arm portion of complex I in *N. crassa* was found to be in association in respirasomes with complexes III and IV [121]. These results indicate that complex I may associate into supercomplexes prior to completion of its assembly. Even in the absence of an assembled complex I, supercomplexes of $CIII_2$/CIV can form [121]. In yeast mitochondria, where complex I is absent, intermediates of complex IV associate with complex III during the assembly process [122], suggesting that supercomplex formation is likely to occur in conjunction with respiratory chain biogenesis. Other respiratory chain complexes may therefore act as scaffolds for complex I intermediates during later stages of complex I assembly while also providing stabilization/protection from degradation following assembly.

A number of other factors influence the stability of supercomplexes following their formation. In particular, cardiolipin, a phospholipid unique to the mitochondrial inner membrane, has been reported to play an important role in promoting and/or stabilizing respiratory chain supercomplexes [123–126]. Mutations in the *taz1* gene that encodes Tafazzin, a putative phospholipid acyltransferase involved in cardiolipin remodeling, lead to destabilization of the complex III_2/complex IV supercomplex in yeast [127]. In humans, defective Tafazzin is associated with Barth Syndrome, an often fatal disease that presents with cardiomyopathy, neutropenia and 3-methylglutaconic aciduria [128]. Cardiolipin defects in lymphoblast mitochondria from Barth Syndrome patients cause instability of the complex I/complex III_2/complex IV supercomplex, resulting in the liberation of complex IV monomer [129]. The supramolecular association of complexes I and III was additionally found to be destabilized in Barth Syndrome patient mitochondria [129]. This suggests that defects in supercomplex assembly and/or stability may contribute to pathogenesis in mitochondrial disease.

16. Model for the assembly of complex I in mammalian mitochondria

Piecing together what we know about human complex I assembly allows us to propose an updated model for its biogenesis (Fig. 4). Older models of complex I assembly describe a unidirectional pathway in which complex I is built from a starting block that continues to completion. However, since nuclear DNA-encoded subunits are likely to be continuously imported into mitochondria, not every subunit that is imported must be incorporated into new assemblies. Instead a subunit may assemble with pre-existing subunits that undergo dynamic transitions between intermediate and fully assembled complex I forms [70]. An exchange process of protein complex components has also been observed in photosystem I (PSI) [130] and the bacterial flagella motor [131]. It appears that nuclear DNA-encoded subunits can integrate rapidly into complex I while mtDNA-encoded subunits first assemble into intermediate complexes that require significant time for their integration into the holoenzyme [70,132]. These findings point toward a mechanism of complex I biogenesis involving both synthesis of mtDNA-encoded subunits to seed new complex I assemblies and exchange of pre-existing subunits with newly imported ones to maintain complex I homeostasis. The model posits that newly imported, nuclear DNA-encoded subunits incorporate into subassemblies of functional modules via a dynamic process. While the exact order of subassembly integration in the biogenesis pathway is enigmatic given its dynamic nature, differentiations can be made between modules that are assembled at early or late stages.

In the model (Fig. 4), subunits NDUFS2 and NDUFS7 incorporate into a subassembly of the Q module. It has been suggested that this subassembly is one of the first complex I assembly intermediates formed and additionally contains subunits NDUFS3 and NDUFS8 [67,83,92]. The mtDNA-encoded subunit ND1, possibly in conjunction with other subunits, may anchor the subassembly of the Q module to the inner membrane, thereby forming an ~400 kDa intermediate [70]. CIA30 is involved in the initial assembly of a separate ~460 kDa membrane subcomplex that likely contains ND2, ND3 and ND6 (as

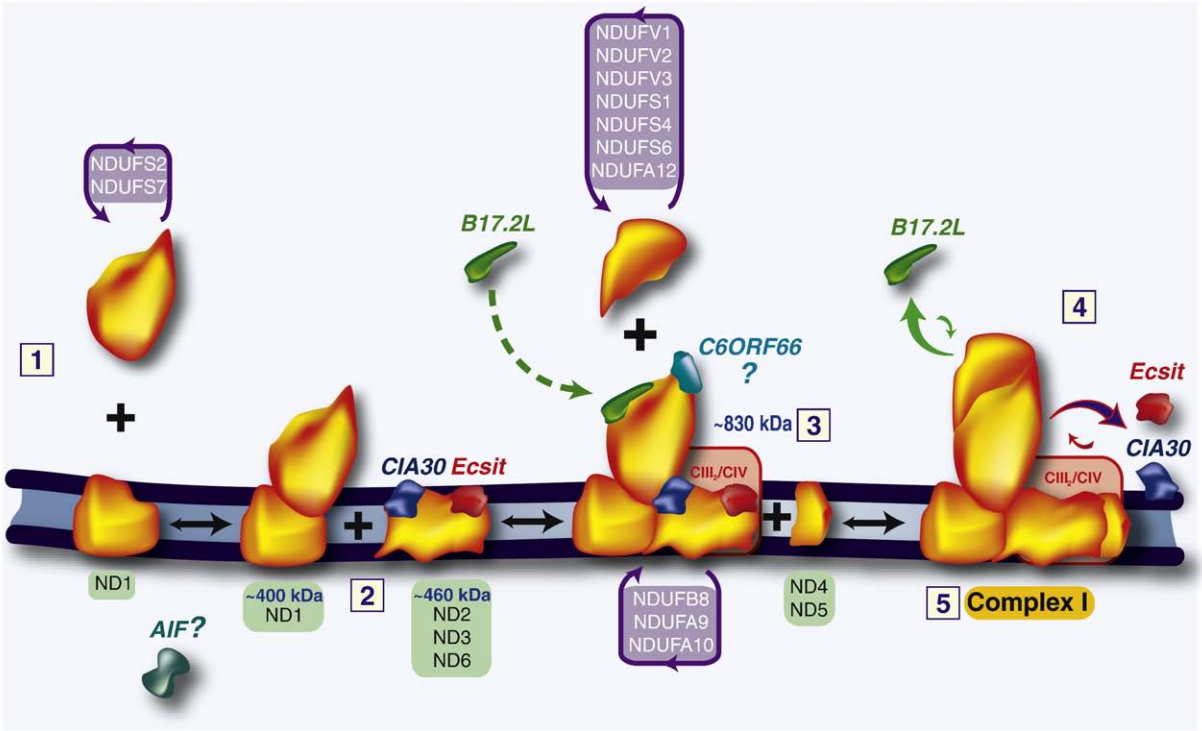


Fig. 4. Model of complex I assembly via dynamic cycling between intermediates and fully assembled complex I. (1) An early subassembly of the Q module consisting of NDUFS2, NDUFS7 and other subunits, is anchored to the membrane by ND1 and other membrane embedded subunits forming an ~400 kDa subcomplex. (2) CIA30, in conjunction with Ecsit, is involved in the assembly of a ~460 kDa subcomplex of the P module that likely contains ND2, ND3 and ND6. (3) The ~400 kDa and ~460 kDa intermediates combine to form a stable ~830 kDa intermediate that is associated with B17.2L and possibly C6ORF66. This intermediate is in a supercomplex with complexes III and IV. Additional nDNA-encoded subunits such as NDUFB8, NDUFA9 and NDUFA10 may be added at this stage. (4) The latter stages of complex I assembly involve the addition of the N module consisting of subunits NDUFV1–3, NDUFS1, NDUFS4, NDUFS6 and NDUFA12, and the completion of the P module via the addition of ND4 and ND5. The assembly of subunits into the matrix arm of complex I correlates with the release of CIA30/Ecsit and B17.2L resulting in (5) the holoenzyme. The role of AIF in the assembly process is unknown and awaits further clarification.

well as other subunits) [91]. Ecsit may act in conjunction with CIA30 at this stage. The membrane arm intermediates come together to form an ~830 kDa intermediate that is associated with B17.2L and perhaps C6ORF66. It is possible that B17.2L, Ecsit, CIA30 and C6ORF66 may be associated with complex I at the same time during a certain stage of the assembly process, however, such an occurrence is likely to involve independent roles of these factors in complex I assembly. At some stage in the course of assembly, complexes III and IV combine with a complex I intermediate to form a supercomplex. This can occur at the stage of the ~830 kDa intermediate, however it is currently unknown if supercomplex association occurs earlier. Additional subunits such as NDUFA9, NDUFA10 and NDUFB8 may be added to the ~830 kDa intermediate during, or subsequent to, its formation [68,70]. Following the formation of the ~830 kDa intermediate, a subassembly of the N module or 'cap' of complex I is added. The 'cap' consists of subunits NDUFV1, NDUFV2, NDUFV3, NDUFS1, NDUFS4, NDUFS6 and other subunits. The assembly process is likely to be completed by the addition of ND4 and ND5 to the large membrane arm which finalizes assembly of the transporter module [70,91].

17. Concluding remarks

We are in an emerging age of systems biology where biochemists are aware that biological processes are extremely complex, interlinked and interdependent. Complex I assembly is not a unidirectional pathway as initially thought. In fact, it is a regulated interplay of protein import, mtDNA protein translation, membrane protein insertion and stabilization, assisted by assembly factors and supercomplexes. Thus, there is still a long way to go in understanding the biogenesis of complex I. Much of this will come down to elucidating the molecular mechanisms of the various assembly proteins. In addition, an improved understanding of complex I assembly will also require studies on the formation of supercomplexes and the factors involved. With continuing research and the speed of identification of new assembly factors increasing, the complex I assembly puzzle can be slowly pieced together.

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