

Chapter 2

Mitochondrial DNA Mutations and Their Effects on Complex I Biogenesis: Implications for Metabolic Disease

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Abstract NADH-ubiquinone oxidoreductase (complex I) is a large, multimeric enzyme complex involved in the generation of ATP by oxidative phosphorylation (OXPHOS). It is comprised of 45 different polypeptide subunits, seven of which are encoded by the mitochondrial genome. For complex I to function efficiently it must be assembled correctly from these subunits in a coordinated manner. Disruption of this assembly process can result in complex I deficiency and a wide range of different mitochondrial disorders, including ophthalmological syndromes and fatal childhood encephalomyopathies. This chapter will describe our current understanding of complex I structure, function, and assembly. In particular, how mutations in mtDNA-encoded subunits disrupt complex I assembly and contribute to human disease pathogenesis will be discussed.

2.1 Introduction

Much of a cell's energy requirements are met by the mitochondria, organelles which generate ATP via a process known as oxidative phosphorylation (OXPHOS). The OXPHOS machinery is comprised of five enzyme complexes which are embedded within the mitochondrial inner membrane; NADH-ubiquinone oxidoreductase (complex I), succinate-ubiquinone oxidoreductase (complex II), ubiquinol-cytochrome c oxidoreductase (complex III), cytochrome c oxidase (complex IV) and F_1F_0 H^+ -ATP synthase (complex V). Electrons derived from the

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oxidation of carbohydrates are transferred via NADH or FADH₂ to complex I or complex II. From here they reduce ubiquinone to ubiquinol (CoQH₂), before being transferred to complex III. The electrons are passed to cytochrome c, then complex IV, and finally to $\frac{1}{2}\text{O}_2$ to give H₂O. The energy released by the electron transfer along the respiratory complexes is used to drive H⁺ ions out of the inner mitochondrial matrix at complexes I, III, and IV. This creates a transmembrane electrochemical gradient $\Delta\psi_m$ that drives complex V to condense ADP and inorganic phosphate (P_i) to ATP.

Disorders of the OXPHOS system are the most common cause of inborn metabolic disease, affecting approximately one in 5,000 live births [1, 2]. They encompass a wide variety of multisystemic degenerative diseases, commonly referred to as mitochondrial encephalomyopathies, which can exhibit various combinations of clinical features. Brain and muscle are usually affected in these disorders, although other tissues that have high energy requirements may also be involved.

We now know that the function of the OXPHOS complexes, and in many cases their assembly, can be affected by pathogenic mutations in both nuclear and mtDNA. Nuclear DNA mutations have been identified in structural subunits of complex I [3], II [4], III [5, 6], IV [7] and V [8] (although nuclear mutations in subunits of complexes III, IV and V are rare). Mutations have also been described in nuclear genes that encode proteins which are not structural subunits of the mature holo-enzymes but actually aid the biogenesis of the OXPHOS complexes. In many cases, mutations in these assembly factors result in disruption of complex assembly and the depletion of steady-state holo-complex levels. Mutations associated with human mitochondrial disease have been identified in assembly factors of complex I [9–17], II [18], III [19–21], IV [22–27] and V [28].

OXPHOS defects can also be caused by mutations in nuclear genes which encode proteins involved in the replication, transcription and translation of mtDNA [29–36] and in genes that are not directly related to OXPHOS function but cause OXPHOS deficiencies. For example, mutations in the *TAZ* gene cause cardiolipin remodeling defects which result in the destabilization of OXPHOS complex structure in Barth Syndrome [37, 38].

In contrast to nuclear DNA, the mtDNA genome is a very different molecule, existing as a double-stranded, circular structure of 16,569 base pairs. It comprises a control region of approximately 1,000 base pairs (D-loop) which contains the heavy (H) and light (L) strand promoters (P_H and P_L) and the H-strand origin of replication (O_H) [39]. MtDNA also encodes 22 tRNAs and two rRNAs which are specific for mitochondrial translation, and 13 polypeptides, all of which are structural subunits of the OXPHOS complexes; ND1-6, ND4L (complex I), Cyt b (complex III), CO1-3 (complex IV), ATP6 and 8 (complex V).

Over 500 different point mutations in mtDNA have been reported, with a number of large-scale deletions and rearrangements also observed (<http://www.mitomap.org>). The first mtDNA mutations were described over 20 years ago in the late 1980s, with a point mutation in the complex I subunit gene *MTND4* identified in a patient with Leber Hereditary Optic Neuropathy (LHON) [40] and

large-scale deletions detected in patients with mitochondrial myopathy [41], Kearns-Sayre Syndrome [42] and progressive external ophthalmoplegia [43]. MtDNA mutations have now been reported in all of the 13 protein coding genes, in both the 12S and 16S rRNA genes, in each of the 22 tRNA genes, and in the non-coding D-loop (although many of these mutations are yet to be confirmed as truly pathogenic).

Mutations in the *MTCYB* gene, which encodes the only mtDNA protein of complex III's 11 subunits, have been described in a number of different mitochondrial disorders, including exercise intolerance [44–47], mitochondrial encephalomyopathy [48], cardiomyopathy [49], and multisystem disorders [50, 51]. These mutations generally result in complex III deficiency (or a combined complex I and III deficiency [52, 53]), and in some cases may also disrupt the biogenesis of the complex by altering its assembly kinetics [54, 55]. Mutations have been described in the mtDNA genes *MTCO1* [56, 57], *MTCO2* [58] and *MTCO3* [59–62], which encode for three of complex IV's 13 subunits. These mutations not only result in complex IV deficiency but can also reduce the levels of mature holo-enzyme by disrupting its biogenesis [62]. Mutations in *MTATP6* and *MTATP8*, genes which encode subunits of the hydrophobic F_0 module of complex V, have also been described in patients with mitochondrial disease [63]. Depending on the mutant load, mutations in *MTATP6* result in either a progressive, adult-onset disorder known as neuropathy, ataxia, and retinitis pigmentosa (NARP) or the severe infantile disorder Maternally Inherited Leigh Syndrome (MILS) [63]. Alternatively, mutations in *MTATP8* have been reported in patients with hypertrophic cardiomyopathy and neuropathy or severe mitochondrial disease [64, 65]. All of these mutations can disrupt complex V assembly, resulting in the accumulation of stalled subcomplexes and deficiencies in enzymatic activity [64, 66–70].

Pathogenic mutations in all seven mtDNA-encoded complex I subunits have been reported, and can result in a wide variety of different clinical phenotypes. This chapter will describe how these mutations affect complex I activity and contribute to mitochondrial disease pathogenesis. Our current understanding of complex I structure, function, and assembly will also be discussed, with particular reference to how mtDNA mutations affect the biogenesis of the complex.

2.2 Complex I Structure

Electron microscopy studies of purified complex I from a variety of species have revealed that the enzyme has an L-shaped structure consisting of a hydrophobic membrane arm and a peripheral arm that protrudes into the mitochondrial matrix [71]. In mammals, complex I is approximately 980 kDa in size and comprises 45 different subunits [72]. Seven of these subunits are encoded by mtDNA, with the remaining 38 subunits encoded by the nuclear genome. The position of each subunit within mammalian complex I has not yet been fully defined, however, treatment of bovine complex I with mild chaotropic agents dissociates the

holo-enzyme into four subcomplexes ($I\alpha$, $I\beta$, $I\lambda$ and $I\gamma$), allowing the identification of subunits within each subcomplex [73–75]. The human complex I subunit nomenclature is based in part according to its subcomplex position, with nuclear encoded subunits designated as either NDUFA ($I\alpha$ subcomplex), NDUFB ($I\beta$ subcomplex) or NDUFC ($I\gamma$ subcomplex). Other subunits are named NDUFs for ‘Fe–S protein’ (although only the subunits NDUFs1, 7 and 8 contain Fe–S clusters) or NDUFV for ‘flavoprotein’ (with NDUFV1 and 2 also containing Fe–S clusters). The seven complex I mtDNA-encoded subunits are given the prefix “ND” (NADH-dehydrogenase).

Our understanding of mammalian complex I structure has also been aided by studying its homolog in bacteria. In *E. coli*, complex I is approximately 550 kDa in size and consists of only 14 subunits. These subunits are considered ‘core’ subunits, in that they are able to form the minimal structure required for efficient electron transfer and proton translocation. Bacterial complex I is formed from three evolutionarily conserved modules; the electron input module (N) and the electron output module (Q), which protrude into the bacterial cytoplasm (mitochondrial matrix in mammals), and the proton translocation module (P), which is embedded within the membrane [76]. The N module consists of the subunits NuoG, NuoF, NuoE (NDUFS1, NDUFV1, NDUFV2) and a flavin mononucleotide (FMN). The module binds and oxidizes NADH, liberating electrons which pass via the FMN to a chain of Iron–Sulfur (Fe–S) clusters. This module has evolved from two separate origins; a soluble NAD⁺-reducing hydrogenase found in purple bacteria [77, 78] and cyanobacteria [79] and a formate hydrogenlyase complex of *E. coli* [80].

The Q module is composed of the NuoI, NuoB, and NuoCD subunits (NDUFS8, NDUFS7, NDUFS3, and NDUFS2), with the genes for *NuoC* and *NuoD* fused in bacteria. This module transfers electrons, which have passed through the N module via the Fe–S clusters, to ubiquinone. The Q module is homologous to present-day soluble Nickel–Iron (Ni–Fe) hydrogenases, with the loss of the Ni–Fe active site and the possible addition of a quinone binding site [81].

The P module is composed of the seven subunits NuoH, NuoN, NuoA, NuoM, NuoK, NuoL and NuoJ, which correspond to the mtDNA-encoded subunits ND1, ND2, ND3, ND4, ND4L, ND5 and ND6 in mammalian complex I. This module is involved in proton translocation across the membrane, with the proton pumping subunits ND2, ND4 and ND5 having evolved from bacterial Na⁺/H⁺ and K⁺/H⁺ antiporters [82].

Recent structural data have provided insights into how the three modules (N, Q and P) act together to couple NADH oxidation, electron transfer, and proton translocation [83, 84], with a two-state, redox driven mechanism proposed [85]. Following oxidation of NADH at the N module, electrons are transferred via a chain of Fe–S clusters to cluster N2, which resides ~ 10 Å from the ubiquinone binding site (Q-site) in the Q module. Upon reduction of ubiquinone at this site, membrane arm proton pumping is induced by long-range conformational energy transfer through an amphipathic α -helix of the ND5 subunit. This helix lies in a perpendicular direction to the membrane arm, and its mechanism of action has been likened to that of the pumping of a steam engine coupling rod.

The amphipathic α -helix is coupled to three discontinuous α -helices belonging to the antiporter-like subunits ND3, ND4, and ND5. Movement of these helices alters the conformation of ionisable channel residues in ND3, ND4, and ND5, thereby inducing proton translocation across the membrane [83, 84, 86].

2.3 Complex I Enzyme Deficiencies in Human Disease

Isolated complex I deficiency is the most common cause of respiratory chain dysfunction, accounting for around 50 % of cases [1, 87]. Pathogenic mutations have been identified in nuclear genes that encode both complex I structural subunits and complex I assembly factors [for review see [3, 88, 89]] and in all seven of the complex I mtDNA-encoded subunit genes (Table 2.1).

Patients with complex I deficiencies can present with isolated symptoms or may exhibit multiple tissue involvement. Of note, a single mtDNA mutation can result in different clinical phenotypes in different patients, and conversely, patients who harbor different mtDNA mutations may all present with the same disorder. For example, LHON is a form of blindness which presents in mid-life as acute or subacute central vision loss due to specific defects of the optic nerve [90]. This disorder has been associated with a number of different mtDNA mutations in the complex I subunit genes *MTND1* [91], *MTND4* [40], *MTND4L* [92] and *MTND6* [93]. However, some mtDNA mutations which cause LHON have also been described in patients with dystonia, a disease that presents in early life (usually childhood) with mental retardation, movement disorders, short stature, and degeneration of the basal ganglia. In some cases a correlation has been observed between the percentage of mutant mtDNA molecules (heteroplasmy) and the severity of disease, that is, a lower percentage results in LHON, whereas a higher percentage results in the more severe dystonia [93–95]. However, this correlation does not always hold, as some individuals who have (near) homoplasmic LHON mtDNA mutations do not develop either LHON or dystonia [96].

Mutations in complex I mtDNA genes are also associated with the multi-symptomatic disorders mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) and Leigh Syndrome. *MTND1* [97], *MTND5* [98] and *MTND6* [99] mutations have been described in patients with MELAS, a heterogeneous mitochondrial disorder with a variable clinical phenotype. Patients can present with myopathy, encephalopathy and features of central nervous system involvement, including seizures, hemiparesis, hemianopia, cortical blindness, and episodic vomiting [100]. In addition, mutations in the *MTND1* [101] or *MTND5* [102] genes have been identified in patients with LHON/MELAS overlap syndrome, highlighting the possible combination of clinical outcomes due to single mtDNA point mutations.

Mutations in *MTND1* [103], *MTND2* [104], *MTND3* [105], *MTND4* [106], *MTND5* [107] and *MTND6* [108] have been described in patients with Leigh Syndrome, an early-onset progressive neurodegenerative disorder characterized by

Table 2.1 Mitochondrial DNA mutations in complex I deficiency

Gene	Disease	Mutation	References
<i>MTND1</i>	LHON	3460G > A, 3635G > A	[91, 116, 158]
	MELAS	3481G > A, 3697G > A, 3946G > A, 3949T > C	[97, 137]
	TIID/CM	3310C > T	[159]
<i>MTND2</i>	LHON	4640C > A	[158]
	LS	4681T > C	[104]
<i>MTND3</i>	LS	10158T > C, 10191T > C	[105, 160–162]
	DYS	10197G > A	[162]
<i>MTND4</i>	LHON	11778G > A	[40, 116, 117]
	LS	11777C > A	[106]
	PEO	11232T > C	[163]
	EXIT	11832G > A	[164]
<i>MTND4L</i>	LHON	10663T > C	[92]
<i>MTND5</i>	LS	12706T > C, 13513G > A	[107, 136, 165]
	MELAS	13063G > A, 13514A > G, 13042G > A	[111, 137, 165–167]
	MELAS/LS	13084T > C	[110]
	LHON/MELAS	13513G > A	[102]
	MELAS/LS/LHON	13045A > C	[112]
	AT/PEO	13094T > C	[103]
	LHON	14484T > C, 14459G > A	[93, 115, 116, 168]
<i>MTND6</i>	LS	14600G > A	[108, 137]
	TIID	14577T > C	[169]
	LS/CM	14487T > C	[137]
	DYS	14459G > A	[93, 115, 170]
	LHON/DYS	14459G > A	[95, 115]

LHON Leber Hereditary Optic Neuropathy; *MELAS* Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-like Episodes, *TIID* Type two Diabetes; *CM* Cardiomyopathy; *LS* Leigh Syndrome; *DYS* Dystonia; *PEO* Progressive External Ophthalmoplegia; *EXIT* exercise intolerance; *AT* ataxia. Mutations listed were scored as pathogenic by Bridges and colleagues [171] using criteria based on biochemical deficiencies, number of independent reports, heteroplasmy, matrilineal variant segregation and conservation [172]. Diseases listed include combined disorders (e.g. LHON/MELAS = LHON/MELAS Overlap Syndrome)

bilateral lesions in one or more areas of the central nervous system. This disorder can present with a range of clinical symptoms, including cardiomyopathy, ataxia, hypotonia and deafness [109]. Leigh Syndrome can also present in conjunction with other mitochondrial diseases, with mutations in *MTND5* associated with MELAS/Leigh Syndrome [110, 111] and MELAS/Leigh/LHON overlap Syndrome [112].

The effects of different mtDNA mutations on complex I activity have been examined by using cytoplasmic hybrids ('cybrids') [113], which are created by fusing cytoplasm fractions containing mitochondria with ethidium bromide treated

human mtDNA-less ρ^0 cells [114]. Using this technique it was found that the 14459G > A *MTND6* mutation, which is associated with LHON, dystonia and Leigh Syndrome, caused a 60 % complex I enzymatic deficiency but only mild respiratory dysfunction on polarographic analysis [115]. Conversely, the 11778G > A *MTND4* LHON mutation resulted in decreased respiration but complex I enzymatic deficiencies were either mild [116] or undetectable [117]. The 14484T > C *MTND6* LHON mutation also resulted in a mild respiratory defect, but again no complex I deficiency was detectable [116].

Although biochemical defects have been assigned to many of the complex I mtDNA mutations, the exact pathophysiological mechanisms involved remain puzzling. In particular, why does one specific mtDNA mutation result in such a range of clinical severity and phenotypes? It is possible that secondary effects may modulate the biochemical defect caused by the primary mtDNA mutation. For example, the altered binding of CoQ₁₀ to complex I when the 14459G > A *MTND6* or 3460G > A *MTND1* mutations are present has been suggested as one possible factor that may contribute to LHON pathogenesis [115, 116]. Impaired CoQ₁₀ reduction results in increased levels of ubisemiquinone and subsequently increased ROS generation, a process which may contribute to the premature death of the optic nerve [118]. This theory is supported by the observed increase in ROS generation in differentiated NT2 neurons containing the 11778G > A *MTND4* or 3460G > A *MTND1* LHON mutations [119]. These LHON mutations may also alter mitochondrial apoptotic signaling, with cybrid cells containing these mutations exhibiting increased sensitivity to Fas-induced apoptosis [120]. In addition, growth of LHON mutant cybrids on galactose media, which forces the cells to utilize OXPHOS to generate ATP, also induced apoptotic cell death [121].

Mutations in mtDNA-encoded complex I subunits have been reported to alter mitochondrial permeability transition pore (PTP) opening. Cell lines harboring either the 14484T > C and 14279G > A *MTND6* LHON mutations, or a *MTND1* frame-shift mutation, exhibit complex I enzymatic deficiencies and reduced levels of mature complex I (in the case of the *MTND1* mutation) [122]. In addition, the threshold voltage for PTP opening was shifted in these mutant cell lines, being induced close to the resting potential. This suggests that increased cell death due to PTP opening may also contribute to disease pathogenesis in patients with LHON mtDNA mutations [122].

Different mtDNA haplogroups may also influence the pathogenicity of certain mtDNA mutations. Studies have shown that Eurasian haplogroup J is preferentially associated with the 11778G > A *MTND4*, 14484T > C *MTND6*, and 10663T > C *MTND4L* LHON mutations, suggesting that this haplogroup exerts an effect on LHON mutation expression [123–128]. Indeed, it has been reported that cell cybrids containing haplogroup H or haplogroup UK mtDNA have different mtDNA and mtRNA levels, resulting in altered mtDNA-encoded protein synthesis and subsequently altered OXPHOS function [129]. Thus, mtDNA haplotype can affect the disease penetrance of the primary pathogenic LHON mutation, in some cases disrupting Complex I activity by modulating its assembly kinetics [130] (see below).

2.4 Defects in Human Complex I Assembly

Much of what we now understand about the assembly of human complex I has come from studies of complex I deficient patient cells. Early experiments using immunoprecipitation in conjunction with pulse-chase analysis of mtDNA-encoded subunits revealed that the loss of the subunit ND4 results in increased turn-over of other mtDNA-encoded complex I subunits and reduced assembly of these subunits into the mature complex [131]. In addition, although NADH: Q1 oxidoreductase activity was completely absent, NADH:Fe(CN)₆ oxidoreductase activity was similar to controls. This suggested that nuclear-encoded subunits were still able to assemble enough of the matrix arm for NADH oxidation to proceed, and that this assembly is independent to that of the membrane arm (which contains the mtDNA-encoded subunits) [131]. Similar results were obtained in mouse cells which harbored a *MTND6* mutation, with complex I activity and assembly of mtDNA-encoded subunits disrupted, but NADH:Fe(CN)₆ oxidoreductase activity remaining intact [132], providing further evidence that the assembly of the matrix arm N module is independent to membrane arm assembly.

Mutations in *MTND5*, which result in complete or near-complete loss of the ND5 subunit, were also shown to affect complex I activity [133]. However, unlike the assembly defect resulting from the loss of ND4, the mtDNA-encoded subunits were still able to assemble into the membrane arm and mature complex I (albeit with reduced efficiency) when ND5 was absent [133]. This suggested that different pathways for the assembly of complex I mtDNA-encoded subunits may exist, and that each subunit plays a different role in complex I activity, assembly and/or stability.

These early experiments provided indirect evidence that mutations in mtDNA-encoded complex I subunits could disrupt assembly, however, the development of blue native (BN)-PAGE allowed for the direct analysis of mitochondrial respiratory complex assembly. By using Coomassie Blue G in conjunction with mild, non-ionic detergents, respiratory complexes could now be resolved on polyacrylamide gels in their native form [134, 135]. Using this technique, a novel, pathogenic mutation in the *MTND6* gene, 14487T > C, was shown to alter the mobility and decrease the amount of fully assembled complex I in fibroblasts from a patient with Leigh Syndrome [108]. Stalled assembly intermediates of ~500 and 800 kDa, which contained nuclear-encoded complex I subunits, were also detected.

In a separate study, pathogenic mutations in the *MTND5* (13513G > A) and *MTND6* (14459G > A) genes were also shown to disrupt complex I assembly, with mature complex I, as detected by BN-PAGE, reduced to ~40 and 20 % of control values, respectively [136]. Of note, the *MTND5* mutation was present at mutant loads of approximately 50 % or less in all patient tissues tested, with only 20 % fully assembled complex I detected in fibroblasts. This suggests that the 13513G > A *MTND5* mutation disrupts complex I assembly and function when present at unusually low mutant loads and may act dominantly. This is in contrast to the studies in mouse cells by Hofhaus and Attardi [133], where an *MTND5* mutation had a relatively mild effect on complex I assembly. Thus, it has become

evident that different mutations in the same mtDNA-encoded subunit can have very different outcomes on the activity and assembly of complex I, which in turn may modulate the severity of the resulting clinical phenotype.

More recent studies have also used BN-PAGE to analyze complex I assembly in the presence of mtDNA mutations. A novel mutation in the *MTND2* gene from a patient with Leigh Syndrome was found to result in complex I enzymatic deficiency and disruption of assembly [104]. Levels of mature complex I were reduced, with the accumulation of stalled membrane arm and matrix arm intermediates. Pathogenic mutations in *MTND1*, *MTND5* and *MTND6* were also shown to result in complex I enzymatic deficiencies, however the assembly of complex I was affected to different degrees [137]. Levels of mature complex I were severely decreased in cybrids derived from patient mitochondria which harbored *MTND1* or *MTND6* mutations, whereas the *MTND5* mutation had little effect on the amount of mature complex I [in this case similar to the study by Hofhaus and Attardi 133]. However, all mutations appeared to increase the amount of stalled assembly intermediates, in particular subcomplexes which contain the nuclear-encoded membrane arm subunit NDUF6 [137].

Studies of three common complex I subunit mutations associated with LHON (3460G > A *MTND1*, 11778G > A *MTND4* and 14484T > C *MTND6*) have revealed that although the steady-state levels of mature complex I are normal, assembly kinetics are affected [130]. Cybrids containing these mutations exhibited increased turn-over of complex I after doxycycline treatment and different synthesis rates of newly formed complex I. In addition, this process was modulated by the mtDNA background, with altered assembly rates and stability of the OXPHOS complexes III and IV. Defects in complex III and IV biogenesis may modulate the assembly/stability of complex I, as complexes I, III and IV are associated together in a large supercomplex or 'respirasome' [138, 139]. Loss of either complex III or IV has been shown to disrupt supercomplex formation, which in turn affects complex I assembly/stability [53, 140–142]. Thus, LHON pathogenic mutations can shift the assembly kinetics of complex I, with the mtDNA haplotype modulating this defect by altering supercomplex (and subsequently complex I) biogenesis [130].

2.5 Assembly Models of Complex I

The first model of complex I assembly was derived from the aerobic fungus *Neurospora crassa* by utilizing pulse-chase labeling of assembly intermediates and the characterization of subcomplexes in mutant strains [143–145]. Compared to human complex I (which is composed of 45 subunits), complex I in *N. crassa* is comprised of only 35 subunits, three of which are not found in the human complex. Therefore, the assembly of complex I may differ substantially between these two species.

To address this issue, human complex I assembly was studied by screening a cohort of complex I deficient patient cells using BN-PAGE, with a set of stalled assembly intermediates identified [146]. From these findings a model of complex I assembly was proposed, whereby both matrix and membrane arm subunits are

found together in early-stage intermediates. Interestingly, this model for human complex I assembly did not correspond to the modular, evolutionarily conserved system proposed for complex I assembly in the fungus *N. crassa*, where matrix and membrane arm subunits are found exclusively in separate intermediates during the early stages of assembly [143].

A subsequent model of complex I assembly utilized a conditional assembly system by blocking, then re-introducing, mtDNA-encoded protein translation [147]. This allowed for the depletion of complex I and the analysis of de novo complex I assembly. Using this system, it was proposed that the peripheral matrix arm and the membrane arm are assembled separately in a semi-sequential process, in this case consistent with the modular assembly found in *N. crassa* [147].

Other studies have utilized a GFP-tagged form of the complex I subunit NDUF33 to monitor the progression of this subunit into the mature holo-enzyme [148]. Interestingly, this system identified intermediates containing both membrane and matrix arm subunits, resulting in a model for complex I assembly that was similar to that originally proposed by Antonicka and colleagues [146]. The assembly of individual, nuclear-encoded subunits has also been monitored using an in vitro mitochondrial import and assembly assay [149]. In the presence of endogenous complex I a number of subunits assembled via different intermediate complexes into the mature holo-enzyme. Conversely, some subunits appeared to assemble directly into mature pre-existing complex I (and its supercomplex forms) [149].

The assembly of mtDNA-encoded subunits into human complex I has also been examined directly by pulse-chase radiolabeling studies [149, 150]. At early chase times, the mtDNA-encoded complex I subunits ND1, ND2, ND3 and ND6 were detected in assembly intermediates in the range of ~400–830 kDa [149]. At later chase times, the subunits ND4 and ND5 assemble into the (almost) mature holo-enzyme, with complete assembly of all seven mtDNA-encoded subunits requiring at least 24 hours [149]. Pulse-chase studies in patient cells which harbor mutations in the nuclear-encoded complex I assembly factor *NDUFAF1* have revealed that loss of *NDUFAF1* results in increased turn-over of ND2, loss of the ~460 kDa assembly intermediate which contains ND2, and the accumulation of a ~400 kDa intermediate which contains ND1 [9]. These findings suggest that ND1 and ND2 are in separate intermediates during the early stages of complex I assembly (corresponding to the proposed assembly pathway of these two subunits in *N. crassa* complex I). This is supported by data from patient cells where mutations are present in the nuclear-encoded complex I assembly factor *C20ORF7* [12]. In this case the opposite is observed; the ~400 kDa intermediate which contains ND1 is lost, with the accumulation of the ~460 kDa ND2-containing intermediate.

The assembly of mtDNA-encoded complex I subunits has also been studied by pulse-chase analyses and the identification of steady-state intermediate complexes in mouse cells with various complex I mtDNA mutations [151]. Using these techniques, five entry points of mtDNA-encoded subunits into the complex I assembly pathway were recently proposed [151]. ND1 was found in an early intermediate complex (point 1), while ND2, ND3 and ND4L were found in a separate early intermediate (point 2). ND4 is subsequently assembled (point 3), followed by ND6 (point 4),

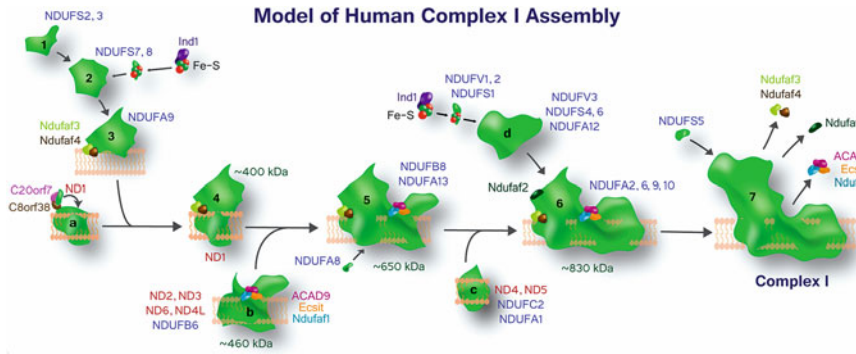


Fig. 2.1 Current model of human complex I assembly. Intermediates 1–6 correspond to the NDUFS3 containing intermediates described by Vogel and colleagues [148]. Entry points of structural subunits (blue, nuclear-encoded; red, mtDNA-encoded) and the various assembly factors involved are indicated. During the early stages of assembly NDUFS2, 3, 7, and 8 form an evolutionarily conserved hydrogenase module (Q module) as part of intermediate 2, with the assembly factor Ind1 inserting Fe–S clusters into NDUFS7 and 8. With the addition of NDUFA9 (and possibly other subunits), intermediate 3 is assembled and subsequently anchored to the membrane by the assembly factors Nduf3 (C3orf60) and Nduf4 (C6orf66). An early membrane arm intermediate, intermediate ‘a’, is assembled from subunits including ND1, whose biogenesis is aided by the assembly factors C20orf7 and C8orf38. Intermediate ‘a’ and intermediate 3 assemble to form the ~400 kDa membrane arm intermediate 4, while a second membrane arm intermediate of ~460 kDa, intermediate ‘b’, is assembled from the subunits ND2, ND3, ND6, ND4L and NDUFB6. This intermediate has been found associated with the assembly factors Nduf1 (CIA30), Ecsit and ACAD9. During the middle stages of complex I assembly, intermediate 4 and intermediate ‘b’ assemble together with NDUFA8, NDUFA13 and NDUFB8 to form the ~650 kDa intermediate 5. At this stage ND4, ND5, and possibly NDUFC2 and NDUFA1 (intermediate ‘c’), are added to form the ~830 kDa intermediate 6. The subunits NDUFA2, 6, 9, and 10 are also added at this stage, with the assembly factors Nduf1, Nduf2 (B17.2L), Ecsit, ACAD9, Nduf3 and Nduf4 remaining associated with this intermediate. During the last stages of assembly the N module is formed from the subunits NDUFV1, 2, 3, NDUFS1, 4, 6 and NDUFA12 (intermediate ‘d’), with Ind1 inserting Fe–S clusters into NDUFV1, 2 and NDUFS1. Lastly, intermediate ‘d’, the subunit NDUFS5, and the ~830 kDa intermediate 6 are assembled together to form mature complex I.

which appears to facilitate the assembly of the ND1 and ND2 containing intermediates. ND5 is assembled last, defining the 5th entry point [151].

From the studies described here, in conjunction with analyses of patient cells that harbor nuclear gene mutations in complex I subunits or complex I assembly factors, a model for human complex I assembly can be proposed (Fig. 2.1). During the early stages of complex I biogenesis, an intermediate is formed from the matrix arm subunits NDUFS2 and 3 (intermediate 1) [148]. NDUFS7 and 8 are added to form intermediate 2, with the assembly factor Ind1 involved in the insertion of Fe–S clusters into these two subunits [152]. Further subunits are added (including NDUFA9) to form intermediate 3 [146, 147]. This intermediate is associated with the assembly factors Nduf3 and Nduf4, proteins which may act to anchor intermediate 3 to the membrane [11].

The mtDNA-encoded subunit ND1 is assembled into membrane arm intermediate ‘a’, possibly with the aid of the assembly factors C20orf7 and C8orf38 [12, 17, 89]. Intermediate ‘a’ is then combined with intermediate 3 to form intermediate 4, an ~ 400 kDa complex which contains parts of both the membrane and matrix arms [88, 146]. A separate membrane arm intermediate (intermediate ‘b’) is formed from mtDNA-encoded subunits ND2, ND3, ND6 [149], ND4L [151] and possibly the nuclear encoded subunit NDUFB6 [89, 147, 152], and is associated with the assembly factors Ndufap1, Ecsit, and ACAD9 [9, 13, 153, 154].

During the middle stages of complex I assembly, intermediate ‘b’ and intermediate 4 combine to form intermediate 5 [148], with the subunits NDUFA13 [148] and NDUFA8 (which inserts from the intermembrane space (IMS) side of the inner membrane [155]) also assembled. The mtDNA-encoded subunits ND4 and ND5 are added [88], with co-evolution analyses predicting an interaction between these two subunits and NDUFC2 [156]. This would suggest that NDUFC2 is also assembled at this point with ND4 and ND5 (intermediate ‘c’). Furthermore, NDUFA1 is predicted to interact with ND1, ND4 and ND5 [156], and may also assemble at this stage to help anchor ND4 and ND5 to the growing membrane arm. Indeed, *in vitro* import studies with isolated mitochondria suggest that NDUFA1 assembles at this stage, along with the subunits NDUFA2, 6, and 10 to form the ~ 830 kDa intermediate 6 [149]. The assembly factors Ndufap2 [10], Ndufap1 [9, 154], Ndufap3 [11], Ndufap4 [11], Ecsit [153] and ACAD9 [13] are also associated with this ~ 830 kDa intermediate.

During the latter stages of complex I biogenesis the ~ 300 kDa matrix arm intermediate ‘d’, which contains the N module, is formed from the subunits NDUFV1, 2, 3, NDUFS1, 4, 6, and NDUFA12 [149], with the assembly factor Ind1 involved in insertion of Fe–S clusters into NDUFV1, 2 and NDUFS1 [152]. Intermediate ‘d’ and intermediate 6 combine, with the addition of further subunits (including NDUFS5 [147]) to form the mature complex.

2.6 Concluding Remarks

By piecing together information from the studies described in this chapter, we have been able to gain insights into the assembly process of complex I and how this relates to its function. Furthermore, we are now developing an understanding of how pathogenic mutations can disrupt complex I assembly during different stages of its biogenesis, thus affecting the activity of the enzyme complex. This new knowledge may prove invaluable for future therapeutic design, where treatment of mitochondrial disorders will require restoration of complex I function by manipulating its biogenesis. This will be of benefit for patients not only with classical mitochondrial diseases but also individuals who develop neurological disorders, such as Parkinson’s Disease [157], where complex I dysfunction has been implicated.

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