


Review

Analysis of Human Mutations in the Supernumerary Subunits of Complex I

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Abstract: Complex I is the largest member of the electron transport chain in human mitochondria. It comprises 45 subunits and requires at least 15 assembly factors. The subunits can be divided into 14 “core” subunits that carry out oxidation–reduction reactions and proton translocation, as well as 31 additional supernumerary (or accessory) subunits whose functions are less well known. Diminished levels of complex I activity are seen in many mitochondrial disease states. This review seeks to tabulate mutations in the supernumerary subunits of humans that appear to cause disease. Mutations in 20 of the supernumerary subunits have been identified. The mutations were analyzed in light of the tertiary and quaternary structure of human complex I (PDB id = 5xtf). Mutations were found that might disrupt the folding of that subunit or that would weaken binding to another subunit. In some cases, it appeared that no protein was made or, at least, could not be detected. A very common outcome is the lack of assembly of complex I when supernumerary subunits are mutated or missing. We suggest that poor assembly is the result of disrupting the large network of subunit interactions that the supernumerary subunits typically engage in.

Keywords: mitochondria; mammalian complex I; NADH dehydrogenase; complex I assembly; complex I structure; complex I deficiency; supernumerary subunits; electron transport chain; mitochondrial dysfunction; Leigh syndrome

1. Introduction

Mutations in the genes that encode complex I are responsible for a large fraction of all mitochondrial diseases. For example, 20–30% of cases in childhood mitochondrial disease (MD) are related to complex I dysfunction [1,2]. There seem to be many reasons for this. Complex I genes make up a large component of the mitochondrial and nuclear genome. Complex I is encoded by seven mitochondrial genes (out of 13 total) and 37 nuclear genes. There are also at least 15 complex I assembly factors [3]. Mitochondrial DNA (mtDNA) is especially prone to mutation due to insufficient DNA repair systems. All of the various functions of complex I can be impacted by mutation. Complex I forms supercomplexes with other members of the respiratory chain, such as complex III (cytochrome *bc₁*) and complex IV (cytochrome *c* oxidase). It is metabolically linked to the citric acid cycle by NADH and to ATP synthesis by proton translocation. Furthermore, it is a major site of superoxide generation in mitochondria. Therefore, mutations that alter or degrade complex I function will typically have wider effects on mitochondrial function.

Complex I is a boot-shaped multi-subunit enzyme embedded in the inner mitochondrial membrane (see Figure 1). Its primary role is to oxidize NADH while reducing ubiquinone and translocating protons across the membrane. One arm extends into the matrix space, and it contains the flavin

appear to assemble first, followed by the stepwise addition of the P-module, associated with the three major subunits ND2, ND4, and ND5. ND5 and the N-module enter the complex last.

In this review, 20 supernumerary subunits for which clinical mutations have been identified are described (see Table 1). Some of the mutations are interpreted in light of the structure of the human complex I (Protein Data Bank file 5xtf) or, in some cases, from other species. Some of the mutations are likely to be null mutations in which no protein is made, but the evidence is not always clear for that. Currently, the understanding of the effects of mutations is limited by a lack of knowledge. For example, how do mutations in one subunit affect the stability or import of that subunit? How does the absence of one subunit affect the expression, import, or stability of other subunits? How do mutations affect the assembly of that subunit or of other subunits? Do supernumerary subunits have roles that can be affected by mutation while assembly remains normal?

Table 1. Clinical missense mutations in supernumerary subunits and their surface contacts. IMS: intermembrane space.

Location	Subunit	Mutated Residues	Primary Contacts
N-module	NDUFA2	p. Lys45Thr p. Glu57Ala p. Asp50Asn + others *	NDUFS1
	NDUFV3	p. Arg26Gln p. Lys56Asn p. Gly103Asp p. Glu276Lys	NDUFV1, NDUFV2
Q-module	NDUFS4	p. Trp114Arg p. Asp119His + others *	NDUFS1, NDUFS3, NDUFS8, NDUFV1, NDUFA6, NDUFA9
	NDUFS6	p. Cys115Tyr + others *	NDUFA9, NDUFA12, NDUFS1, NDUFV2, NDUFS2, NDUFS8
	NDUFA9	p. Arg321Pro p. Arg360Cys	NDUFS3, NDUFS7, NDUFS4, NDUFS6, NDUFA6
ND1- module	NDUFA1	p. Gly8Arg p. Arg37Ser p. Gly32Arg p. Pro19Ser	ND1, NDUFA8
	NDUFA3	Deletion of one allele	ND1, ND3, NDUFS8, NDUFA8, NDUFA13
	NDUFA13	p. Arg57His	ND1, ND6, NDUFS2, NDUFS5, NDUFA3, NDUFA8
LYR proteins	NDUFA6	p. Arg64Pro + others *	NDUFAB1, NDUFS1, NDUFS3, NDUSA9
	NDUFB9	p. Leu64Pro p. Arg47Leu	NDUFB1, NDUFB3, NDUFB4, NDUFB5, NDUFB6
	NDUFS5	p. Pro96Ser	ND2, ND4L, NDUFB5, ND6, NDUFB13
IMS proteins	NDUFA8	p. Arg47Cys p. Glu109Lys p. Arg135Gln	NDUFA13, NDUFA3, NDUFA1
	NDUFB10	Glu79 p. Cys107Ser	NDUFB5, NDUFB6, NDUFB11
ND2-module	NDUFA10	p. Gln142Arg p. Leu294Pro p. Gly99Glu + others	ND2, NDUFS2
	NDUFC2	p. His58Leu + others *	ND2, NDUFC1, NDUFB5, NDUFA8

Table 1. Cont.

Location	Subunit	Mutated Residues	Primary Contacts
ND4-module	NDUFA11	p. Ala132Pro p. Thr106Ile (isoform two) + others *	ND2, ND4, ND5, NDUFB5
	NDUFB11	p. Arg134Ser p. Glu219Lys + others	ND4, NDUFB10
ND5-module	NDUFB3	p. Trp22Arg + others	NDUFB9, NDUFAB1, NDUFB2, ND5
	NDUFB6	Promoter mutation	NDUFB5, NDUFAB1, NDUFB9, ND5, NDUFB10, NDUFB7
	NDUFB8	p. Pro76Gln p. Cys144Trp p. Tyr62His + others *	ND5, NDUFB4, ND4, NDUFAB1, NDUFB7, NDUFB9, NDUFB10

* Other mutations include nonsense mutations, splicing mutations, start codon mutations, insertions, and deletions. Mutations are defined by the change in the protein (p.) and the amino acid substitution, Lys45Thr, lysine at position 45 changed to threonine. See text for more details.

2. Review of Mutations in the Supernumerary Subunits

2.1. N-Module Subunits

The first subunits to be described are two subunits that primarily have contacts to core subunits of the N-module (see Supplementary File Table S1 for a Table of Subunit Interactions). Overall, they are rather isolated from other supernumerary subunits, as shown in Figure 2. Beyond those similarities, their properties appear divergent.

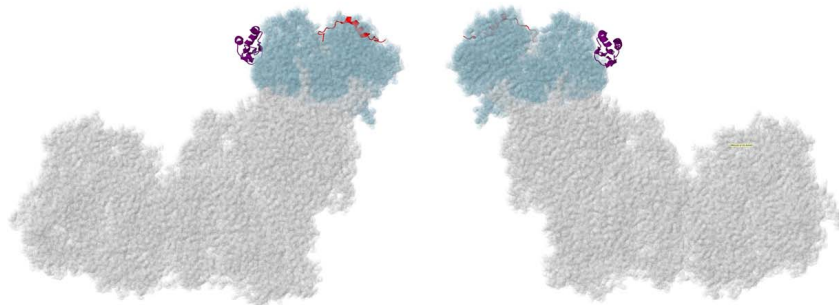


Figure 2. Location of NDUFA2 and NDUFV3. Most of Complex I is colored gray. Core subunits in the N-module arm are colored light blue (NDUFV1, NDUFV2, and NDUFS1). NDUFA2 and NDUFV3 are shown in ribbons, with NDUFA2 colored purple and NDUFV3 colored red. The two views are rotated 180° relative to each other. The structure is from PDB id = 5xtl [9].

2.1.1. NDUFA2

The gene for NDUFA2 is located on chromosome 5. The protein contains 99 amino acids with a mass of about 10.9 kDa before the processing of the amino-terminal methionine. This subunit is bound to the matrix arm in a unique fashion, with exclusive contacts to core subunit NDUFS1. It is bound to C-terminal domains of NDUFS1, and it is at least 40 Å from any of the FeS clusters. It has a compact structure that resembles a thioredoxin fold and includes a flat four-stranded beta-sheet (see Figure 3). Two Cys residues, 24 and 58, are found in the loops of the beta-sheet, but they do not appear to be oxidized in the existing structures of mammalian complex I. Contact with NDUFS1 is primarily with the beta-sheet region, including both Cys residues. In culture, NDUFA2 knockout cell lines have been found to lack complex I activity, and an analysis by blue native (BN) gel electrophoresis showed a limited assembly of the N-module—in particular, the loss of core subunits NDUFS1, NDUFV1,

and NDUFV2 and supernumerary subunits NDUF54, NDUF56, NDUFA7, and NDUFV3. In a cell line with an NDUFA2 knockout, complex I assembly is slightly affected, with a prominent band in BN gel electrophoresis but apparently missing some subunits, probably the N-module. Complex I activity has been found to be extremely low, consistent with a missing NADH binding site [11].

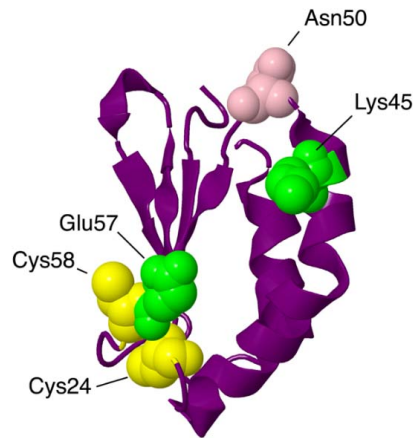


Figure 3. Structural features of NDUFA2 from the N-module. The protein is portrayed in a purple-colored ribbon. Sites of mutations are shown in space-filling views: Lys45Thr and Glu57Ala are mitochondrial disease mutations (colored lime). Asn50Asp has been found in breast cancer patients (colored pink). Two Cys residues are colored yellow.

An individual with Leigh syndrome and hypertrophic cardiomyopathy was discovered to be homozygous with a mutation, c.208 + 5 G > A, in the NDUFA2 gene that caused a reduction in correct splicing [12]. Normally, NDUFA2 is coded by three exons, but in this individual, most of the transcripts lacked exon 2, causing a frameshift. No protein or transcript was found, making it a null mutation. The patient died of cardiovascular arrest at eleven months.

Two individuals with leukoencephalopathy were discovered to have mutations in NDUFA2 [13]. One was homozygous for p.Lys45Thr, and the other had compound heterozygous mutations, p.Lys45Thr and a deletion at Asn76, c.225del, and p.Asn76Metfs*4, thus causing a frameshift and a stop codon after 4 codons. The first was found to have complex I deficiency and lacked an assembled complex I in BN gel electrophoresis. This individual also had a systemic deficiency in carnitine due to a homozygous mutation in SLC22A5. She developed focal epilepsy at six years, was wheelchair-bound at nine years, and was last evaluated at 12 years. The second patient had a similar presentation, including abnormal white matter in the brain. She also had movement difficulties and was last evaluated at age four. Lys45 is found at the interface with NDUF51 and has close contacts with several NDUF51 residues, including Gly376, Asp380, and Ser672. It is likely that the binding of NDUFA2 to NDUF51 is destabilized.

In 2020 [14], another individual was described with a homozygous mutation in NDUFA2, p.Glu57Ala. This residue is at the interface with NDUF51 and makes a possible ion pair with Arg382 of NDUF51. It also makes nonbonding contacts with Gly661, Ala662, Asn663, Tyr664, Leu381, Arg382, and Ser383, suggesting a likely disruption of binding. This individual had abnormal white matter in the brain, microcephaly, seizures, and movement disorders. She was last evaluated at four years of age.

In a screen of breast cancer patients [15], a homozygous p.Asp50Asn mutation in NDUFA2 was detected in one individual. This residue is not at the interface with NDUF51, and so it perhaps does not disrupt the assembly of the enzyme. The significance of this finding is uncertain.

2.1.2. NDUFV3

The gene for NDUFV3 is found on chromosome 21. This was the first subunit of human complex I known to have multiple isoforms [16–18], which can be found by alternative splicing. It is not an

essential protein, as shown by knockout studies in cell culture [11]. The loss of this gene does not prevent the assembly of complex I, as seen in BN gels, and cells retain the ability to grow in a galactose medium, indicative of oxidative phosphorylation activity. Exogenous NDUFV3 has been seen to exchange with fully assembled complex I, suggesting that it can be incorporated last into the enzyme. The more common isoform has 108 amino acids (74 after the cleavage of the transit peptide) and a mass of 11.8 kDa, while the second isoform has 473 amino acids and a mass of about 51 kDa. Isoform two has an additional exon between the second and the last exons of the shorter form. The additional exon contains a serine-rich region that was found to be phosphorylated [19]. NDUFV3 is not present in fungal complex I from *Yarrowia lipolytica* [7].

NDUFV3 was originally identified in the flavoprotein fraction of bovine heart complex I and now can be seen to have primary contacts with NDUFV1 and NDUFV2 in the human enzyme. It also has limited contact with NDUFS1 and NDUFS4. In the human model of complex I (PDB id = 5xtd) [9], only 33 amino acids are visible, corresponding to residues 74–106. Residues 82–5 are alpha-helical. The longer isoform has an insertion at residue 56 and retains the same C-terminus as isoform one. Therefore, both isoforms can likely bind in the same fashion to other complex I subunits. Both isoforms have been seen in a variety of tissues, but isoform two is more common in cultured cells and in brain tissue [16]. Others have found that isoform one is more prevalent in bovine and murine complex I of heart tissue, as well as that increased K_m values for NADH consumption by complex I correlate with an increased extent of the short form in heart tissue [18]. In a cross-linking study of native mouse heart mitochondria [20], both isoforms were identified in cross-links with a surface peptide of malate dehydrogenase, found near the NAD^+ binding site. The binding of malate dehydrogenase and several other enzymes to porcine complex I was demonstrated in 1984 [21]. Other cross-links identified with NDUFV3 were with NDUFV1, NDUFV2, Cox7A1, and Atp5a1—the latter two supporting the proximity of the complexes of oxidative phosphorylation.

Sequence variations have been discovered in NDUFV3 among individuals with decreased levels of complex I activity, but none have been shown to be causative for disease. In one such cohort, four individuals with mutations in NDUFV3 were found [22]. The first individual had p.Arg26Gln, which would occur in the mitochondrial transit peptide and thus might affect import. A second mutation, was found in the same individual in the mitochondrial DNA polymerase G gene POLG, p.Gly11Asp. The second individual had the mutation p.Lys56Asn, which occurs at the splice junction and might affect expression, but this individual also carried a mutation in AMACR, an enzyme associated with branched-chain fatty acid metabolism, p.Val185Ala. The third individual had the mutation p.Gly103Asp, which would occur in both isoforms, in the region that binds to other subunits. Gly103 contacts Trp166 of NDUFS4 in a non-bonding interaction, and it is adjacent to several charged residues. Arg104 of NDUFV3 is near Arg169 of NDUFS4 and Asp426 of core subunit NDUFS1. Glu105 of NDUFV3 and Glu72 of NDUFV2 are nearby. Ser106 is also in this region, and it was found to be phosphorylated in a screen of human cancer cells [23]. Therefore the p.Gly103Asp mutation appears likely to be disruptive to the binding of NDUFV3. The fourth individual had the mutation p.Glu276Lys, which is found only in the long form, near the sites of phosphorylation. This individual also carried a p.Gly154Ser mutation in the core subunit NDUFS8, which is a buried residue, and that substitution is more likely to be deleterious.

2.2. Q-Module Subunits

NDUFS4, NDUFS6, and NDUFA9 are found in the Q-module, as shown in Figure 4. They interact with each other and with many other subunits in the matrix arm. They appear to have roles in assembling and stabilizing the N- and Q-modules.

2.2.1. NDUFS4

The gene for NDUFS4 is found on chromosome 5. NDUFS4 is a protein that was initially found in the iron–sulfur protein fraction of bovine heart complex I. It is synthesized as 175 amino acids,

and after the cleavage of the mitochondrial transit peptide, the mature protein is 133 amino acids with a mass of about 15.3 kDa. As shown in Figure 1, it is located in the matrix arm of complex I and contacts core subunits NDUFS1, NDUFS3, NDUFS8, and NDUFV1, as well as supernumerary subunits NDUFA6 and NDUFA9. It fits between two lobes of core subunit NDUFS1. It can be understood as a globular protein with a mixed alpha/beta architecture and three extensions that reach towards the chain of FeS clusters (see Figure 5). The C-terminus, in particular, is within about 9 Å of FeS clusters N3 and N1b, found in NDUFV1 and NDUFS1, respectively. A loop ending in Met87 is within 10 Å of FeS clusters N4 and N5 of NDUFS1, and a second loop including Met112 is pointing toward the last three FeS clusters—N6a, N6b, and N2—but is at least 13 Å away. NDUFS4 has been found to be phosphorylated at residue Ser173, near the C-terminus [24]. It has been suggested that this improves the net import of the subunit by inhibiting its return to the cytoplasm. In a knockout cell line of cultured human cells, the loss of NDUFS4 had a mild effect on assembly of complex I, causing it to migrate as a smaller-than-normal-sized complex in BN gel electrophoresis, probably lacking the N-module [11].

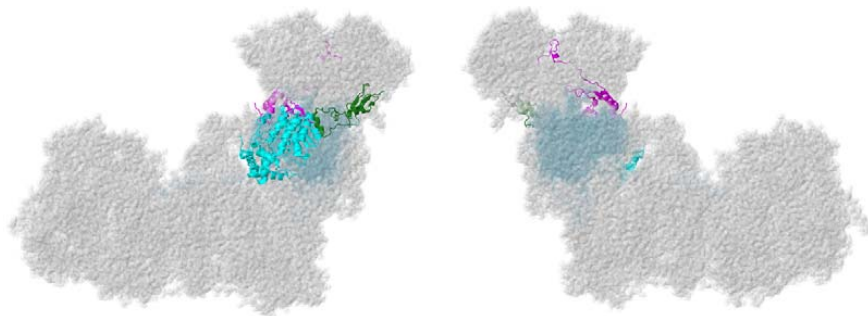


Figure 4. Location of NDUFS4, NDUFS6, and NDUFA9. Most of Complex I is colored gray. Core subunits in the Q-module are colored light blue (NDUFS2, NDUFS3, NDUFS7, and NDUFS8). NDUFS4, NDUFS6, and NDUFA9 are shown in ribbons, with NDUFS4 colored magenta, NDUFS6 colored green, and NDUFA9 colored cyan. The two views are rotated 180° relative to each other. The structure is from PDB id = 5xtd [9].

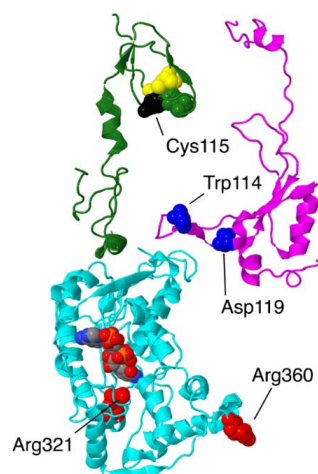


Figure 5. Structural features of 3 interacting subunits from the Q-module. The proteins are portrayed in ribbons. NDUFS4 is colored magenta. The sites of two mutations in this subunit, Trp114Arg and Asp119His, are shown in space-filling and are colored blue. NDUFS6 is colored green. The zinc bind residues are shown in space-filling, with Cys colored yellow and His colored green. The site of the mutation, Cys115Tyr is colored black. NDUFA9 is colored cyan, and its bound ligand NADP⁺ is shown in space-filling with CPK colors (e.g., carbon gray, nitrogen blue, oxygen red, phosphorus orange). The sites of 2 mutations, Arg321Pro and Arg360Cys, are shown in space-filling and are colored red.

Three human mutations in NDUF54 from Leigh syndrome patients were examined biochemically [25]: a duplication of AAGTC at position 466–470 of the coding sequence, a single G deletion at position 289 (in Trp97), and a nonsense mutation, c.44 G > A, p.Trp15X, in the first exon of the gene. Nonsense-mediated decay apparently eliminated the transcript in the second case. In the third case, c.44 G > A, it was later determined that three alternative splice variants were produced [26]. In all three cases, the mutations were homozygous, and it was demonstrated that little or no protein was made. An analysis of these null mutants from cultured fibroblasts showed that complex I did not assemble by BN gel electrophoresis and that little or no activity could be measured [25].

Two clinical missense mutations have been identified in this subunit, p.Trp114Arg [27] and p.Asp119His [28], both resulting in Leigh syndrome. Several frameshift mutations have been identified, including the homozygous c.221delC (p.Thr74Ifs*17) and the compound heterozygous mutations c.462delA (p.Lys154Nfs*34) and c.99-1 G > A (p.Ser34Ifs*4), which also appear to cause Leigh syndrome [22]. Both missense mutations are found in the loop nearest to the N2/N6ab FeS clusters. Perhaps more importantly, this extension of NDUF54 is part of a junction of three subunits, with NDUF9A, the NADP⁺ binding protein, and an extension of core subunit NDUF53. The individual with the p.Trp114Arg mutation was found to be homozygous for this allele, while both parents were found to be heterozygous [27]. This individual was diagnosed with myocarditis, respiratory failure, delirium, and basal ganglia abnormalities in the brain as seen by magnetic resonance imaging (MRI), and she was found to have complex I deficiency. Trp114 is a conserved residue that has close contact with Gln228 of NDUF53 and is near several residues of core subunit NDUF58. In the human structure (PDB id = 5xtf), the glutamine oxygen from the sidechain is pointing into the tryptophan ring, but it seems that the amino group would more logically assume that position. In conclusion, it seems likely that the p.Trp114Arg mutation would be disruptive and might impact the transfer of electrons through complex I.

The individual with the p.Asp119His mutation was compound heterozygous, with a second allele containing a Lys154 frame shift mutation, and he died in his third year [28]. While these mutations were found in a heterozygous situation (mother, father, and brother), there was no clinical presentation, suggesting that both mutations contributed to declining health. In a different study, the transcript for the frameshift allele was shown to exist, indicating that nonsense-mediated decay did not occur and suggesting that the protein was made. Asp119 is primarily contacted by other residues in NDUF54, including an ion pairing with Arg75. The closest contact with subunit NDUF9A is residue Lys45 at a distance of over 5 Å. Therefore, it is likely that the Asp119His substitution would be deleterious. If we assume that the frameshift mutation would produce a protein that is truncated at residue 154, that would remove a small but highly conserved domain that normally interacts with NDUFV3, and with core subunits NDUFV1, NDUFV2, and NDUF51, in the vicinity of the FeS clusters N3 and N1b. Even this loss of about 20 amino acids would appear to be disruptive, and it seems possible that the truncated protein might be found at lower-than-normal levels due to instability.

2.2.2. NDUF56

The gene for NDUF56 is located in chromosome 5 and contains four exons. The protein has 124 amino acids before the N-terminal 28 are cleaved upon entry to the mitochondrion. The original mass is 13.7 kDa. The protein is bound to the matrix arm and has two domains connected by a short alpha-helix, residues 67–75 (see Figure 5). The N-terminal domain, residues 29–66, is closer to the membrane and primarily contacts subunits NDUF9A and NDUF12. The C-terminal domain, residues 76–124, contains several short beta-strands and is in contact with core subunits NDUF51, NDUFV2, NDUF52, and NDUF58. A zinc ion is bound by three cysteines and one histidine that are part of a C–X₈–H–X₇–C–X₂–C motif with Cys87, Cys112, Cys115, and His96. The significance of the bound zinc is not clear, but this subunit is found not only in the fungal *Y. lipolytica* enzyme [29,30] but also in some bacterial enzymes (e.g., *Paracoccus denitrificans*) [31]. In cell culture, the knockout of NDUF56 results in a mild assembly defect in which complexes of somewhat smaller size with a

reduced oxidative phosphorylation capacity are seen [11]. In *Y. lipolytica*, the knockout of the NDUFS6 gene results in a complex lacking NDUFA12 and the N4 FeS cluster. Complementation with constructs lacking one of the zinc binding residues failed to regenerate the wild-type activity, suggesting a role in the incorporation of this FeS cluster [29].

The first mutations associated with NDUFS6 were described in 2004 [32]. Three individuals presented with lethal neonatal mitochondrial complex I deficiency and died within days of birth. Two were siblings with homozygous mutations, c.186 + 2 T > A, which affected splicing, leading to 26 bp of intronic RNA incorporated at the exon 2/3 junction, causing a frameshift and a protein predicted to be 71 amino acids. A small amount (3%) of normally spliced transcript was also found. The third individual was also homozygous, with a deletion of 4.75 kb that removed exons 3 and 4. This individual died five days after birth.

A mutation in one of the residues that bind zinc, p.Cys115Tyr, was found in two different families [33]. The affected individuals, two from each family, presented with fatal neonatal lactic acidemia and died within a few days of birth. All were homozygous for this allele.

Two additional mutations in NDUFS6 were identified, first in 2017 [34] and then again in 2019 [35] when a more extensive analysis was done. Both were characterized as having Leigh syndrome, and the individuals were compound heterozygous, carrying both mutant alleles, c.309 + 5 G > A and c.343 C > A (p.Cys115Arg). The individual analyzed in 2019 lived for eleven months. It was determined that the splicing error led to a transcript missing exon 3, but that a small amount of normally spliced form was present. Complex I assembly was very limited, as indicated by BN gel electrophoresis. The mutation of Cys115 to Arg or Tyr could disrupt the folding of the protein and its binding to the matrix arm, which could have led to the loss of the FeS cluster N4, as was shown to happen in the fungal *Y. lipolytica* enzyme [29].

2.2.3. NDUFA9

The gene for NDUFA9 is located on chromosome 12. It is encoded as a protein of 377 amino acids, about 42.5 kDa, but the N-terminal 35 amino acids are cleaved upon entry to the mitochondrion. The protein contains two domains, one being a Rossmann fold with an NADP⁺ bound to the C-terminal ends of the parallel beta-strands of the central sheet. A second domain, which contacts the NADP⁺, is largely alpha-helical. The second domain is largely C-terminal, and the Rossmann fold is largely N-terminal, except for two helices have been domain-swapped (see Figure 5). This protein is related to a family of short chain dehydrogenases, as first identified by Fearnley and Walker [36]. It is also one of the proteins that undergoes conformational changes in the active/deactive transition, as seen in mouse (PDB id = 6g2j, 6g72) [37] and sheep open and closed structures [38]. NDUFA9 is found at the junction of the Q-module and the membrane arm. It primarily has contact with core subunits NDUFS3 and NDUFS7, as well as with NDUFS4, NDUFS6, and NDUFA6. It also has minor contact with the core subunits ND1, ND3, ND6, NDUFS1, and NDUFS8. There is no evidence that this subunit exhibits any enzyme activity.

A human cell line was established with a knockout of NDUFA9 using transcription activator-like effector nucleases (TALEN) technology [39]. A clear assembly defect was discovered in which an 880 kDa complex was transiently seen, but a more stable 600 kDa complex was later found. The latter complex had the membrane arm subunits and some of the Q-module subunits of the matrix arm. In a more comprehensive study of all supernumerary subunits [11], the same group later established that levels of N-module subunits decreased the most in cell lines with the NDUFA9 knockout. The offered interpretation was that NDUFA9 is important for stabilizing the binding of the N-module to the Q-module during assembly. The complex seen in cells with the NDUFA9 knockout was smaller than those seen when NDUFS4 or NDUFS6 were knocked out, indicating that the Q-module was also destabilized.

Two missense mutations have been discovered in patients. The first was p.Arg321Pro, a homozygous mutation found in a boy of consanguineous parents [40]. He had complications in respiration after

birth, with lactic acidosis and vision and hearing loss. He died after one month of respiratory failure, and it was classified as Leigh syndrome. An analysis of cultured fibroblasts showed very low levels of NDUFA9, and several other subunits, as well as essentially no assembled complex I in BN gel electrophoresis. Arg321 is found in the NADP⁺ binding pocket near the nicotinamide end but not in contact with it. It does not contact any other subunits, as it is completely buried. Therefore, it is likely that the substitution to Pro would disrupt the folding of the subunit and render it unable to properly bind to the assembling complex I.

The second discovered mutation also involved a homozygous Arg mutation, p.Arg360Cys [41]. This patient first exhibited symptoms of dystonia at age seven, leading to a loss of speech and becoming wheelchair-bound. His conditions stabilized in adulthood. A biochemical analysis did not reveal any metabolic disease, but an MRI of his brain revealed atrophy consistent with Leigh syndrome. An analysis of fibroblast mitochondria revealed reduced levels of complexes I and IV by BN gel electrophoresis, as well as reduced levels of several complex I subunits by immunoblotting. Complex I activity was 17–61% of control samples. Complementation with wild-type NDUFA9 confirmed that this mutation was causative for the reduced complex I activity. Arg360 is near the C-terminus of the subunit and is sandwiched between Trp361 of NDUFA9 and Tyr78 of ND6, a core subunit. A similar arrangement is seen in the mouse structure of the active conformation [37], except that Glu80 of ND6 is also involved. This junction between NDUFA9 and ND6 appears to be disrupted in the deactive conformation, as shown in the mouse structure [37] in which the C-terminus of NDUFA9 is not visible and is perhaps disordered. A similar situation has been seen in the higher resolution structures from sheep [38] in which the loops between NDUFA9 and ND6 are formed in the closed structures (PDB id = 6zko and 6zkc), while these loops, including Arg360 are disordered in the open structures (PDB id = 6zkd, 6zke, 6z kf, 6z kp, and 6zkr). Therefore, it is possible that even if the p.Arg360Cys subunit can assemble, it might be less able to undergo conformational changes during turnover, and so activity would decrease.

2.3. ND1-Module Subunits

Three membrane subunits from the “heel” of complex I are described next: NDUFA1, NDUFA3, and NDUFA13, as shown in Figure 6. These subunits are part of the ND1-module for assembly. Each crosses the membrane once. NDUFA3 interacts with the transmembrane domain of NDUFA13, while NDUFA1 interacts with NDUFA13 in the IMS.

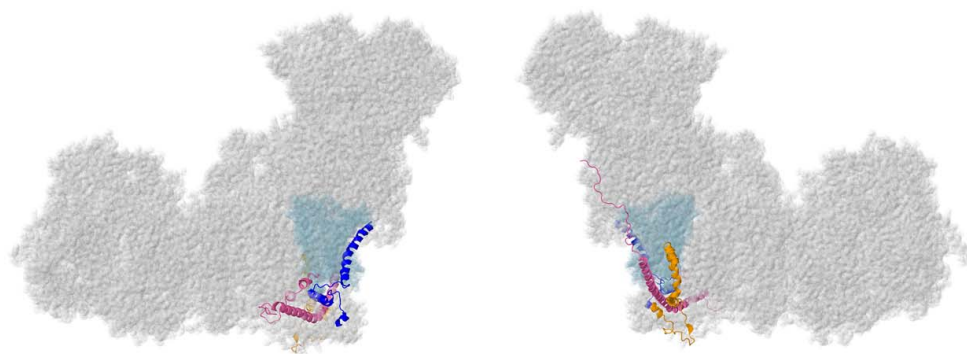


Figure 6. Location of NDUFA1, NDUFA3, and NDUFA13. Most of Complex I is colored gray. Core subunits in the ND1-modules are colored light blue (ND1). NDUFA1, NDUFA3, and NDUFA13 are shown in ribbons, with NDUFA1 colored blue, NDUFA3 colored orange, and NDUFA13 colored pink. The two views are rotated 180° relative to each other. The structure is from PDB id = 5xt d [9].

2.3.1. NDUFA1

The NDUFA1 gene is located on the X chromosome. The NDUFA1 subunit, also known as MWFE, contains 70 amino acids and has a mass of 8.0 kDa. It is a single-pass transmembrane protein that lies at the junction of the membrane and matrix arms of complex I. The N-terminus lies at the

matrix surface. The protein consists of two separate domains: residues 1–31 form an alpha-helix that is situated in a groove between the first and seventh transmembrane helices of core subunit ND1. The C-terminal domain lies in the IMS and contains an alpha-helix (residues 42–56) and a short 3–10 helix (residues 65–70). This domain primarily contacts NDUFA8 (see Figure 7). A Ser residue is phosphorylated at position 55, but the significance of this modification remains unknown [42]. In a knockout strain of cultured human cells, the loss of NDUFA1 resulted in a reduced expression of complex I, and it migrated as a smaller-than-normal-sized complex in BN gel electrophoresis [11]. NDUFA1 mainly contacts core subunit ND1 and supernumerary subunit NDUFA8, but it also weakly contacts core subunit ND6 and supernumerary subunits NDUFS5, NDUFS8, and NDUFA13.

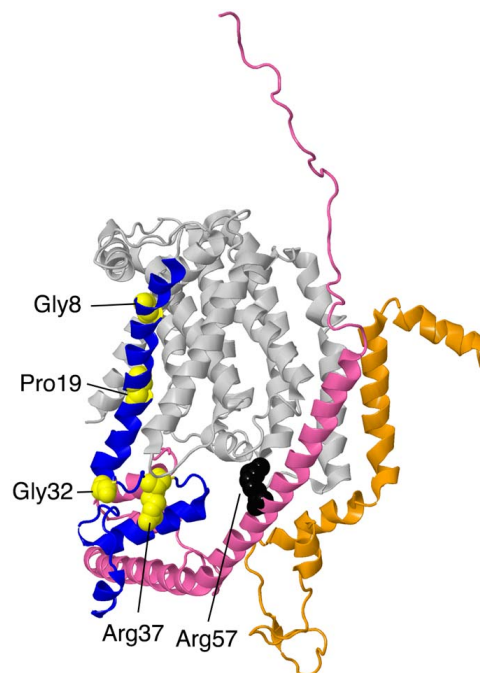


Figure 7. Structural features of ND1-module subunits. The proteins are portrayed in ribbons. ND1 is in the background and colored light gray. NDUFA1 is colored blue with the sites of 4 mutations, Gly8Arg, Pro19Ser, Gly32Arg, and Arg37Ser, shown in space-filling and colored yellow. NDUFA3 is shown in orange. No point mutations have been discovered yet. NDUFA13 is colored pink. The site of one mutation, Arg57His, is shown in space-filling and colored black.

Four clinical mutations have been identified in this subunit: p.Gly8Arg, p.Arg37Ser, p.Gly32Arg, and p.Pro19Ser, and so would be hemizygous in males. The p.Gly8Arg mutation was identified in two half-brothers diagnosed with Leigh syndrome who both died in infancy [43]. The brothers had different biological fathers but shared a mother who carried the p.Gly8Arg mutation. The older brother developed psychomotor retardation at nine months and generalized hypotonia and choreoathetosis. At 19 months of age, brainstem lesions were observed, and the patient died from cardiorespiratory failure. The younger brother presented with axial hypotonia, vertical rolling nystagmus, choreoathetosis, and bilateral lesions. He developed respiratory insufficiency at 13 months of age and died at 14 months of age from cardiorespiratory arrest. The family pedigree shows that three maternal uncles had died of an unknown disease. The severity of the disease is reflected in the structural importance of the Gly8 residue on NDUFA1. Gly8 lies in the conserved hydrophobic N-terminal region of NDUFA1. Gly8 is in loose contact with residues Thr23, Lys26, and Leu43 in core subunit ND1. The substitution of the glycine for an arginine would be disruptive to interactions in this region.

The p.Arg37Ser mutation was identified in a boy who was diagnosed with generalized hypotonia, myoclonic epilepsy, and cerebellar atrophy [43]. His clinical evolution stabilized, and he was still living at 10 years old. His mother was a heterozygous carrier for the p.Arg37Ser mutation. BN gel

electrophoresis demonstrated a low level of complex I formation, and muscle cells showed 15–30% of the normal level of complex I activity. The loss of a positive charge may disrupt the local structure or affect the phosphorylation of the subunit at Ser55. Arg37 contacts two residues from supernumerary subunit NDUFA8: it forms a hydrogen bond with Ser22 and is in contact with Gln92. It is also located near Asp90 and Gly93 from NDUFA8. Furthermore, according to mouse active (PDB id=6g2j) and inactive (PDB id = 6g72) structures [37], the residue may engage in different intermolecular interactions depending on the state of complex I, although this was not seen when comparing open and closed structures from the ovine enzyme [38].

The p.Gly32Arg mutation has been well-studied. Three separate clinical studies have been performed on this mutation. The first study [44] focused on two male patients who were maternal cousins from the same healthy non-consanguineous family. One patient experienced deterioration of motor and verbal skills at age four and an unsteady gait, retinitis pigmentosa, and cerebellar atrophy at age seven. His cousin developed an ataxia and proximal muscle weakness at age five and myoclonic seizures and bilateral sensorineural hearing loss at age 10. Both probands had mothers who were heterozygous carriers. Both men had lived into their thirties when the study was conducted. A six-year-old boy was also found to carry the p.Gly32Arg mutation [45]. Though the age of onset was not recorded, he experienced episodic neuroregression and encephalopathy but was seizure-free. His family pedigree was unknown. This finding is in concordance with the fact that the p.Gly32Arg mutation is not as deleterious as the p.Gly8Arg mutation. In 2011, a female patient with a heterozygous p.Gly32Arg mutation was identified [46]. She did not show signs of deficiency until 11 months of age, when she developed an X-linked respiratory chain deficiency in skeletal muscle tissue. She developed frequent upper airway infections and experienced somnolence and muscle hypotonia during these illnesses. However, at age five, she showed nearly normal psychomotor development. Though there was only a 25% expression of the normal allele in her skeletal muscle tissue, she showed a relatively mild clinical phenotype. It is hypothesized that X-inactivation due to selection advantage may have favored the expression of the normal allele. Neither her mother nor her father had the p.Gly32Arg mutation, suggesting that the mutation arose spontaneously. The fact that all four patients carrying the p.Gly32Arg mutation survived into adulthood may be attributed to the fact that Gly32 in NDUFA1 lies in the membrane near the cytoplasmic side and is not located at the interfaces of NDUFA1 and other subunits.

In 2014, a patient diagnosed with Leigh syndrome and mitochondrial respiratory chain disorder (MRCD) was discovered to have the p.Pro19Ser mutation [47]. The age of onset was five years old, but before 10 months of age, the boy had already experienced hypotonia, nystagmus, generalized epilepsy, and high blood lactate and pyruvate levels. Pro19 in NDUFA1 is a highly conserved in vertebrates and lies in the N-terminal membrane spanning helix (Pro7–Arg28). The residue contacts Leu9, Pro12, and Met91 of core subunit ND1 in the membrane region of the bilayer, and the substitution of the polar serine for proline could be deleterious.

2.3.2. NDUFA3

The NDUFA3 gene is located on chromosome 19. The NDUFA3 protein is a 9.3 kDa protein that consists of 83 amino acids. It is a single-pass protein located in the inner mitochondrial membrane. It lies in the junction between the membrane and matrix arms of complex I and contains a kinked transmembrane alpha-helix domain that spans residues 17–35 and 37–49. During processing, the initiator methionine is cleaved, and Ala at position 2 is N-acetylated by analogy with the bovine enzyme [48]. NDUFA3 contacts core subunits ND1, ND3, and NDUFS8. Additionally, it contacts supernumerary subunits NDUFA8 and NDUFA13 (see Figure 7). In a knockout strain of cultured human cells, the loss of NDUFA3 resulted in a reduced expression of complex I and migrated as a smaller-than-normal-sized complex in BN gel electrophoresis [11]. An earlier study demonstrated the role of NDUFA3 in the assembly of the Q-module of complex I [49].

Though no clinical mutations in *NDUFA3* have been identified yet, it has been shown that the full deletion of the *NDUFA3* gene, along with other genes on chromosome 19, results in retinitis pigmentosa (RP), a disorder caused by photoreceptor cell degeneration. It is characterized by vision loss following night-blindness. In 2006, a family was identified as carrying a 30 kb deletion in chromosome 19 [50]. The family members afflicted with RP contained at least one copy of chromosome 19 that lacked a region containing genes *NDUFA3*, *TFPT*, *PRP31*, and the *OSCAR* promoter. The ages of onset of the RP symptoms varied from 3 to 30 years of age. Surprisingly, the patients were not afflicted by any disease other than RP. From this study, the absence of symptoms relating to complex I deficiencies suggests that only one copy of the gene for *NDUFA3* may be sufficient for complex I activity.

Similarly, in 2011, another family was discovered to harbor a 112 kb deletion that encompassed the *PRP31* gene and five of its upstream genes: *TFPT*, *OSCAR*, *NDUFA3*, *TARM-1*, and *VSTM-1* [51]. Only one family member, a 33-year-old female, was diagnosed with RP. Once again, the RP patient showed no signs of complex I deficiency, and the authors suggested that one copy of *NDUFA3* was sufficient for complex I activity.

2.3.3. *NDUFA13*

The *NDUFA13* gene is located on chromosome 19. *NDUFA13* is a unique complex I subunit because it functions as a cell-death regulatory protein, *GRIM-19*, as well. It has been suggested that *GRIM-19* regulates *STAT3*, a signal transducer and transcription activator, and is involved in interferon- β - and retinoic acid-induced cancer cell death [52]. For this reason, although *NDUFA13* is mainly found in the inner mitochondrial membrane as a single-pass membrane protein, it can also be translocated to the nucleus to serve its apoptotic purpose. *NDUFA13* is a 16 kDa protein synthesized as a 144-residue polypeptide, but the initiator methionine is removed during processing. It has a transmembrane alpha helix that spans residues 30–51 (see Figure 7). *NDUFA13* has a longer alternate isoform that exists as a 222-residue polypeptide. *NDUFA13* shares a large contact surface area with core subunits *ND1*, *ND6*, and *NDUFS2*, *NDUFS5*, *NDUFA3*, and *NDUFA8*. It weakly contacts *ND3*, *NDUFS8*, and supernumerary subunits *NDUFA1* and *NDUFA7*. In a knockout line of cultured human cells, the loss of *NDUFA13* resulted in a reduced expression of complex I, and it migrated as a smaller-than-normal-sized complex in BN gel electrophoresis [11]. Furthermore, when homozygous *GRIM-19*-deficient mice were generated, embryonic lethality resulted [53].

At least two diseases have been associated with mutations in *NDUFA13*. In 2015, a pair of sisters were discovered to harbor the first pathogenic germinal mutation identified in the short isoform one of *NDUFA13*, p.Arg57His (c.170 G > A in exon 2) [54]. Both sisters were homozygous for the mutation and presented with early onset hypotonia, dyskinesia, auditory neuropathy, and severe optic neuropathy. The patients were siblings who had been born from a consanguineous marriage. Their parents and elder sister were heterozygous for the p.Arg57His mutation and asymptomatic. The sisters were still living in 2015, the elder sister being 12 years old at the time. Though the sisters experienced early-onset neurological symptoms, the progression of neurological symptoms has been slow. Arg57 is a highly conserved residue across species. Upon biochemical analysis, *NDUFA13* expression was reduced by a mean of 70% in the two sisters. Additionally, *NDUFA9* and *NDUFB8* expression was reduced by 95% and 90%, respectively. Due to the absence of intermediary complex I structures in the BN gel electrophoresis, the p.Arg57His mutation was concluded to cause major instability in *NDUFA13* and prevent complex I assembly. It was suggested that the varied clinical symptoms in these sisters could be attributed to the dual function of *NDUFA13/GRIM-19*.

Somatic mutations in the long isoform two of *NDUFA13* have been implicated in Hurthle cell thyroid tumors. Isoform two of *NDUFA13* has 222 amino acids instead of 144 amino acids like isoform one. In 2005, four unrelated patients with Hurthle cell thyroid carcinomas were found to harbor four different heterozygous missense mutations that affected the N-terminal sequence of *NDUFA13* [55]. Because the patients' transcripts arose from an alternate reading frame and coded for isoform two, a structural analysis of these mutations cannot be performed.

2.4. LYR Family Subunits

Two subunits from the LYR family are described next: NDUFA6 and NDUFB9, as shown in Figure 8. These proteins have the LYR sequence motif, which helps them bind to one of the two acyl carrier proteins, NDUFAB1. They also bind the substrate 4'-phosphopantethiene for the acyl carrier proteins. They are found on the matrix side of the membrane arm.

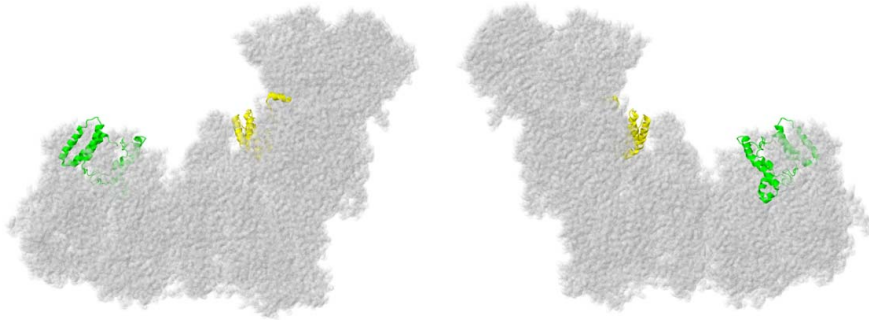


Figure 8. Location of NDUFA6 and NDUFB9. Most of Complex I is colored gray. They are shown in ribbons, with NDUFA6 colored yellow and NDUFB9 colored lime. The two views are rotated 180° relative to each other. The structure is from PDB id = 5xtf [9].

2.4.1. NDUFA6

The gene for NDUFA6 is located on chromosome 22. The encoded protein has 128 amino acids, with a mass of about 15.1 kDa. This subunit is found on the matrix arm. Its tertiary structure resembles a four-helix bundle in which the fourth helix is pulled away from the other three (see Figure 9a). Helices 1 and 2 interact with one of the two NDUFAB1 subunits, which are also known as the acyl carrier proteins and presumed to be involved in fatty acid biosynthesis. The short C-terminal helix interacts with core subunit NDUF51, while core subunit NDUF53 and supernumerary subunit NDUFA9 interact with both the N-terminal helices and the extended C-terminus. Thus, NDUFA6 bridges the N- and Q-modules. NDUFA6 is a member of the LYR family [56], whose members are known to interact with large mitochondrial complexes. Leu35 of the LYR motif fits inside the bundle of three helices, while Tyr36 and Arg37 contact NDUFAB1. In knockout cell lines, the assembly of full-size complex I was diminished, as seen in BN gel electrophoresis, and the levels of all subunits associated with the N-module of the peripheral arm were decreased by two-fold [11].

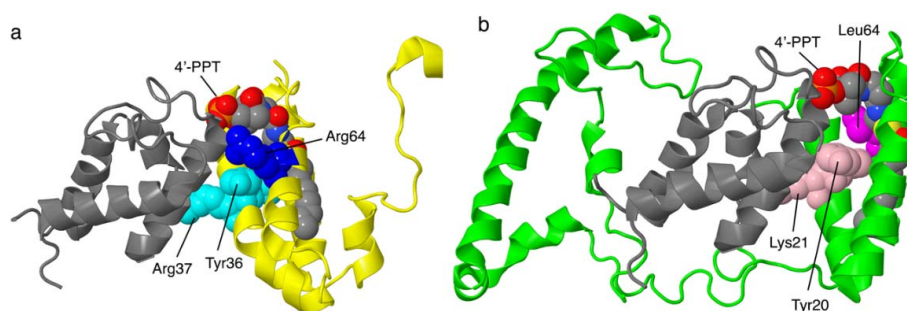


Figure 9. Structural features of the LYR proteins, NDUFA6 and NDUFB9. (a) NDUFA6 (colored yellow) is shown with its partner NDUFAB1 (colored gray), known as one of the two acyl carrier proteins (ACPs). NDUFA6 binds the 4'-phosphopantethiene (4'-PPT) analog substrate for the ACP. Tyr36 and Arg37 of the LYR motif are shown in space-filling and colored cyan. The site of the Arg64Pro mutation is shown in space-filling and colored blue. (b) NDUFB9 (colored lime) is shown with its partner NDUFAB1 (colored gray), the other one of the two ACPs. NDUFB9 binds the 4'-PPT analog substrate for the ACP. Tyr20 and Lys21 of the LYR motif are shown in space-filling and colored pink. The site of the Arg64Pro mutation is shown in space-filling and colored magenta.

Mutations in *NDUFA6* have been described for four individuals, though only recently [57]. The most severe of the illnesses occurred in an individual with compound heterozygous mutations: p.Arg64Pro and c.265 G > T, a nonsense mutation at Glu89. An analysis of tissue demonstrated almost no fully assembled complex I and almost no in-gel activity in BN gel electrophoresis. This individual died after about two days. Tissue from two other individuals was analyzed and showed low levels of assembled complex I with activity in BN gel electrophoresis. Residue Arg64 is ion-paired with Asp111 of *NDUFAB1* and is near Asp92 of *NDUFAB1* and Tyr36 of the LYR motif of *NDUFA6*. The adjacent Ser112 of *NDUFAB1* is part of the catalytic site of the acyl carrier enzyme and is bound by a substrate in the human structure (PDB id = 5xtf) [9].

The second individual was homozygous for a two-nucleotide deletion at Glu111, c.331_332del, resulting in 35 altered amino acids before a stop codon appeared [57]. This transcript was found at normal levels, thus indicating the lack of nonsense-mediated decay, presumably because it occurs in the last of three exons near the normal termination codon. If this transcript was translated, the protein would lack the C-terminal alpha-helix that interacts with core subunit *NDUFS1*. The third individual was homozygous for a c.3 G > A, substitution in the start codon. It was suggested that a transcript starting from an upstream Met codon, as predicted by another isoform, might have permitted some expression of a functional protein. Both individuals showed low levels of fully assembled complex I with activity, and their outcomes were only somewhat better than the first, with abnormal white matter in the brain, a loss of movement and vision, and seizures. The fourth individual was compound heterozygous with two frameshift mutations: p.Met104Cysfs*35 and p.Leu119Tyrfs*20. This child died in infancy, and no biochemical analyses were performed.

2.4.2. *NDUFB9*

The gene for *NDUFB9* is located on chromosome 8. It is encoded as a protein of 179 amino acids, with a mass of about 21.8 kDa. The N-terminal Met is likely removed, and other possible modifications include the acetylation of Ala2 [58] and the phosphorylation of Ser85 [59]. *NDUFB9* is a peripheral protein that is bound to the matrix side of core subunit ND5, with significant contacts to *NDUFAB1*, one of the acyl carrier proteins (see Figure 9b), and to *NDUFB3*, *NDUFB4*, *NDUFB5*, and *NDUFB6*. The protein is U-shaped, with each arm consisting of a three-helix bundle and pointing away from ND5. *NDUFB9*, along with *NDUFA6*, is a member of the LYR protein family, characterized by a Leu–Tyr–Arg motif near the N-terminus. In the case of *NDUFB9*, the motif is Leu19–Tyr20–Lys21, and it is found in the N-terminal alpha-helix. The acyl carrier protein *NDUFAB1* fits between the two helical domains and binds to Tyr20 and Lys21 of this motif. In a cell line in which *NDUFB9* was knocked out, complex I failed to assemble [11].

In 2012, a large-scale mutation-screening identified two patients who carried *NDUFB9* mutations [60]. One patient, who presented with lactic acidemia and muscular hypotonia, was homozygous for the missense p.Leu64Pro. This residue is found in the third helix and packs against the first helix in the N-terminal three-helix bundle. Complex I activity was about 39% of the normal activity. After lentiviral rescue with a wild-type *NDUFB9* gene, both complex I activity and levels of other complex I subunits were restored to normal levels. A second individual was found to carry the heterozygous mutation Arg47Leu, but this mutation was not found in a sibling with similar symptoms and could not be rescued by the complementation with a wild-type *NDUFB9* gene. Therefore, it was concluded that illness was due to another gene.

2.5. *Subunits of the Twin C–X₉–C Family in the IMS*

Complex I has four proteins that are members of the twin C–X₉–C family and three with identified mutations that are described next: *NDUFS5*, *NDUFA8*, and *NDUFB10*, as shown in Figure 10. All are found in the IMS. They are substrates of the IMS oxidoreductase *CHCHD4*, also known as hMia40, which is essential for the export of FeS clusters from the mitochondrial matrix [61].

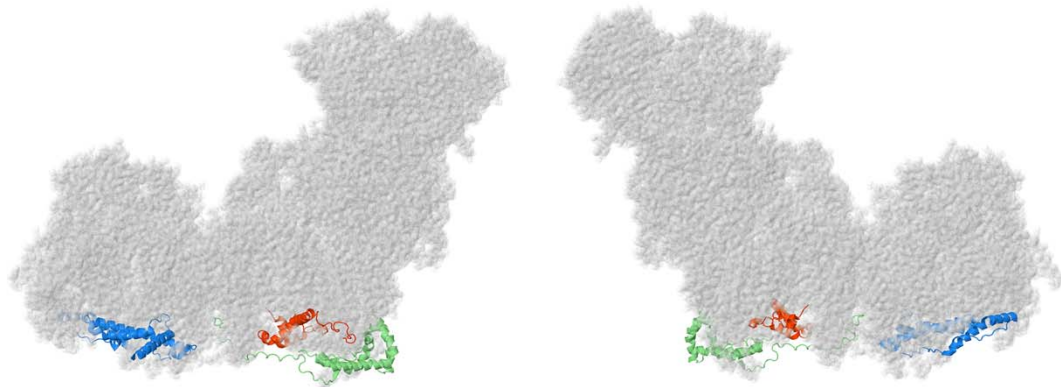


Figure 10. Location of NDUF55, NDUFA8, and NDUFB10. Most of Complex I is colored gray. NDUF55, NDUFA8, and NDUFB10 are shown as ribbons, with NDUF55 colored red, NDUFA8 colored green, and NDUFB10 colored blue. They are found facing the intermembrane space. The two views are rotated 180° relative to each other. The structure is from PDB id = 5xtf [9].

2.5.1. NDUF55

The gene for NDUF55 is found on chromosome 1. The protein contains 106 amino acids, with a mass of 12.5 kDa. In cultured human cell lines, the knockout of NDUF55 completely eliminates the assembly of complex I [11]. Though this protein was initially identified with the iron–sulfur protein fraction of bovine complex I, it is now known to be localized to the IMS and does not contact any proteins with FeS clusters. NDUF55 is bound to the surface of the membrane and lacks transmembrane helices. It has a limited tertiary structure and includes a helix–coil–helix with two C–X₉–C sequence motifs that form two disulfides between the two alpha-helices at residue pairs 33–66 and 43–56. Another alpha-helix is formed by residues 70–86, but the rest of the protein has little regular secondary structure. The N-terminal domain (2–30) primarily contacts core subunits ND2 and ND4L, and supernumerary subunit NDUFB5, while the C-terminal domain (70–106) primarily contacts core subunit ND6, and NDUFB13.

One missense mutation in NDUF55 has been reported in a compound heterozygous individual, p.Pro96Ser [22]. This individual has a second mutation in core subunit NDUF58, p.Arg2Cys, of unknown consequence. These mutations were identified in a cohort of 103 patients with complex I deficiency. Pro96 in NDUF55 is highly conserved among vertebrates and appears as the third Pro in an unusual sequence motif of Pro-Pro-Pro-His-His near the C-terminus. In human complex I (Video S1), Pro96 contacts Thr129 of NDUFA13. There is a nearby ion pair between Lys101 of NDUF55 and Glu89 of NDUFA13. Somewhat different interactions can be seen in the mouse complex I in both active and inactive conformations [37]. Therefore, it seems likely that this missense mutation would disrupt local structure, but it is not clear that it would be deleterious to complex I assembly. The mutation in NDUF58, p. Arg2Cys, should also be considered, as it might be defective, for example, in translation initiation.

2.5.2. NDUFA8

The gene for NDUFA8 is located on chromosome 9. The protein has 172 amino acids with a mass of about 20 kDa, and the initial methionine is cleaved. NDUFA8 is found in the IMS and has large contact surfaces with supernumerary subunits NDUFA13, NDUFA3, and NDUFA1, and it is near core subunit ND1. It has two pairs of alpha helices, each having two pairs of Cys residues that likely form disulfides (see Figure 11). They have characteristic spacings of either 9 or 11 amino acids between the cysteine residues. In the human structure (PDB id = 5xtf), not all disulfides are shown as formed, but it is likely that they do form in the IMS: C36–C66, C46–C56, C78–C110, and C88–C100. In the mouse structures (PDB id = 6g7j and 6g72), they are shown as disulfides [37]. The N-terminal residues 2–22 are extended and interact with NDUFA13, NDUF55, and NDUFA1. The C-terminal region extends from residues 113 to 172 without a regular secondary structure. It makes an interesting junction with

NDUFC2 and NDUFB5 in a conserved multi-centered ion pair including Arg166 of NDUF8, Asp86 of NDUFC2, and Glu153 of NDUFB5.

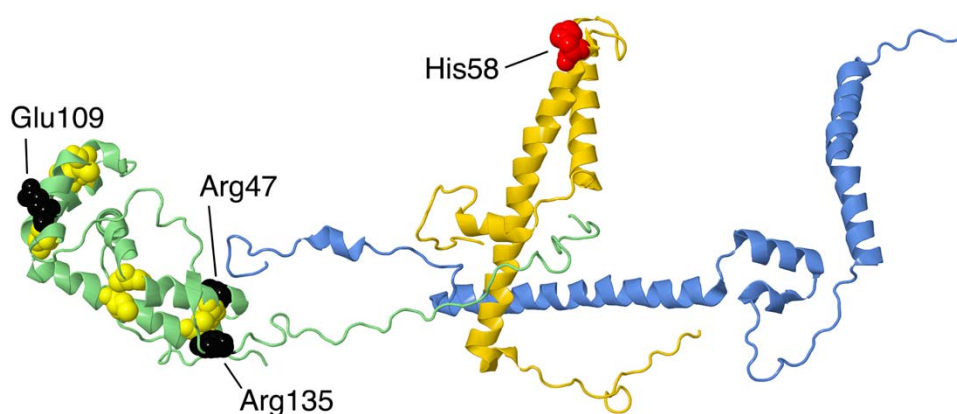


Figure 11. Structural features of NDUF8, a member of the twin C-X₉-C. The proteins are portrayed in ribbons. NDUF8 is colored green, NDUFC2 is colored gold, and NDUFB5 is colored blue. In NDUF8, the eight Cys residues that form disulfide bonds are shown in space-filling and colored yellow. The sites of three mutations, Arg47Cys, Glu109Lys, and Arg135Gln, are shown in space-filling and colored black. NDUFB5 makes contact near Arg47, and both NDUFB5 and NDUF8 contact NDUFC2. The site of one mutation in NDUFC2, His58Leu, is shown in space-filling and colored red.

At least three missense mutations have been described in NDUF8. The p.Arg47Cys mutation was described in a patient that was homozygous, having received a mutant allele from each parent, who were heterozygous and asymptomatic [62]. This individual presented with developmental delay and epilepsy, and they were bed-ridden by age 26. Fibroblasts showed reduced levels of NDUF8, as well as other complex I subunits, and reduced complex I activity. The mutation creates an additional Cys residue next to Cys46, which possibly leads to incorrect disulfide bond formation. In addition, Arg47 has multiple interactions within the NDUF8 subunit, as well as with NDUF13 and NDUFB5, which would be disrupted in the mutant p.Arg47Cys.

The second individual was compound heterozygous with p.Glu109Lys in NDUF8 and p.Ala224Val in core subunit NDUF2 [63]. The transcript for the mutant NDUF8 subunit was not found, and so the mutation might have affected its stability. Ala224 in the core subunit NDUF2 is highly conserved and is found near Cys 153 and 160 of a FeS cluster, and so this substitution might also have been deleterious. The infant showed neonatal hypotonia and died at two months.

The third individual was found in a screen of patients with reduced complex I levels [22]. Two potential deleterious mutations were discovered. First was a heterozygous mutation p.Arg135Gln in NDUF8, and second was a homozygous mutation p.Leu229Pro in C20orf, a spindle assembly factor for microtubules. Since the individual showed decreased levels of complex I activity, the NDUF8 mutation was implicated, but it was not clear whether the spindle assembly factor had any impact on complex I levels. Arg135 is highly conserved, and it makes an ion pair with Glu59, also a conserved amino acid of NDUF8. Glu59 is near the disulfide formed by Cys46 and Cys56. This ion pair is exposed on the IMS and does not contact any other subunits.

2.5.3. NDUFB10

The gene for NDUFB10 is located on chromosome 16. The NDUFB10 protein has 172 amino acids and a mass of 20.8 kDa. It lacks a mitochondrial import sequence. It is one of four IMS proteins found in complex I, and all have four cysteine residues that form two disulfide bonds. In NDUFB10, two cysteine residues, Cys78 and Cys107, are found near the ends of a long alpha-helix (M77–E111). These cysteine residues form disulfides with nearby cysteines, connected to the long helix by loops of 6–11 residues. Cys78 forms its disulfide with Cys71, and Cys107 forms its disulfide with Cys119. In addition, there is

another cysteine, Cys145, conserved among vertebrates, and of unknown significance. The import of NDUFB10 is dependent upon its oxidation by CHCHD4/Mia40 and its formation of correct disulfide bonds [64]. NDUFB10 also contains three additional alpha-helices, two C-terminal ones and one N-terminal one. These extensions allow it to contact many subunits in the vicinity of ND4 and ND5. It primarily contacts NDUFB5, NDUFB6, and NDUFB11, with lesser contact to ND4, ND5, and NDUFC2. In an analysis of cultured cells, the knockout of NDUFB10 resulted in a loss of complex I assembly and, especially, the ND4/5 modules.

One individual with mutations in NDUFB10 was studied [64]. She was compound heterozygous with a paternally inherited termination codon at Glu70 of NDUFB10 and a maternally inherited p.Cys107Ser substitution. This infant presented with lactic acidosis and cardiomyopathy and survived for only about one day. An analysis revealed reduced levels of complex I activity, reduced levels of in-gel assays of complex I in BN gels, and the presence of the Cys107Ser protein in the cytosol. The transcript of the nonsense allele was not found by RT-PCR. Cys107 is not near any other subunits, the closest being NDUFB5, NDUFB6, and NDUFB11. Given the healthy status of the parents, it appears that the Cys107S mutant does not have a dominant negative phenotype. Rather, it primarily does not enter the mitochondrion, and if another allele is available, complex I can assemble without difficulty.

2.6. Subunits of the ND2-Module

The next two subunits to be described are members of the ND2-module: NDUFA10 and NDUFC2, as shown in Figure 12. NDUFA10 is a globular protein bound on the matrix side, while NDUFC2 is a membrane protein. These two subunits have contacts on the matrix side of the membrane.

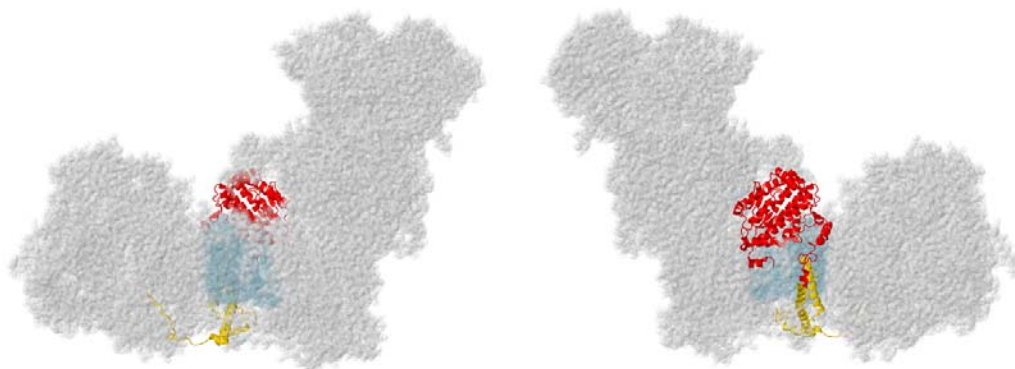


Figure 12. Location of NDUFA10 and NDUFC2. Most of Complex I is colored gray. Core subunits in the ND2-module are colored light blue (ND2). NDUFA10 and NDUFC2 are shown in ribbons, with NDUFA10 colored red and NDUFC2 colored gold. The two views are rotated 180° relative to each other. The structure is from PDB id = 5xttd [9].

2.6.1. NDUFA10

The gene for NDUFA10 is found on chromosome 2. The encoded protein has 355 amino acids and a mass of about 40.8 kDa, with 35 N-terminal amino acids cleaved upon entry to the mitochondrion. Structurally, NDUFA10 has been identified as a member of the deoxynucleoside kinase (dNK) family [8], with the dNK domain (also known as the PF01712 family in the Pfam database), although it is unlikely to be an active enzyme. The protein is compact with a four-stranded parallel beta-sheet surrounded by numerous alpha-helices (see Figure 13). The human structure (PDB id = 5xttd) [9] did not reveal any ligands, but adenosine nucleotides have been found at the C-terminal ends of the beta-sheet in a pocket surrounded by alpha-helices in the mouse (PDB id-6g2j) [37] and sheep [65] structures. NDUFA10 is found on the matrix side of the membrane and primarily interacts with core subunits ND2 and NDUFS2. These interactions with the extended N-terminus of NDUFS2, and the matrix-side peripheral helices of ND2 are all centered over the C-terminal sector of ND2, which contains the broken helix that

is part of the proton translocation pathway. NDUFA10 also makes limited contact with the N-terminal regions of NDUFC1, NDUFC2, and NDUFB11 on the matrix side.

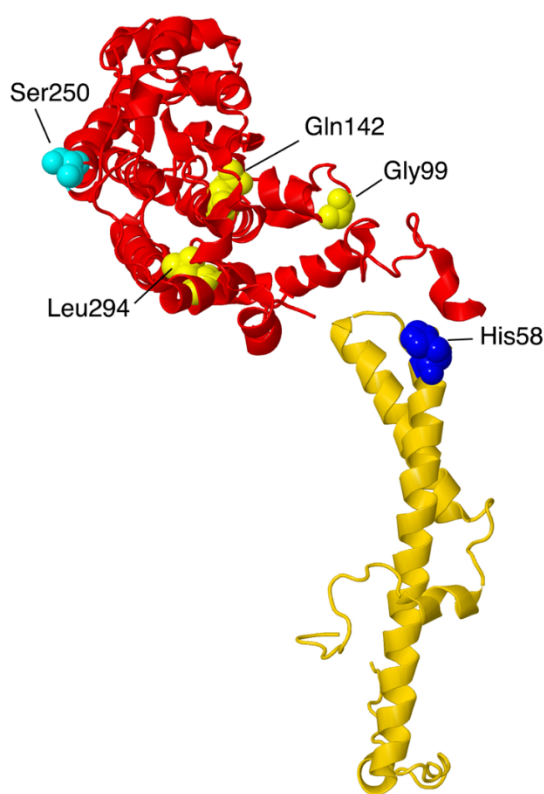


Figure 13. Structural features of two subunits from the ND2-module. NDUFA10, colored red, and NDUFC2, colored gold, are portrayed in ribbons. The site of one mutation, His48Leu in NDUFC2, is shown in space-filling and colored blue. The sites of three mutations in NDUFA10—Gly99Glu, Gln142Arg, and Leu294Pro—are shown in space-filling and colored yellow. The site of phosphorylation by PINK1, Ser250, is shown in space-filling and colored cyan. NDUFA10 likely binds an adenosine nucleoside, not shown.

In knockout human cell line BN gel electrophoresis, only faint bands of complexes containing primarily membrane subunits are seen [11]. Perhaps because of its contacts with a Q-module, core subunit NDUFS2, and a core membrane subunit ND2, along with subunits at the interfaces of ND2/ND4 (NDUFC1 and NDUFC2) and of ND4/ND5 (NDUFB11), it appears to be a key subunit in assembly. Residue Ser250, which is found on the matrix surface, was reported to be phosphorylated by the kinase PINK1 [66], a kinase that is known to be imported into the mitochondrial matrix space [66,67]. This is a conserved amino acid, and phosphorylation appears to be important for complex I activity.

Three reports of clinical mutations in NDUFA10 have been described. The first patient had compound heterozygous mutations including one allele in which the start codon was changed to GTG, and a second in which p.Gln142Arg occurred [68]. This individual showed developmental problems at 10 months and was eventually diagnosed with Leigh syndrome. It is likely that the first mutation would significantly impair the translation unless an alternative start codon were available. The second mutation is found in the interior of the protein, and so the Arg likely disrupts the packing of the protein, especially because of the positive charge.

The second patient was a boy with Leigh syndrome who, in a screen, was determined to have compound heterozygous mutations: p.Leu294Pro and c.383_384insTAA (p.Ser218delinsI5) [27]. These were found to be inherited from the father and mother, respectively. The latter mutation would likely lead to a degraded mRNA or protein. The former mutation p.Leu294Pro resides on an alpha-helix

at an interior location. The substitution of Leu by Pro might be disruptive, as this alpha-helix contacts two distinct regions of ND2. The immunoblotting of fibroblasts showed reduced levels of NDUFA10.

The third patient was initially diagnosed as having a nonlethal infantile mitochondrial disorder, and the mutation was discovered in a screen [69]. This individual developed brain lesions and was considered to have Leigh syndrome. His parents were third cousins, and DNA sequencing revealed him to be homozygous for p.Gly99Glu, while both parents were heterozygous for the same mutation. A deltoid muscle biopsy showed significant reduction of complex I activity (about 40–70% of normal levels). This amino acid is packed against the N-terminal amino acids of NDUFC1, and so the introduction of the negatively-charged Glu could disrupt the assembly of the membrane arm.

2.6.2. NDUFC2

The gene for NDUFC2 is located on chromosome 11. The NDUFC2 protein is 119 amino acids, with a mass of 14.2 kDa. It is a double-pass transmembrane protein that contacts core subunit ND2, with both termini on the IMS side of the membrane. From the N-terminus, the protein enters the membrane in a non-helical stretch from Ser 19 to Arg29, and then residues 29–47 are alpha-helical. The second crossing of the membrane is alpha-helical from residues 56–97, extending into the IMS. NDUFC2 also contacts NDUFC1, NDUFB5, and NDUFA8 (see Figure 11), and it is part of the ND2 assembly module. NDUFC1 has a special role in sealing NDUFC2 from the lipid bilayer, with its single transmembrane helix parallel to the long helix of NDUFC2 and very limited contact to any other subunits in the membrane. The extension of the long alpha-helix into the IMS, residues 84–97, is the region that contacts NDUFA8 and NDUFB5 (see Figure 11). On the matrix side it contacts NDUFA10 (see Figure 13).

Mutations of NDUFC2 in three patients from two families were reported in 2020 [70]. The patients presented with symptoms of Leigh syndrome and reduced complex I activity but had different outcomes. In both families, the parents were healthy consanguineous first cousins and were heterozygous for the mutations. In one family, the mutation was a deletion of 22 base pairs near the C-terminus at residue His116, p.His116_Arg119delins21. This would cause a frame shift and elimination of the normal stop codon at position 120. It was not clear how long the new reading frame would be. The transcript of this gene was identified at low levels, but the immunoblotting of fibroblast samples was negative, suggesting the degradation of the protein or a lack of expression. Little or no complex I was seen in native gels. Complexome profiling found evidence of Q-module assembly and ND4-module assembly but little else of complex I subunits. The daughter had no seizures and survived until at least age six, while her brother passed away at three years of age.

In the second family, the mutation was p.His58Leu. This patient had normal transcript levels but only slightly more evidence of complex I assembly. His58 is found on the matrix side of the membrane and starts the long helix that crosses the membrane. It sits between Val44 of NDUFC1 and Trp353 of NDUFA10 and is very near the phosphate group of a bound lipid. In the mouse structure (PDB id = 6g2j), the corresponding His59 is even more tightly packed between NDUFC1 and NDUFA10 residues, suggesting that a Leu substitution could be deleterious. This child had impaired growth and seizures and passed away at eight months.

NDUFC2 has a unique position in complex I. It is embedded in the membrane near the ND2-ND4 junction but contacts two subunits that are embedded at distant sites, with long extension to NDUFC2: NDUFA8 is found at the “heel” of the complex I boot, in the IMS, while NDUFB5 is found at the junction of ND4-ND5. Interactions in this network of subunits appear to be essential for complex I assembly.

2.7. Subunits Form the ND4-Module

Two subunits from the ND4-module are described next: NDUFA11 and NDUFB11, as shown in Figure 14. Both are membrane proteins but are found on alternate sides of ND4 and do not contact each other.

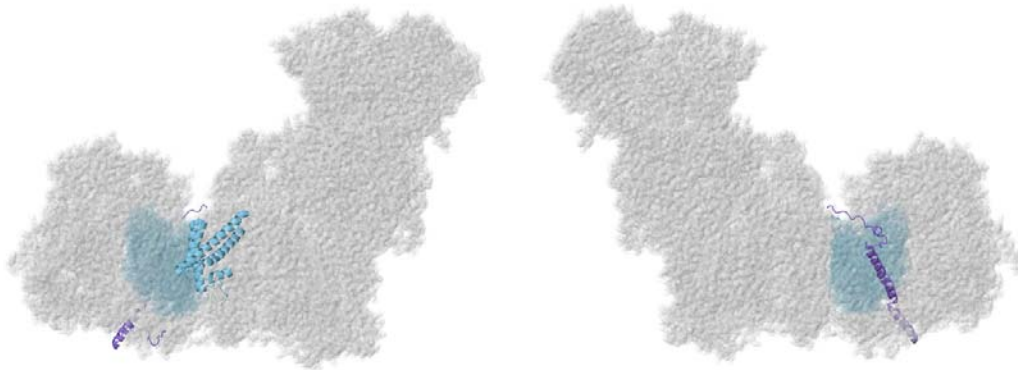


Figure 14. Location of NDUFA11 and NDUFB11. Most of Complex I is colored gray. Core subunits in the ND4-module are colored light blue (ND4). NDUFA11 and NDUFB11 are shown in ribbons, NDUFA11 colored blue and NDUFB11 colored purple. The two views are rotated 180° relative to each other. The structure is from PDB id = 5xtf [9].

2.7.1. NDUFA11

The NDUFA11 gene is located on chromosome 19. NDUFA11 is a 14.7 kDa protein with four transmembrane helices and little exposure outside the membrane. NDUFA11 has two isoforms. The first isoform contains 140 amino acids after the cleavage of the initiator methionine and is N-acetylated at Ser2. It consists of five alpha helices: a short helix is found on the IMS side, followed by membrane spanning helices 3–11, 17–45, 48–82, 87–106, and 107–137. NDUFA11 contacts the core subunits ND2, ND4, and ND5, as well as the supernumerary subunit NDUFB5. The second isoform is rare and contains 228 amino acids. In a previous study, it was found that the knockdown of NDUFA11 by RNA interference (siRNA) in human cell culture led to partially assembled subcomplexes visualized by blue native gels [71]. It was concluded that NDUFA11 acts like an assembly factor in complex I. Furthermore, it was shown that, in a knockout strain of cultured human cells, the loss of NDUFA11 resulted in no expression of complex I in BN gel electrophoresis [11].

The first identified NDUFA11 clinical mutation, a G to A mutation at the exon-intron junction (exon 1-IVS1) donor splice site, c.99 + 5 G > A, was identified in 2008 [72]. Six patients from three unrelated families were products of consanguineous marriages and were found to be homozygous for the mutation. They were clinically affected with either fatal infantile lactic acidemia or encephalomyopathy. Though heterozygotes were identified in two families, all family members besides the patients were healthy. The patient from Family A and the two patients from Family B had similar clinical presentations. They all developed severe metabolic acidosis and hyperlactatemia within 10–24 h of age. All three patients from Families A and B died from acidosis within 6–40 days of age. In Family C, three patients were homozygous for this splice mutation. They experienced slow psychomotor development, hypertrophy of myocardial walls, acidosis, and generalized brain atrophy. Two out of the three patients from Family C died at 18 months and four years of age, but the third patient from this family lived until at least six months of age. The presence of both wild-type and mutant mRNA transcripts in the patients' fibroblasts indicates that the varied clinical presentation may have been caused by variable splicing that produced variable mutant/wild-type transcript ratios. They suggested that normal splicing would have produced a protein that was responsible for the detected activity, while alternative splicing would have yielded a nonfunctional protein.

In 2019, a two more NDUFA11 clinical mutations, p.Ala132Pro and p.Thr106Ile, were found in isoform two of NDUFA11 [73]. These mutations were found simultaneously in a compound heterozygote patient with mitochondrial myopathy. The patient developed late-onset symptoms of a neuromuscular disorder, bilateral hearing loss, saccadic eye movements, and proximal leg weakness. The good health of the patient's offspring and the absence of neuromuscular disease in the patient's family history suggested that these two mutations were spontaneous. In contrast to isoform one, NDUFA11 isoform two is rare and appears in skeletal muscle. Thus, a mutation in isoform two may

only lead to mild muscular impairment. The structure of this rare isoform of NDUFA11 has not yet been determined, so we cannot confirm that the mutation causes a structural disruption within this subunit.

2.7.2. NDUFB11

Similar to NDUFA1, the NDUFB11 gene is located on the X chromosome. It has three exons. It is synthesized as a 153 amino acid protein with a mass of about 17.3 kDa, but its first 29 amino acids serve as a transit peptide that is cleaved off during processing. NDUFB11 is a single-pass membrane protein with the N-terminus on the matrix side and one helix that spans residues 80–107, followed by another helix into the IMS of residues 115–132 (see Figure 15). Its N- and C-termini are extended and point in the same direction, like a letter C. It has a large contact surface with core subunit ND4 and NDUFB10, but it also weakly contacts core subunit NDUFS2, NDUFA10, NDUFB5, and NDUF2. In a knockout line of cultured human cells, the loss of NDUFB11 resulted in almost no assembly of complex I [11]. Furthermore, NDUFB11 short-hairpin RNA (shRNA) knockdown in HeLa cells on complex I resulted in decreased expression of subunit NDUF8, the failure of the membrane arm and the holocomplex to assemble, and decreased oxygen consumption. Moreover, NDUFB11 reduction was associated with decreased cell growth and increased apoptosis. For this reason, cell death caused by NDUFB11 mutations may be responsible for embryonic lethality in males and developmental defects in female patients [74].

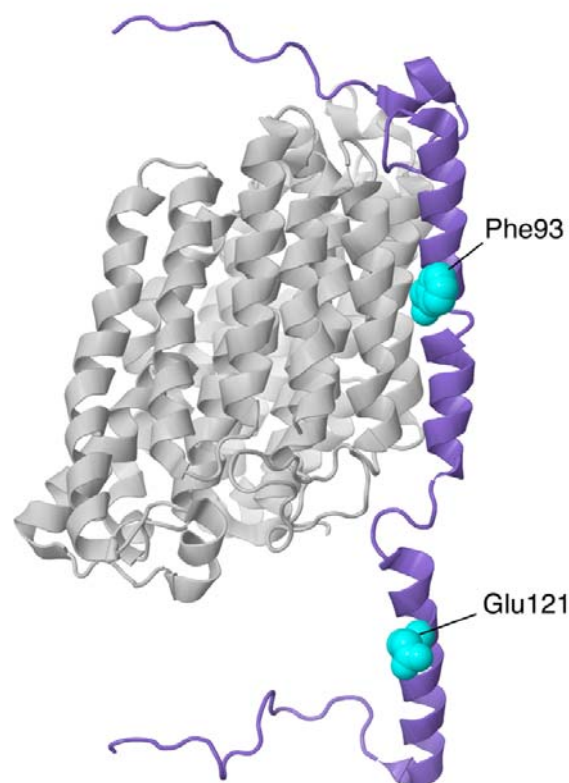


Figure 15. Structural features of NDUFB11 of the ND4-module. The proteins are shown as ribbons, with NDUFB11 colored purple and ND4 colored light gray. The sites of two mutations are shown in space-filling, Phe93 (a deletion) and Glu121Lys (in the IMS).

Two studies conducted in 2015 found that nonsense mutations in NDUFB11 are associated with histiocytoid cardiomyopathy (histiocytoid CM) and microphthalmia with linear skin defects syndrome. Clinical characteristics of histiocytoid CM, an arrhythmogenic disorder, are incessant ventricular tachycardia, cardiomegaly, and sudden death within the first two years of life. The study did not

expand on specific patient symptoms or survival. In the first study, two unrelated female patients with histiocytoid CM were identified to harbor de novo nonsense mutations in NDUFA11 [74]. The first patient had a mutation that changed Tyr108 to a stop codon (c. 324 T > G). The second patient had a mutation that changed Trp85 to a stop codon (c. 255 G > A). Both mutated residues are located in exon 2 of NDUF11. The authors concluded that these de novo mutations result in a dominant haploinsufficient phenotype, which contrasts with the Mendelian recessive inheritance pattern of many complex I deficiencies.

In the second study, two female patients were discovered with microphthalmia and linear skin defects syndrome (MLS) [75]. MLS is an X-linked disease found exclusively in females and is embryonically lethal in males. Both patients presented with linear skin defects but not microphthalmia. Patient 1 was heterozygous for a de novo nonsense mutation, c.262 C > T in exon 2, that changed Arg88 to a premature stop codon. Besides MLS and histiocytoid CM, she experienced additional symptoms such as axial hypotonia, failure to thrive, oncocytic metaplasia, and histiocytoid cardiomyopathy. She died at six months of age from cardiac arrest. This mutation was also identified in a later study [76]. Patient 2 was heterozygous for an inherited one base-pair deletion, c.402delG in exon 3, that caused a p.Arg134Ser mutation and changed Val136 to a premature stop codon. She was observed to have corpus callosum agenesis and dilated lateral ventricles during the fetal stage and experienced seizures, cardiomyopathy, myopia, nystagmus, severe psychomotor developmental delay, and muscular hypotonia after birth. She was still living at seven years of age. Her mother was a healthy carrier of the mutation. Though the authors of the previous study [74] concluded that one copy of wild-type NDUF11 was not sufficient for normal cell function, the existence of a heterozygous, healthy carrier of a NDUF11 deletion contradicts this conclusion. We conclude that the variable outcomes for females might be due to mosaicism; phenotypic differences may depend on which tissues receive the wild-type NDUF11 due to X-inactivation. After Patient 2's birth, her mother was pregnant with another fetus who had the same frameshift mutation. The fetus had to be aborted due to severe intrauterine growth retardation. Only wild-type NDUF11 transcripts were found in the fibroblasts and leukocytes of Patient 1 and Patient 2, indicating that the two mutations resulted in no transcript or expression of NDUF11.

Another NDUF11 mutation was found in 2016 [27]. A male patient who harbored a hemizygous de novo mutation (c.361 G > A, p.Glu121Lys) presented with lethal infantile mitochondrial disorder (LIMD), heart and respiratory failure, and complex I deficiency. There was no NDUF11 expression in the patient's fibroblasts, and he died 55 h after birth. Glu121 is a highly conserved residue. It lies on the intermembrane space side of complex I and sits between Arg124 and Tyr117 of the same subunit and near His50 of NDUF10.

Additionally in 2016 [77], another mutation in NDUF11 was discovered in five males from four families in a screen of patients with congenital sideroblastic anemia. The mutation was a three-nucleotide deletion, c.276_278del, p.F93del, occurring in three consecutive phenylalanine codons (TTC). These three Phe occur in the membrane helix between ND4 and NDUF5 (see Figure 15). It is possible that the deletion could be partially accommodated by the chain of three consecutive Phe residues. The subjects ranged from 2 to 76 years of age, and many had associated symptoms of short stature, congenital optic atrophy, myopathy, and lactic acidosis.

2.8. Subunits from the ND5-Module

Finally, three subunits are next described from the ND5-module: NDUF3, NDUF6, and NDUF8, as shown in Figure 16. These subunits do not interact directly with each other, but they serve to enclose the distal end of complex I on all sides. In particular, NDUF8 clamps the lateral helix of core subunit ND5.

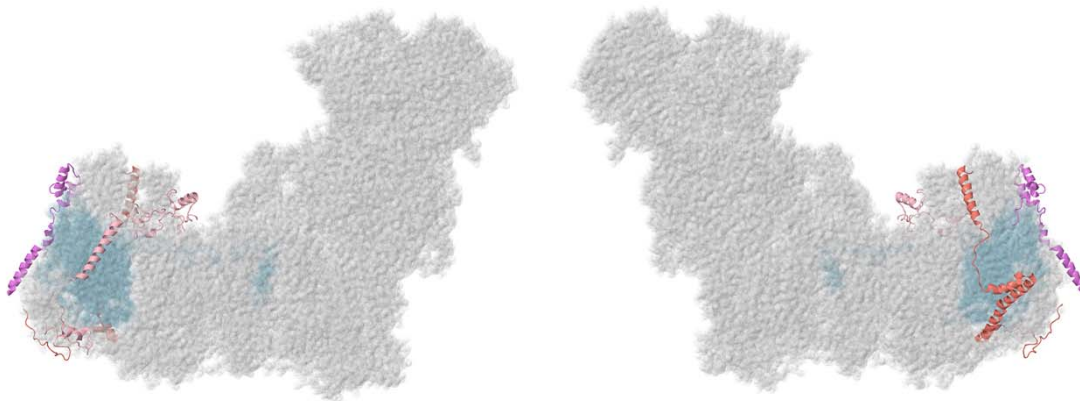


Figure 16. Location of NDUFB3, NDUFB6, and NDUFB8. Most of Complex I is colored gray. Core subunits in the ND5-module are colored light blue (ND5). NDUFB3, NDUFB6, and NDUFB8 are shown in ribbons, with NDUFB3 colored violet, NDUFB6 colored salmon, and NDUFB8 colored pink. The two views are rotated 180° relative to each other. The structure is from PDB id = 5xtf [9].

2.8.1. NDUFB3

The gene for NDUFB3 is found on chromosome 2. The encoded protein has 99 amino acids with a mass of about 11.4 kDa. The N-terminal Met appears to be cleaved. NDUFB3 is found at the distal end of complex I near core subunit ND5. It has a small N-terminal domain composed of three alpha-helices on the matrix side, and the C-terminus forms an alpha-helix, residues 62–89, that crosses the membrane. NDUFB3 contacts NDUFB9 and NDUFAB1 on the matrix side, as well as NDUFB2 and core subunit ND5, primarily in the membrane region. In knockout cell lines, the absence of NDUFB3 was found to result in the near total loss of complex I assembly and a reduction in the level of subunits from both N- and ND5 assembly modules [11].

Two mutations have been identified in the gene for NDUFB3 among over 10 individuals. In a report from [78], p.Trp22Arg appeared as a homozygous mutation in a girl who exhibited intrauterine growth retardation and premature birth at 31 weeks. She died at four months of age. Very low levels of complex I activity (<15%) were measured in fibroblasts, and this phenotype was rescued by lentivector complementation. A second individual was identified with compound heterozygous mutations: p.Trp22Arg and p.Gly70X [79]. The level of complex I activity was <25% of normal, and the individual exhibited muscular hypotonia, developmental delay, and lactic acidosis. In fibroblasts, complex I activity could be recovered by the ectopic expression of wild-type NDUFB3, though not either mutant form. The health status of this individual was not reported. In contrast, two more recent reports have identified the p.Trp22Arg mutation in older and seemingly healthier individuals. Ten children, up to 10 years of age, were identified as homozygous carriers of p.Trp22Arg in NDUFB3 [80]. These individuals with short statures and prominent foreheads ranged from 10 months to 10 years in age, and their complex I activity levels ranged from 25 to 35% of normal levels. Various levels of assembled complex I were seen from these individuals using blue native gel electrophoresis. More recently, a 32-year-old previously diagnosed with non-alcoholic steatohepatitis was discovered to be homozygous p.Trp22Arg [81]. This individual had reduced levels of complex I activity (<30% of normal) and suffered from oculomotor dysfunction with optic nerve anomalies, episodes of lactic acidosis during surgical interventions, and progressive fatigue.

The latter two studies emphasized that the context of these two mutations in NDUFB3 appears to be significant. The level of complex I activity, and its assembly, is likely on the borderline between tolerable and deleterious. Trp22 is found in a conserved region of NDUFB3 on the matrix side of the membrane. The NH side chain of Trp is in position to H-bond to the OH side chain of Tyr85 of NDUFAB1. It also makes nonbonding interactions with Arg45 and Gln46 of NDUFB2. Therefore, the mutation to the positively charged Arg is likely to be disruptive. The second mutation, p.Gly80X,

occurs in the last exon and so is not likely to trigger nonsense-mediated decay. This residue occurs in the transmembrane region, and the stop codon would truncate the protein and eliminate the transmembrane helix. Gly70 is flanked by Lys69 and Lys72, which probably demarcates the matrix end of the lipid bilayer. Thus, it seems possible that the truncated NDUF3 would retain some ability to assemble and provide normal interactions on the matrix side.

2.8.2. NDUF6

The NDUF6 gene is located on chromosome 9. NDUF6 is a single-pass membrane protein with its N-terminus found on the matrix side. It is a 15 kDa protein with 127 amino acids, with residues 6–26 forming an alpha helix on the matrix side and residues 56–92 forming an alpha-helix that crosses the membrane. Its initiator methionine is removed during processing. Furthermore, it contains an N-acetylated threonine at residue 2 [58] and a N6-acetylated lysine at residue 24 by analogy with mouse [82]. NDUF6 contacts NDUF5, NDUFAB1, and NDUFB9 on the matrix side, core subunit ND5 in the membrane, and supernumerary subunits NDUF7 and NDUF10 in the IMS. In a knockout strain of cultured human cells, the loss of NDUF6 resulted in no assembly of complex I [11].

It has been established that insulin-resistance and type 2 diabetes are associated with reduced NDUF6 expression [83]. To date, only one polymorphism has been found in NDUF6 [84]. This polymorphism, rs629566 (A/G or G/G), is located in the promoter region of the NDUF6 gene. It changes the DNA sequence at position 544 from CA to CG. This polymorphism introduces a fourth potential methylation site in the promoter of the NDUF6 gene. The introduction of a fourth methylation site resulted in increased DNA methylation at the NDUF6 gene promoter and decreased NDUF6 mRNA expression in the muscle of elderly patients. As predicted, elderly patients with the rs629566 G/G genotype had lower NDUF6 mRNA expression than elderly patients with the A/G genotype. Surprisingly, young patients with the A/G polymorphism had the same level of NDUF6 mRNA expression as young patients with the wild-type A/A gene. Moreover, young patients with the G/G genotype actually had the highest levels of NDUF6 mRNA. Thus, it was concluded that age significantly affects NDUF6 DNA methylation and influences NDUF6 expression.

Furthermore, it has been suggested that NDUF6 is a possible tumor suppressor of metastatic clear cell renal cell carcinoma (ccRNC) [85]. In an analysis of 50 primary ccRNC samples, copy number alteration at the 9p24.3–p13.3 region (the chromosomal region in which NDUF6 gene is located) was found to strongly correlated with poor ccRNC prognosis. Patients missing this chromosomal region had higher cancer recurrence rates, higher metastasis rates, and lower survival rates. It was determined that NDUF6 expression was downregulated in primary ccRNC samples due to gene copy number loss. More focused experimentation revealed that when NDUF6-encoded lentiviruses were transduced into a ccRNC cell line, cell proliferation was suppressed. Consequently, it was observed that siRNA knockdown of NDUF6 led to cell proliferation with the same cell line. The study concluded that loss of region 9p24.1–p13.3 results in NDUF6 downregulation, which causes cell proliferation in metastatic ccRNC tumors.

2.8.3. NDUF8

The NDUF8 gene is located on chromosome 10. The NDUF8 subunit is initially synthesized with 186 residues and a mass of about 22 kDa, but the first 28 residues serve as a transit peptide and are cleaved off during processing. NDUF8 is a single-pass protein with the C-terminus in the IMS and the N-terminus in the matrix (see Figure 17). It is located near the end of complex I, and its single transmembrane span lies near ND5. The helix residues (126–151) are not conserved, but the small domains on each side of the helix are much more conserved. On the matrix side NDUF8 mainly contacts core subunit ND5 and NDUF4, but it also contacts core subunit ND4 and NDUF9. On the IMS side, NDUF8 contacts NDUF7 and NDUF10. In a knockout strain of cultured human cells, the loss of NDUF8 resulted in no assembly of complex I [11].

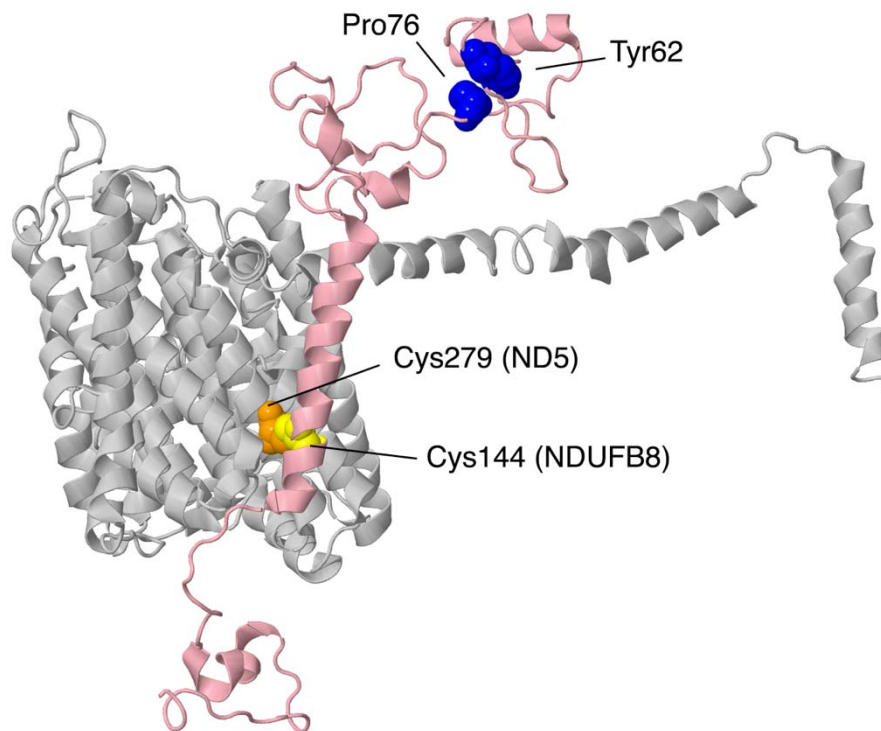


Figure 17. Structural features of NDUFB8 from the ND5-module. The sites of three mutations in NDUFB8 are shown in space-filling. NDUFB8 is colored pink, while the sites of Pro76Gln and Tyr62His are shown in blue on the matrix side, and Cys144Trp is shown in yellow. The Cys279 of ND5 is shown in orange, and it appears to form a disulfide with Cys144 of NDUFB8.

In 2018, two unrelated compound heterozygous patients with biallelic NDUFB8 mutations were identified to have Leigh-like encephalomyopathy [86]. At three months of age, Patient 1 showed symptoms of failure to thrive, muscle hypotonia, and elevated lactate levels. Patient 2 began to show these symptoms at six months of age. Similar to patients with Leigh syndrome, both patients showed symmetrical basal ganglia and capsula interna lesions. Hypertrophy of the left cardiac ventricle was observed in Patient 1, and he died at 15 months of age. Patient 2 was still alive at six years of age.

Patient 1 harbored a p.Pro76Gln mutation and a p.Cys144Trp mutation. Pro76 is located within a highly conserved region. The residue lies on the matrix side above the lateral helix of core subunit ND5, and it is near core subunit ND4. Cys144 is found within the membrane domain. Though it is weakly conserved, it appears to form a disulfide with Cys279 of chain ND5. Surprisingly, it was discovered that the p.Cys144Trp mutation generated a new exonic splicing silencer (ESS). The Human Splicing Finder algorithm was used to identify the ESS, and Sanger sequencing revealed that exon 4 (Met105–Val156) was skipped in the NDUFB8 transcript, thus confirming the splice defect. Nevertheless, 14% of total cDNA from Patient 1 contained the p.Cys144Trp missense mutation, indicating that while skipping exon 4 is favored, normal splicing also occurs at a lower frequency. Upon the analysis of respiratory chain activity in Patient 1's muscle tissue and BN gel electrophoresis in a muscle biopsy sample, isolated complex I deficiency was confirmed.

Patient 2 carried a p.Tyr62His mutation and a loss-of-function deletion Glu63Aspfs*35 (c. 189delA). Tyr62 is completely surrounded by mostly conserved residues of NDUFB8 and is located in the same conserved region as Pro76; the two residues are only 4–5 Å distant from each other. Furthermore, the side chain hydroxyl group of Tyr62 forms a hydrogen bond with the side chain and backbone oxygen atoms of Asp74 in NDUFB8. Thus, although His is a fairly conserved substitution for Tyr, His substitution would likely be disruptive at this residue. Complex I deficiency in Patient 2 was observed through the BN gel electrophoresis of muscle homogenate. The complementation of both mutant cell lines was tested using the lentiviral expression of wild-type NDUFB8. Cells from Patient 1

showed normal levels of complex I activity by in-gel assays after BN gel electrophoresis, and normal levels of the NDUFB8 subunit by immunoblotting. Cells from Patient 2 did not grow well enough for such analysis, but both cell lines showed complementation in assays using microrespirometry and flow cytometry.

3. Conclusions

This review has discussed 20 of the 30 supernumerary subunits of human complex I. Missense and various other point mutations have been described for 17 of the subunits. One subunit, NDUFV3, has several known amino acids substitutions, but there is no evidence that they are causative for disease. All of the mutations, the diseases they are associated with, and their effects on assembly of complex I are summarized in Table 2. The gene of one subunit NDUF3 was completely deleted, along with several neighboring genes. This provided evidence for haplosufficiency, since no mitochondrial disease characteristics occurred in those individuals. The gene for another subunit NDUFB6 had a mutation in the promoter, resulting in a change in the expression level.

Table 2. Summary of mutations with associated diseases and effects on assembly.

Gene	Mutation (DNA)	Mutation (Protein)	Diagnosis	Assembly ¹	Reference
NDUFA2	c.A170 C	p.Glu57Ala	Microcephaly Leukoencephalopathy	NT	[14]
	1. c.134 A > C 2. c.225del	1. p.Lys45Thr 2. p.Asn76Metfs*4 ²	Leukoencephalopathy	NT	[13]
	c.134 A > C	p.Lys45Thr	Leukoencephalopathy	• Smaller complex	[13]
	c.208 + 5 G > A	mRNA not detected	Leigh syndrome, hypertrophic cardiomyopathy	•	[12]
NDUFV3	1. c.77 G>A 2. POLG: c.32 G > A	1. p.Arg26Gln 2. POLG: p.Gly11Asp	Complex I deficiency	NT	[22]
	1. c.168 A > C 2. POLG: c.2492 A > G 3. ND1 m3946 A > G	1. pLys56Asn 2. POLG: p.Tyr831Cys 3. ND1: p.Glu214Lys	Complex I deficiency	NT	[22]
	c.308 G > A	p.Gly103Asp	Complex I deficiency	NT	[22]
	1. c.826 G > A 2. NDUF58:c.460 G > A	1. p.Glu276Lys 2. NDUF58: p.Gly154Ser	Complex I deficiency	NT	[22]
NDUFS4	1. c.168 A > C 2. AMACR: c.554 T > C	1. pLys56Asn 2. AMACR: p.Val185Ala	Complex I deficiency	NT	[22]
	AAGTC at 466–470 duplication	frameshift	Leigh-like disease	•	[25]
	c.G289*del	p.Tyr97* ²	Leigh-like disease	•	[25]
	c.G44 A	Splicing variants, no protein detected	Leigh-like disease	•	[25]
	1. c.355 G > C 2. c.462delA	1. p.Asp119His 2. p.Lys154Asnfs*34	Leigh syndrome	Smaller size	[28]
	1. c.99-1 G > A 2. c.462delA	1. p.Ser34Ilefs*4 2. p.Lys154Asnfs*34	Leigh syndrome	NT	[22]
	c.340 T > C	p.Trp114Arg	Leigh syndrome	NT	[27]
	c.221delC	p.Thr74Ilefs*17	Leigh syndrome	NT	[22]

Table 2. Cont.

Gene	Mutation (DNA)	Mutation (Protein)	Diagnosis	Assembly ¹	Reference
NDUFS6	c.344 G > A	p.Cys115Tyr	Neonatal lactic acidemia	NT	[33]
	c.186+2 T > A	Affected splice site, eventually leading to premature termination	Lethal infantile mitochondrial disease	NT	[32]
	1. c.343 C > A 2. c.309 + 5 G > A	1. p.Cys115Arg 2. Loss of exon 3, but some normally spliced	Leigh syndrome	•	[34,35]
NDUFA9	c.962 G > C	p.Arg321Pro	Leigh syndrome	•	[40]
	c.1078 C > T	p.Arg360Cys	Leigh syndrome	••	[87]
NDUFA1	c.22 G > C	p.Gly8Arg	Leigh syndrome	••	[43]
	c.55 C > T	p.Pro19Ser	Leigh syndrome	•	[47]
	c.111 G > C	p.Gly32Arg	Mitochondrial encephalopathy	••	[44,46,88]
	c.251 G > C	p.Arg37Ser	Myoclonic epilepsy	••	[43]
NDUFA13	c.170 G > A	p.Arg57His	Mitochondrial encephalopathy	•	[54]
NDUFA6	1. c.191 G > C 2. c.265 G > T	1. p.Arg64Pro 2. p.Glu89*	Auditory and optic neuropathy	• Lacking N- and Q-modules	[57]
	c.331_332del	p.Glu111Serfs*35	Mitochondrial-related infantile death	• Lacking N- and Q-modules	[57]
	c.3 G > A	p.Met1Ile	Brain disorder	• Lacking N- and Q-modules	[57]
	1. c.309del 2. c.355del	1. p.Met104Cysfs*35 2. p.Leu119Tyrfs*20	Leukoencephalopathy	NT	[57]
NDUFB9	c.140 G > T	p.Arg47Leu. (heterozygous)	Complex 1 deficiency	•••	[60]
	c.191 T > C	p.Leu64Pro	Complex 1 deficiency	•	[60]
NDUFS5	1. c.286 C > T 2. NDUFS8:c.4 C > T	1. p.Pro96Ser 2. NDUFS8: p.Arg2Cys	Complex I deficiency	NT	[22]
NDUFA8	1. c.404 G > A 2. C20orf: c.686 T > C	1. p.Arg135Gln 2. C20orf: p.Leu229Pro	Complex I deficiency	NT	[22]
	c.139 C > T	p.Arg47Cys	Microcephaly and epilepsy	•	[62]
	1. c.325 G > A 2. NDUFS2: c.671 C > T	1. p.Glu109Lys, mRNA not found 2. p.Ala224Val	Neonatal hypotonia and epilepsy	NT	[63]
NDUFB10	1. c.319 T > C 2. c.206_207insT	1. p.Cys107Ser 2. p.Glu70X	Fatal lactic acidosis, cardiomyopathy	•	[64]
NDUFA10	1. c.1 A > G 2. c.425 A > G	1. p.Met1Val 2. p.Gln142Arg	Leigh syndrome	•	[68]
	1. c.891 T > C 2. c.383_384insTAA	1. p.Leu294Pro 2. p.Ser218delinsIS	Leigh syndrome	NT	[27]
	c.296 G > A	p.Gly99Glu	Leigh syndrome	NT	[69]
NDUFC2	c.346_*7del	p.His116_Arg119delins21	Leigh syndrome	•	[70]
	c.173 A > T	p.His58Leu	Leigh syndrome	•	[70]

Table 2. Cont.

Gene	Mutation (DNA)	Mutation (Protein)	Diagnosis	Assembly ¹	Reference
NDUFA11	c.99 C + 5 G > A	G to A mutation at exon 1-IVS1 splice junction	Encephalocardiomyopathy and fatal infantile lactic acidemia	NT	[72]
	1. c.317 C > T 2. c.394 G > C	1. p.Thr106Ile (isoform two) 2. p.Ala132Pro	Neuromuscular disorder	NT	[73]
NDUFB11	c.324 T > G	p.Tyr108*	Histiocytoid cardiomyopathy, and microphthalmia	NT	[74]
	c. 255 G > A	p.Trp85*	Histiocytoid cardiomyopathy, and microphthalmia	NT	[74]
	c. 262 C > T	p.Arg88*	Histiocytoid cardiomyopathy, and microphthalmia	NT	[75]
	c.402delG	p.Arg134Serfs*2	Microphthalmia and cardiomyopathy	NT	[75]
NDUFB3	c.361 G > A	p.Glu121Lys	Lethal infantile mitochondrial disorder	•	[27]
	c.276_278del	p.F93del	Congenital optic atrophy and myopathy	NT	[77]
	1. c.64 T > C 2. c.208 G > T	1. p.Trp22Arg 2. p.Gly70X	Muscular hypotonia and lactic acidosis	•	[79]
NDUFB8	1. c.64 T > C	p.Trp22Arg	Muscular hypotonia and lactic acidosis	••	[78,81,89]
	1. c.227 C > A 2. c.432 C > G	1. p.Pro76Gln 2. p.Cys114Trp	Leigh-like disease	•	[86]
	1. c.184 C > G 2. c.189delA	1. p.Tyr62His 2. p.Glu63Aspfs*35	Leigh-like disease	••	[86]

¹ Assembly: ••• normal or near normal; •• intermediate level; • little or no assembly. NT: not tested. When 2 mutations are listed, they are two alleles of the same gene, or if indicated, one is a second gene. See the text for more details. ² p.Asn76Metfs*4 is a frameshift mutation at codon 76 that converts Asn (AAT) to Met (ATG) with a stop codon appearing 4 codons downstream. p.Tyr97* is a nonsense mutation at Tyr76 to TAG.

Among the 17 subunits that were found to have missense or other point mutations, nearly all were homozygous or compound heterozygous and therefore lacked a normal allele. This supports the likely general trend of haplosufficiency. One exception might be in NDUFB11, which is found on the X chromosome. We suggest that mosaicism in female patients due to X-inactivation might explain variable outcomes from heterozygous null mutations. To resolve such questions, it is important to extensively sequence the genome to identify other possible mutations related to the disease state. Another discrepancy was found to occur in NDUFB3, in which one girl with one homozygous mutation died in infancy [78]; meanwhile, several boys with the same mutation, in the hemizygous form, survived into adolescence and adulthood [80]. Deep sequencing might be the answer, again to try to identify other mutations.

Among the missense mutations, many are found at subunit interfaces. Such mutations are predicted to negatively impact assembly, at least local assembly. Others are found at interior sites of the proteins, and it can be predicted that protein folding will be disrupted and lead to a nonfunctional state, an effect similar to that of a gene knockout. The results of the knockouts of each of the supernumerary subunits in cell culture [11] have demonstrated that most are essential for complex I assembly. The same general trend can be seen with missense mutations in the supernumerary subunits. Essential subunits might include those that serve to stabilize the structure of complex I or that might act as chaperones in the assembly process. In recent years, analyses of complex I assembly have increased, but there have not been a great number of mutants analyzed at sufficient depth. The network of interactions among

supernumerary subunits, as well as with core subunits, suggests that the instability of complex I might reflect a cooperative assembly and binding of the subunits. The loss of one supernumerary subunit could impact multiple other subunits such that assembly is not completed. Even if complex I is able to assemble, its stability might be marginal, eventually leading to a loss of function.

In conclusion, the increasingly high-resolution structures of complex I from human and other species have helped to interpret the possible roles of the supernumerary subunits and the loss of function due to mutation. Unbiased DNA sequencing will be necessary to find all of the mutations that might exist in various disease states, as illustrated by the mutations discovered in NDUFB11 to be associated with congenital sideroblastic anemia [77]. In this way, other rare diseases might become better understood and treated.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2075-1729/10/11/296/s1>, Table S1: Interactions of Human Complex I Subunits, Video S1: Structure of Human Complex I.

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