

## Review Article

# Blackout in the powerhouse: clinical phenotypes associated with defects in the assembly of OXPHOS complexes and the mitoribosome

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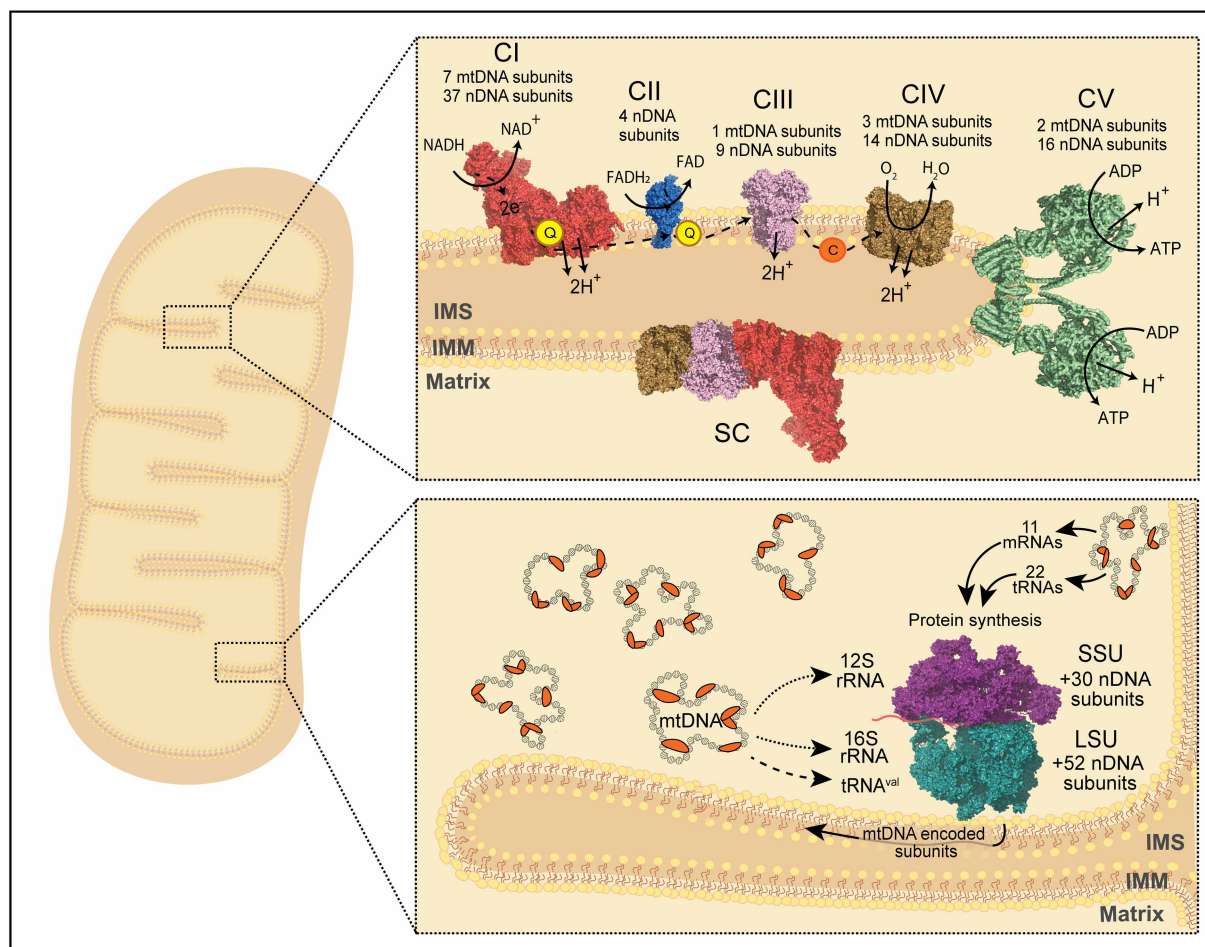
Mitochondria produce the bulk of the energy used by almost all eukaryotic cells through oxidative phosphorylation (OXPHOS) which occurs on the four complexes of the respiratory chain and the  $F_1F_0$  ATPase. Mitochondrial diseases are a heterogeneous group of conditions affecting OXPHOS, either directly through mutation of genes encoding subunits of OXPHOS complexes, or indirectly through mutations in genes encoding proteins supporting this process. These include proteins that promote assembly of the OXPHOS complexes, the post-translational modification of subunits, insertion of cofactors or indeed subunit synthesis. The latter is important for all 13 of the proteins encoded by human mitochondrial DNA, which are synthesised on mitochondrial ribosomes. Together the five OXPHOS complexes and the mitochondrial ribosome are comprised of more than 160 subunits and many more proteins support their biogenesis. Mutations in both nuclear and mitochondrial genes encoding these proteins have been reported to cause mitochondrial disease, many leading to defective complex assembly with the severity of the assembly defect reflecting the severity of the disease. This review aims to act as an interface between the clinical and basic research underpinning our knowledge of OXPHOS complex and ribosome assembly, and the dysfunction of this process in mitochondrial disease.

## Introduction

Mitochondria are ubiquitous organelles that have their own DNA and translation machinery. The human mitochondrial (mt) DNA is a circular double-stranded molecule containing 16 569 base pairs and 37 genes [1]. Two of these genes encode mitochondrial ribosomal RNAs (mt-rRNA) which are required for the assembly of the mitochondrial ribosome (mitoribosome). Another 22 genes encode mitochondrial transfer RNAs (mt-tRNAs) which are responsible for deciphering RNA sequences during protein translation. The mt-tRNA for valine ( $tRNA^{val}$ ) is also present as a structural component of the mitoribosome [2–4] (Figure 1, lower inset). The remaining 13 mitochondrial genes encode highly hydrophobic transmembrane proteins that are translated on mitoribosomes and assembled into complexes I, III, IV and V of the oxidative phosphorylation (OXPHOS) system [5–8] (Figure 1, upper inset). Besides the 37 genes encoded on mtDNA, over a thousand other proteins encoded on nuclear DNA (nDNA) are translated in the cytosol and imported into the mitochondria via dedicated protein import machinery [9,10]. Mitochondria are responsible for generating the majority of cellular ATP via OXPHOS, which occurs on the mitochondrial respiratory chain (MRC) and the  $F_1F_0$ -ATPase. The MRC consists of four multiprotein complexes (complexes I–IV) embedded in the inner mitochondrial membrane (IMM) and two electron carriers called coenzyme Q (CoQ, Q) and cytochrome *c* (Cyt C, C). Complexes I, III and IV are also found together in higher order structures known as supercomplexes or respirasomes [11], though the precise function this coalescence is not fully clear [12]. The

Received: 1 May 2020  
 Revised: 29 September 2020  
 Accepted: 5 October 2020

Version of Record published:  
 5 November 2020



**Figure 1. The oxidative phosphorylation (OXPHOS) system and the mitoribosome.**

*Upper inset*, the OXPHOS system consists of complexes I, II, III and IV of the respiratory chain and complex V (the  $F_1F_0$ -ATP Synthase). Complexes I, III and IV also exist in large assemblies known as supercomplexes (SC). Subunit compositions of individual complexes are indicated. mtDNA, mitochondrial DNA; nDNA, nuclear DNA; Q, Coenzyme Q; C, Cytochrome c. *Lower inset*, the mitoribosome synthesises the 13 mtDNA-encoded subunits that are present within the OXPHOS complexes. It consists of two major parts, the small subunit (SSU) and large subunit (LSU). The SSU contains one mtDNA-encoded rRNA (12S rRNA) and 30 nDNA encoded proteins. The LSU contains one mtDNA-encoded rRNA (16S rRNA), 52 nDNA encoded proteins and a single mtDNA-encoded tRNA<sup>val</sup> molecule that has a structural role in mitoribosome. All 22 mtDNA-encoded tRNAs, including tRNA<sup>val</sup>, are involved in the synthesis of the 13 mtDNA-encoded proteins from 11 mRNAs. Two mRNAs, MT-ND4/MT-ND4L and MT-ATP6/MT-ATP8 share open reading frames and the proteins are transcribed as bicistronic elements. Orange elements in mtDNA represent mtDNA associated protein TFAM. IMS, intermembrane space; IMM, inner mitochondrial membrane; Matrix, mitochondrial matrix. Models for complexes I, II, III, IV, V and mitoribosome generated from PDB: 5LDW, 1ZOY, 1BGY, 5B1A, 5ARE and 3U9M, respectively.

MRC as a whole, as well as the two electron carriers are involved in a series of redox reactions that creates a proton gradient across the IMM, which is in turn used by the fifth complex, complex V or the  $F_1F_0$ -ATPase, to synthesise ATP.

The MRC, complex V and the mitoribosome are built through a series of orchestrated steps that require the help of other proteins not part of the mature complex, often called assembly factors. Not all assembly factors directly promote assembly, the controlled coalescence of subunits into complexes through stabilisation of assembly intermediates, but many instead provide critical services in the form of maturation and delivery of cofactors such as heme, copper and iron–sulfur clusters (Fe–S), post-translational modification of subunits and regulation of translation. Furthermore, transcription of mtDNA, mitochondrial mRNA processing, mitochondrial tRNA maturation, mitoribosome assembly and translation all directly influence OXPHOS complex

biogenesis function [5–8]. Dysfunction in any of the aforementioned processes can lead to mitochondrial disease. Given the central importance of mitochondria to cellular function, disease can affect any tissue in the body, though tissues and organs of high energy demand such as heart, brain and muscle are frequently affected. Furthermore, some patients present with tissue or organ specific phenotypes (e.g. cardiomyopathy) while others exhibit multi-system disorders or delayed onset, reflecting the different requirements for mitochondrial energy production in different cell types, the function of affected protein, their tissue-specific expression or their being encoded on nuclear or mitochondrial DNA [5–8]. There are over 300 known disease genes, located in either nuclear DNA (nDNA) or mitochondrial DNA (mtDNA), implicated in mitochondrial diseases ([5,13], this review). Broadly speaking, these can be separated into two categories: genes that are involved in primary energy generation (i.e. subunits or assembly factors of the OXPHOS system, mitoribosome, involved in transcription, mtDNA homeostasis and related systems) and genes that have a secondary function (i.e. protein quality control, membrane structure and metabolite transport) [5,14,15]. Of the over 190 known primary disease genes, more than half lead to defective assembly of the OXPHOS complexes or the mitoribosome ([5], this review).

The current diagnostic paradigm for mitochondrial disease involves a combination of whole exome (WES) or whole genome (WGS) sequencing, with linkage studies and homozygosity mapping [5,13]. In the case of mtDNA mutations, diagnosis can be even more challenging considering that the mtDNA is present in multiple copies per cell and might contain a heterogeneous population of mutated and non-mutated mtDNA, a phenomenon termed heteroplasmy. In the case of mitochondrial disease, the ratio of mutated to non-mutated mtDNA is termed mutation load, which is particularly important in the onset and tissue-specific manifestation of mitochondrial diseases [16]. Diagnostic yield when using strategies combined with WES or WGS is only 30–68% [17–20], suggesting that other novel disease genes and variants remain to be discovered. The principal challenge in the current diagnosis paradigm is validation of variants of uncertain significance (VUS) detected in patients with suspected mitochondrial disease [21]. To address this, investigators often turn to functional studies. Studies such as these, sometimes continuing long after confirmed diagnosis, have been incredibly informative to our understanding of mitochondrial complex assembly. The results of these studies can later provide information invaluable to those attempting to solve new undiagnosed cases. For example, we now know from cultured cell line knockout studies that loss of proteins required for some steps of complex I assembly does not greatly impact assembly and function of the enzyme, whereas loss of proteins required for other steps leads to catastrophic failure in complex biogenesis [22]. Perhaps unsurprisingly, in patients null mutations have been described in genes required for the former step, but not the second (see the below sections for detail). Armed with this information, an investigator may be able to prioritise a novel VUS in a gene associated with one of these steps for follow-up studies. It is, therefore, the intention of this review to present a common ground to both basic researchers interested in the assembly of mitochondrial complexes, as well as more clinically focused audiences, in the hope that both will benefit from each other's ongoing efforts to understand the function and impacts of dysfunction in these complex molecular machines.

Due to the large number of steps in the biogenesis of these complexes, most of the following sections have been separated into sub-sections describing distinct stages of assembly and the impacts of disease on this process. Subunits (i.e. structural proteins found in the fully assembled complex) in the below sections are indicated in bold typeface, whereas assembly factors and other proteins are listed in regular typeface. The nomenclature for some subunits and assembly factors is complicated, different groups may use different symbols and for most complexes the subunit naming convention differs between humans and other organisms. For the purposes of this review, we have chosen to refer to the subunits and assembly factors by their Human Genome Organisation (HUGO) approved gene symbol [23], though have aimed to include the commonly used protein symbol in parenthesis at first mention. Finally, due to the large number of case reports for some disease genes we have chosen to cite the first example for each commonly observed phenotype. Further case reports can be found by referring to the relevant entry in the Online Mendelian Inheritance in Man database (<https://www.omim.org>), with the OMIM entry number cited for each gene in the relevant table.

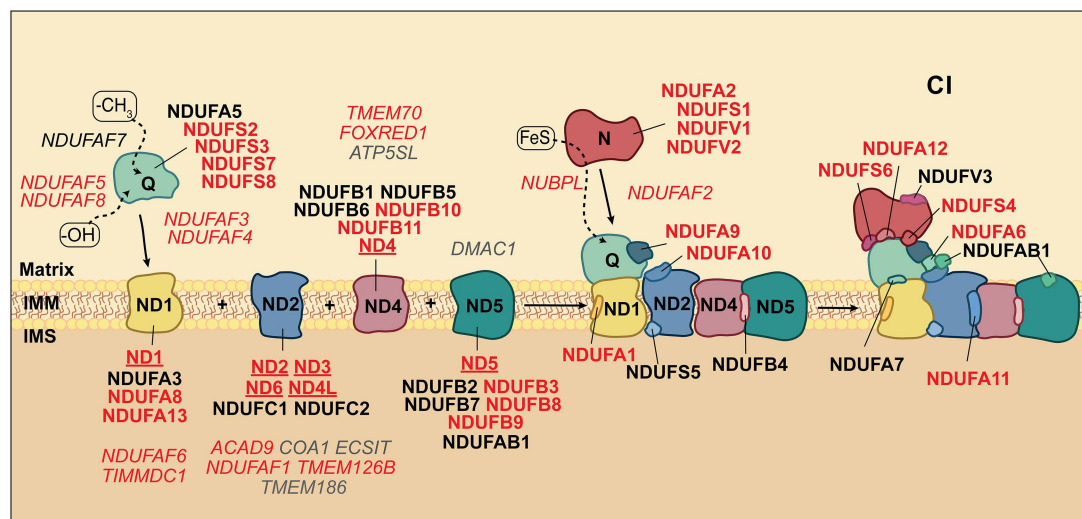
## Complex I

Mitochondrial complex I (NADH, Ubiquinone reductase) is the first complex in the MRC. The fully assembled complex I is L-shaped with a hydrophobic arm embedded in the IMM and a hydrophilic arm extending into the mitochondrial matrix [24,25]. The latter contains the catalytic N-module, which is involved in oxidation of NADH to NAD<sup>+</sup>, and the Q-module which transfers electrons via Fe–S clusters to Coenzyme Q [26,27]. This

in turn triggers conformational changes leading to pumping of protons across the membrane arm (ND-modules) and creation of the proton-motive force used by complex V [28]. Complex I consists of 45 subunits (44 distinct subunits as one subunit, **NDUFAB1** is present in two locations within the complex) [29]. Of the 44 distinct subunits, 14 are classified as core subunits since they possess catalytic function and homologues are present in all organisms with complex I, including bacteria [30,31]. Of the 14 core mammalian subunits, 7 are encoded on mtDNA: **ND1**, **ND2**, **ND3**, **ND4**, **ND4L**, **ND5** and **ND6**, all of which are present in the membrane arm, while the remainder are encoded on nuclear DNA. Four are located in the Q-module: **NDUFS2**, **NDUFS3**, **NDUFS7**, **NDUFS8**; and three in the N-module: **NDUFS1**, **NDUFV1**, **NDUFV2**. The remaining 30 subunits (prefixed **NDUFA**, **NDUFB**, **NDUFC** and **NDUFAB1**) are known as accessory or supernumerary subunits, with 25 of them being characterised as essential for the assembly and stability of the complex [22]. The assembly of complex I occurs through the sequential addition of modules seeded by core subunits to which accessory subunits are added. Although the order of module coalescence is still not fully understood, the assembly pathway for complex I is one of the most well studied of all mitochondrial complexes and there is an increasingly well accepted model (Figure 2) [22,24,32].

## Assembly of the Q and ND1 modules

The Q-module subassembly contains nuclear-encoded subunits **NDUFA5**, **NDUFS2**, **NDUFS3**, **NDUFS7** and **NDUFS8** and appears to be one of the earlier modules to assemble (Figure 2) [22,24,32]. With the exception of **NDUFA5**, all of the above subunit genes have been associated with Leigh syndrome, leading to early childhood death (Table 1). In line with this, their gene-editing based ablation in the commonly used human embryonic kidney cell (HEK293T) model system leads to complete loss of complex assembly and activity [22]. The assembly factors **NDUFAB5** and **NDUFAB7** are required for maturation of the Q-module [33–35] and they contain the S-adenosylmethionine (SAM)-dependent methyltransferase domains [33,36] that provide post-translational modifications to subunits within the Q-module. **NDUFAB5** is responsible for hydroxylation of an arginine residue in **NDUFS7** [34] and requires the assembly factor **NDUFAB8** for its stabilisation, though its molecular function in this capacity is not yet clear [37,38]. On the other hand, **NDUFAB7** is required for dimethylation of an arginine residue in **NDUFS2** [33,35]. While the function of these modifications is unknown, their loss results in complex I dysfunction [39,40]. There have been no patients reported for **NDUFAB7**, however, a



**Figure 2. Schematic depicting the complex I assembly pathway showing known mitochondrial disease genes in red, mtDNA-encoded subunits underlined and assembly factors in italics.**

Complex I assembly is characterised by coalescence of distinct modules assembled individually prior to final assembly into large intermediates and eventually the functional complex. The Q-module joins the ND1 module and coalesces with ND2, ND4 and ND5 modules. The N-module (harbouring the site of NADH oxidation) is the last module to join the complex in the assembly pathway. Fe-S, iron-sulfur. IMS, intermembrane space; IMM, inner mitochondrial membrane; Matrix, mitochondrial matrix.

**Table 1. Defects affecting biogenesis of the Q and ND1 modules**

Part 1 of 2

| Gene              | A. S. F. | Types of genetic variants and protein impact   | Clinical presentations and relevant information  | Ref.            | OMIM   |
|-------------------|----------|--|--|-----------------|--------|
| <i>Q-module</i>   |          |  |  |                 |        |
| <i>NDUFS2</i>     | X        | Various missense leading to uncertain impact or near absence of protein.   | Leigh syndrome, optic atrophy, hypertrophic cardiomyopathy, elevated blood lactate.  | [380,381]       | 602985 |
| <i>NDUFS3</i>     | X        | Missense leading to uncertain protein impact.  | Leigh syndrome, optic atrophy.   | [382]           | 603846 |
| <i>NDUFS7</i>     | X        | Predominantly missense but also intronic (nonsense) leading to truncated protein or uncertain impact on protein.         | Leigh syndrome, hypotonia, may present normal blood lactate and pyruvate.  | [383,384]       | 601825 |
| <i>NDUFS8</i>     | X        | Missense leading to uncertain impact on protein or reduced levels.   | Ranging from severe Leigh syndrome, elevated blood lactate and pyruvate, hypotonia, early death to slowly progressive neurological disease at the end of first decade of life.                     | [385–387]       | 602141 |
| <i>NDUFA3</i>     | X        | Missense leading to uncertain protein impact.  | Severe lactic acidosis, optic atrophy, respiratory failure and variable brain involvement including Leigh syndrome, myoclonic seizures, macrocephaly and cavitating leukoencephalopathy.           | [41,388,389]    | 612911 |
| <i>NDUFA4</i>     | X        | Missense leading to decreased protein levels or uncertain protein impact.  | Leigh syndrome, hypotonia, elevated blood and cerebrospinal fluid lactate, may present severe infantile cardiomyopathy.  | [42,390]        | 611776 |
| <i>NDUFA5</i>     | X        | Predominantly missense but also intronic (nonsense) leading to uncertain protein impact.                                 | Most common presentation of Leigh syndrome, but also fatal lactic acidosis, hyponatremia, hypotonia and bilateral optic neuropathy. Variable survival depending on variant. Decreased CI activity. | [36,391–393]    | 612360 |
| <i>NDUFA8</i>     | X        | Nonsense (frameshift and intronic) and missense leading to uncertain protein impact.                                     | Leigh syndrome, may present optic atrophy and elevated blood lactate.  | [38]            | 618461 |
| <i>ND1 module</i> |          |  |  |                 |        |
| <i>MT-ND1</i>     | X        | Multiple, predominantly missense variants with protein levels dependant on variant. and mutant mtDNA load.               | LHON, MELAS.   | [43–45]         | 516000 |
| <i>NDUFA1</i>     | X        | Missense leading to decreased protein levels.  | X-linked. Variable presentation from severe Leigh syndrome to developmental delay, hypotonia, elevated blood lactate and myoclonic epilepsy and survival to childhood.                             | [48]            | 300078 |
| <i>NDUFA8</i>     | X        | Missense leading to reduced proteins levels.   | Psychomotor retardation, severe quadriplegia, elevated blood lactate, cerebral atrophy, hypertonia, epilepsy with survival to adulthood.   | [394]           | 603359 |
| <i>NDUFA13</i>    | X        | Missense or nonsense (frameshift) leading to reduced protein levels.   | Hypotonia, dyskinesia, sensorial impairments or Leigh syndrome and mild hypertrophic cardiomyopathy.   | [395,396]       | 609435 |
| <i>NDUFA6</i>     | X        | Predominantly missense but also frameshift and intronic leading to decreased protein levels or uncertain protein impact. | Leigh syndrome is the most common presentation, but also Fanconi syndrome, lactic acidosis, bilateral striatal necrosis and progressive dystonia in childhood.                                     | [47,66,397,398] | 612392 |

Continued

**Table 1. Defects affecting biogenesis of the Q and ND1 modules**

Part 2 of 2

| Gene           | A. S. F. | Types of genetic variants and protein impact   | Clinical presentations and relevant information                                | Ref. | OMIM   |
|----------------|----------|--|--|------|--------|
| <i>TIMMDC1</i> | X        | Intronic variant causing frameshift and early termination leading to decreased protein levels. | Infantile muscular hypotonia, developmental delay, neurological deterioration. | [46] | 615534 |

S., subunit; A.F., assembly factor; LHON, Leber's hereditary optic neuropathy; MELAS, mitochondrial encephalopathy, lactic acidosis and stroke-like episodes.

number have been reported for *NDUFAF5* and *NDUFAF8* (Table 1). For *NDUFAF5*, the majority of patients harbour missense mutations leading to impaired complex assembly. The phenotype is predominantly Leigh syndrome, however, many patients have survived until adolescence or adulthood (Table 1). *NDUFAF8* was only recently reported to be a disease gene, with the patients described having presumably null mutations leading to a classic Leigh syndrome phenotype [38]. The assembly factors *NDUFAF3* and *NDUFAF4* are required for maturation of the Q-module and have been found in association with both *NDUFS3* and *NDUFS3* subunits [41] as well as the Q-module intermediate [32]. While the precise molecular function of *NDUFAF3* and *NDUFAF4* are not known, they appear to be essential for progression to the next assembly stage [22,32,42]. Consistent with this, patients for both have presented with severe infantile disease due to predominantly missense, presumably null variants (Table 1).

Following association with *NDUFAF3* and *NDUFAF4*, the assembled Q-module joins with the mtDNA-encoded **ND1** subunit and the assembly factor *TIMMDC1* [32]. Numerous mutations in *ND1* have been reported leading to Leber hereditary optic neuropathy (LHON) and mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) (Table 1). Generally, these patients have high mutant loads or are in some cases homoplasmic (100%) for the variants [43–45]. Like most mutations in mtDNA, variants in *ND1* generally lead to adult onset disease. *TIMMDC1* is only a recently identified disease gene with a single homozygous intronic variant detected, leading to a splicing defect and no detectable protein [46] (Table 1). The assembly factor *NDUFAF6* is also required for *ND1* biogenesis. While many missense variants in the *NDUFAF6* gene lead to Leigh syndrome, it does not appear to be stably associated with an intermediate and its precise function is not yet known [47]. Once the nascent Q–ND1 module is formed, it subsequently incorporates the subunits *NDUFA3*, *NDUFA8* and *NDUFA13* [32] to produce the final Q–ND1 intermediate. Both *NDUFA8* and *NDUFA13* are disease genes with missense variants leading to complex I deficiency, however, the few patients identified have survived into young adulthood (Table 1). The reason for only missense variants leading to reduced protein levels being reported for *NDUFA8* and *NDUFA13* might be explained by the fact that loss of either protein products in HEK293T cells leads to complete loss of complex assembly and activity [22], suggesting that complete absence of these proteins might be not tolerated. Finally, while *NDUFA1* is a subunit of the ND1 module, it is not added to the complex I until late during assembly [32]. Many missense variants in *NDUFA1*, encoded on the X chromosome, have been linked to complex I deficiency with phenotypes ranging from mild (survival into childhood) to severe (Leigh syndrome and death in early childhood) [48] (Table 1).

## Assembly of the ND2 module

In the early steps of the assembly of the ND2 module, the subunits **ND2**, *NDUFC1* and *NDUFC2* associate with assembly factors *ACAD9*, *ECSIT*, *NDUFAF1* and *COA1* (Figure 2). To this subcomplex, **ND3** and the assembly factors *TMEM126B* and *TMEM186*, and subsequently **ND4L** and **ND6** are added [32,49]. The aforementioned assembly factors are also known to form the mitochondrial complex I intermediate assembly (MCIA) complex which is essential for the assembly of the ND2 module [49]. *COA1*, which was originally suggested to be a complex IV assembly factor [50], has recently been shown to be involved in the early stages of **ND2** translation [51]. Patients have been described for all mtDNA-encoded subunits present in this intermediate (**ND2**, **ND3**, **ND4L** and **ND6**). The phenotype spectrum is broad and depends on both the variant itself and mutant load, however, Leigh syndrome, LHON and associated phenotypes are predominant (Table 2). Although *ND4L* is transcribed as a bicistronic mRNA with *ND4* (of the ND4 module) [52] no variants have been described that affect the both proteins. All assembly factors present in the MCIA complex, with the

**Table 2. Defects affecting biogenesis of the ND2 module**

| Gene            | A. S. F. | Types of genetic variants and protein impact   | Clinical presentations and relevant information  | Ref.          | OMIM   |
|-----------------|----------|--|--|---------------|--------|
| <i>MT-ND2</i>   | X        | Predominantly missense but also nonsense and deletion leading to frameshift and protein impact dependant on variant and mutant mtDNA load. | Variable onset and presentation from Leigh syndrome, LHON, mild exercise intolerance, myalgia with survival into late adulthood reported.  | [171,399,400] | 516001 |
| <i>MT-ND3</i>   | X        | Missense and protein impact dependant on variant and mutant mtDNA load.  | Variable onset and presentation including severe Leigh syndrome, encephalopathy, lactic acidosis, LHON, hypotonia, ataxia, seizures, dystonia with survival into adulthood reported.                                     | [401–406]     | 516002 |
| <i>MT-ND4L</i>  | X        | Missense leading to unclear protein impact.  | LHON. Decreased CI activity.   | [407,408]     | 516004 |
| <i>MT-ND6</i>   | X        | Missense and protein impact dependant on variant and mutant mtDNA load.  | Most common presentation of LHON and dystonia with variable onset, but also MELAS with survival into childhood and severe Leigh syndrome or adult onset Leigh-like syndrome.   | [409–413]     | 516006 |
| <i>NDUFA9</i>   | X        | Missense leading to decreased protein levels.  | Variable phenotype of childhood-onset of progressive dystonia developing neuropathy and Leigh syndrome without acidosis in adulthood or severe respiratory and metabolic acidosis, retinitis pigmentosa and early death. | [64,69]       | 603834 |
| <i>NDUFA10</i>  | X        | Predominantly missense but also insertion leading to decreased protein levels.   | Leigh syndrome combined with hypertrophic cardiomyopathy, hypotonia.   | [65,66]       | 603835 |
| <i>NDUFA11</i>  | X        | Missense and intronic with unclear protein impact.   | Variable presentation from mild late-onset myopathy to fatal infantile lactic acidosis, encephalocardiomyopathy, hypotonia, bilateral optic atrophy  | [67,68]       | 612638 |
| <i>NDUFAF1</i>  | X        | Missense leading to decreased or unclear protein levels.   | Hypertrophic cardiomyopathy, failure to thrive, developmental delay, lactic acidosis, hypotonia, leukodystrophy.   | [55–57]       | 606934 |
| <i>ACAD9</i>    | X        | Predominantly missense but also duplication leading to frameshift with unclear protein impact.   | Variable phenotype from mild growth retardation, exercise intolerance, cardiac hypertrophy surviving to adulthood to severe hypertrophic cardiomyopathy, encephalopathy and lactic acidosis.                             | [414,415]     | 611103 |
| <i>TMEM126B</i> | X        | Missense but also nonsense (frameshift) leading to unclear protein impact.   | Most common presentation of myalgia and exercise intolerance with survival to adulthood but also hypertrophic cardiomyopathy, renal tubular acidosis and severe muscle weakness. Decreased CI assembly.                  | [416,417]     | 615533 |

S., subunit; A.F., assembly factor; LHON, Leber's hereditary optic neuropathy; MELAS, mitochondrial encephalopathy, lactic acidosis and stroke-like episodes.

exception of ECSIT and newly identified members COA1 and TMEM186 [49], are known disease genes (Table 2). The function of NDUFAF1 (formerly CIA30) was first linked to complex I assembly through the use of fungal [53] and then mammalian [54] model systems, and eventually led to the identification of patients presenting with cardiomyopathies [55–57]. Fibroblasts from *NDUFAF1* patients have reduced levels of complex I and defective assembly of the ND2 module [55]. *ACAD9* was originally thought to be involved in  $\beta$ -oxidation

based on sequence homology with other members of the acyl-CoA family as well as its ability to bind acetyl-CoA substrates *in vitro* [58]. The association with cardiomyopathy [59], demonstration of its interaction with other MCIA members and requirement for complex I assembly [60] solidified its role as an assembly factor. *ACAD9* is one of the most common causes of complex I deficiency and while it typically presents as cardiomyopathy, the phenotype can be quite varied with milder presentations such as mild growth retardation and exercise intolerance also noted (Table 2) [61]. Interestingly, while it is now clear that *ACAD9* deficient cells lack  $\beta$ -oxidation defects, riboflavin supplementation has been shown to lessen symptoms in patients and improve complex I activity [61]. *ACAD9* is a flavoprotein (riboflavin is a precursor to flavin adenine dinucleotide; FAD), however, a catalytically dead mutant retains the ability to rescue complex I assembly, suggesting that *ACAD9* has a secondary role in mitochondrial function [62]. *TMEM126B* was identified as an assembly factor through complexome profiling studies [63] with patients later being identified. The role of *TMEM126B* in complex I assembly is still not completely clear, although we know this protein has multiple transmembrane domains and may act as an anchor for the other MCIA subunits, despite their levels remaining unaffected by loss of *TMEM126B* [49]. Patients harbouring mutations in *TMEM126B* also present milder phenotypes compared with those with mutations in *NDUFA1* and *ACAD9* (Table 2).

At this stage of complex I biogenesis there appears to be multiple parallel routes to the final complex, either the ND2 module joins with the Q–ND1 intermediate or the ND4 intermediate [32] (Figure 2). Once either the Q–ND1–ND2 subcomplex or ND2–ND4 subcomplex is assembled the ND2 module subunits *NDUFA9*, or *NDUFA10* and *NDUFS5* are respectively incorporated. *NDUFA11* appears to be added later during assembly once the membrane arm is fully built [32]. Despite the timing of these subunit additions, all are critical for complex I assembly in gene-edited HEK293T cells [22]. Patients have been identified for *NDUFA9*, *NDUFA10* and *NDUFA11* (Table 2) and generally present with severe infantile Leigh syndrome or encephalocardiomyopathy and isolated complex I deficiency [64–68], though there have been reports of milder *NDUFA9* and *NDUFA11* cases presenting with childhood-onset progressive dystonia [69] and late-onset myopathy respectively [68].

## Assembly of the ND4 and ND5 modules

The assembly steps of the ND4 and ND5 modules are less well characterised than the other modules, with assembly factors only identified in recent years. In the case of the ND4 module, a subcomplex containing four accessory subunits *NDUFB5*, *NDUFB6*, *NDUFB10* and *NDUFB11* assemble early and are followed by the addition of *NDUFB1* and the mtDNA-encoded subunit *ND4*. Like for *NDUFA10* and *NDUFS5* (discussed in the ND2 module section), *NDUFB4* seems to be incorporated into the ND4 module once the intermediate ND2–ND4 subcomplex is formed [32]. In our HEK293T model system, loss of all nuclear subunits present in the ND4 module leads to turnover of almost all complex I subunits, leaving only an intermediate containing Q/ND1 subunits module intact [22]. As such, defects in genes associated with the ND4 module lead to severe disease (Table 3). *ND4*, *NDUFB10*, *NDUFB11* are known disease genes and patients with variants in *ND4* largely present with similar clinical features to those with mutations in other mtDNA-encoded complex I subunits, with LHON the dominant phenotype but also Leigh syndrome and encephalopathy have been reported. Indeed, one *ND4* variant (11778A), accounts for more than half of the reported primary cause of LHON in Caucasian families [70] and over 90% in the Chinese families [71]. Although *ND4* and *ND4L* (found in the ND2 module) are transcribed as a bicistronic mRNA [52], no cases have been described where both proteins are affected. For *NDUFB10* there is a single known case that presented with severe neonatal cardiomyopathy leading to infantile death. The patient harboured a compound frame shift variant leading to early termination and a missense (p.C107S) variant leading to a mutation in the conserved CX<sub>n</sub>C motif important for import of the protein into mitochondria [72]. Consistent with an import defect, tissues had reduced but not absent levels of *NDUFB10* and accumulation of complex I intermediates. Similarly, variants in *NDUFB11* also typically lead to infantile cardiomyopathy, though the presentation is complicated by the gene being present on the X chromosome (Table 3) [66,73,74]. Interestingly, female patients appear to suffer strong skewing of X-chromosome inactivation toward the mutant allele [73].

The assembly factors *TMEM70*, *FOXRED1* and *ATP5SL* are found associated with a near complete ND4 module [22,32,75]. While their function in assembly of this module is not clear yet, all three interact with subunits of the ND4 module and their loss, either in model systems or patient cell lines [22,76] leads to accumulation of membrane assembly intermediates. While no patients have been identified for *ATP5SL*, both *TMEM70* and *FOXRED1* are known mitochondrial disease genes (Table 3). *FOXRED1* was identified through high-

**Table 3. Defects affecting biogenesis of the ND4 and ND5 modules**

Part 1 of 2

| Gene              | S. | A.<br>F. | Types of genetic<br>variants and protein<br>impact  | Clinical presentations<br>and relevant information  | Ref.            | OMIM   |
|-------------------|----|----------|---|---|-----------------|--------|
| <i>ND4 module</i> |    |          |   |   |                 |        |
| <i>MT-ND4</i>     | X  |          | Missense with unclear protein impact.   | LHON, early onset Leigh syndrome, late-onset encephalopathy, may present lactic acidosis. Phenotype severity may correlate with mutation load.  | [418–421]       | 516003 |
| <i>NDUFB10</i>    | X  |          | Missense and nonsense leading to impaired protein import.   | Fatal lactic acidosis and cardiomyopathy.   | [72]            | 603843 |
| <i>NDUFB11</i>    | X  |          | Nonsense but also missense and deletion leading to reduced protein levels.  | X-linked gene. Encephalopathy, cardiomyopathy, MIDAS, LIND, sideroblastic anaemia   | [66,73,74]      | 300403 |
| <i>FOXRED1</i>    |    | X        | Missense, frameshift and nonsense leading to unclear protein impact   | Variable phenotype from severe neonatal lactic acidosis with early death to Leigh syndrome, hypotonia, lactic acidosis, hypertrophic cardiomyopathy and survival into adolescence and adulthood. Decreased CI activity                        | [77,78,422]     | 613622 |
| <i>TMEM70</i>     |    | X        | Clinical cases are presented at the complex V defects section   |   |                 |        |
| <i>ND5 module</i> |    |          |   |   |                 |        |
| <i>MT-ND5</i>     | X  |          | Predominantly missense but also deletion leading to frameshift with protein impact dependant on variant and mutant mtDNA load | Variable onset MELAS, Leigh syndrome, LHON. May present as combination of the previous phenotypes with hypotonia, failure to thrive, cardiomyopathy, renal failure, myopathy. Phenotypes may vary according to age of onset and mutation load | [86,87,423–427] | 516005 |
| <i>NDUFB3</i>     | X  |          | Missense but also nonsense leading to unclear protein impact  | Variable phenotype from mild short stature, distinctive facial appearance surviving into childhood and decreased CI assembly to severe encephalopathy, myopathy, hypotonia, lactic acidosis, failure to thrive                                | [88–90]         | 603839 |
| <i>NDUFB8</i>     | X  |          | Predominantly missense but also frameshift  | Leigh syndrome, fatal infantile lactic acidosis,  | [91]            | 602140 |

Continued

**Table 3. Defects affecting biogenesis of the ND4 and ND5 modules**

Part 2 of 2

| Gene          | S. | A.<br>F. | Types of genetic<br>variants and protein<br>impact | Clinical presentations<br>and relevant information                           | Ref. | OMIM   |
|---------------|----|----------|--|--|------|--------|
|               |    |          | leading to decreased<br>protein levels             | respiratory failure, cardiac<br>hypertrophy, hypotonia,<br>failure to thrive |      |        |
| <i>NDUFB9</i> | X  |          | Missense leading to<br>decreased protein levels    | Early onset progressive<br>hypotonia, increased blood<br>lactate             | [92] | 601445 |

S., subunit; A.F., assembly factor; LHON, Leber's hereditary optic neuropathy; MIDAS, mitochondrial dysfunction-associated senescence; LIMD, lethal infantile mitochondrial disease; MELAS, mitochondrial encephalopathy, lactic Acidosis and stroke-like episodes.

throughput sequencing of a complex I deficient cohort [77] and since then many patients have been characterised [77–79]. The phenotype is variable, with both severe (Leigh syndrome, cardiomyopathy) and mild cases having been identified, presumably dependent on the severity of the particular variant(s). Interestingly, FOXRED is an FAD-dependent oxidoreductase, and although it is not clear if FAD binding is important in its function, Rendon and co-workers [79] identified a patient with relatively mild symptoms (epilepsy and severe psychomotor retardation) that suffered a combined complexes I and II defect, suggesting FOXRED1 may be involved in the biogenesis of multiple flavoprotein containing complexes. TMEM70 is an assembly factor implicated in the assembly of both complexes I and V, with functional studies showing that its loss leads to accumulation of assembly intermediates for both complexes [75,80]. In line with this dual role, patients commonly have cardiac and brain involvement and defects in both OXPHOS complexes [81] or isolated complex I or V deficiencies, varying according to tissue type [81–84] (Tables 3 and 14).

The ND5 module contains the core subunit **ND5** and accessory subunits **NDUFB2**, **NDUFB3**, **NDUFB7**, **NDUFB8**, **NDUFB9** and **NDUFAB1**. **NDUFAB1** is unique in that it is found in two pools, a soluble non-complex I associated pool where it associates with many different proteins containing the LYR motif [85] and a complex I associated pool [24]. In the case of the latter it is found twice, associating with the two LYR motif containing complex I subunits, **NDUFB7** of this module and **NDUFA6** of the N-module (discussed below) (Figure 2). Interestingly, loss of **NDUFAB1** in the gene-edited HEK293T model system leads to cell death (whereas loss of the other accessory subunits leads to a presumed shift to glycolysis), and while there is a severe complex I assembly defect the essential role of **NDUFAB1** appears to be related to its non-complex I associated pool [22]. Little is known about assembly of the ND5 module with the only intermediate observed containing all known subunits [32]. The recently identified assembly factor DMAC1 [22] is thought to be required for ND5 module assembly as it has been shown to interact with newly synthesised **ND5** and other subunits of the module [22], however, its molecular function is not yet known. Interestingly the stability of both the ND2 and ND5 modules appears to rely on the presence of a properly built ND4 module but not vice versa [22]. Defects in **ND5** lead to the typical late onset (LHON, MELAS) phenotypes observed in patients with mutations in mtDNA-encoded complex I subunits (Table 3) although there have been many reports of more severe infantile presentations [86,87]. Variants have been identified in **NDUFB3**, **NDUFB8** and **NDUFB9**. Patients present with typical severe childhood phenotypes underpinned by complex I dysfunction (e.g. encephalopathy, myopathy, hypotonia), dependent on the variants impact on protein function [88–92]. In line with the requirement of these genes for complex I assembly in the HEK293T model system [22], in all patients, residual levels of the presumably semi-functional proteins have been detected [88–92].

### Assembly of the N-module

The N-module, which is the site of NADH oxidation, is probably pre-assembled into two subcomplexes containing the **NDUFV1** and **NDUFV2** subunits and **NDUFS1** and **NDUFA2** subunits before incorporation with the Q-ND1 subcomplex (Figure 2) [32]. Defects have been identified in all four genes encoding these subunits (Table 4). In line with their central role in complex I enzyme function [24,25], patients present with severe disease, typically Leigh syndrome in infants, although some variants in **NDUFA2** which is not thought to be directly involved in NADH catalysis, lead to less severe disease and survival into childhood [93–102]. The

**Table 4. Defects affecting biogenesis of the N-module**

| Gene           | A. S. F. | Types of genetic variants and protein impact   | Clinical presentations and relevant information   | Ref.             | OMIM   |
|----------------|----------|--|---|------------------|--------|
| <i>NDUFA2</i>  | X        | Majority missense but also intronic and frameshift with unclear protein impact.                                  | Variable phenotype from severe Leigh syndrome, hypertrophic cardiomyopathy, severe lactic acidosis to cystic leukoencephalopathy and survival into childhood. Impaired CI assembly/activity.  | [93,94]          | 602137 |
| <i>NDUFA6</i>  | X        | Frameshift, nonsense and missense leading to unclear protein impact.   | Variable phenotype from optic atrophy, motor regression and survival into childhood to severe fatal lactic acidosis and brain abnormalities, hypotonia, seizures. Decreased CI assembly/activity.   | [106]            | 602138 |
| <i>NDUFA12</i> | X        | Nonsense leading to complete absence of protein.   | Leigh syndrome, dystonia, hypotonia, normal hearing and vision.   | [107]            | 614530 |
| <i>NDUFS1</i>  | X        | Predominantly missense but also nonsense with unclear protein impact.  | Variable severe phenotypes including Leigh syndrome, bilateral optic atrophy, hyperlactatemia, mental retardation, macrocytic anaemia, cavitating leukoencephalopathy, dystonia, hypotonia mostly leading to early death. Decreased CI assembly/activity. | [95–98]          | 157655 |
| <i>NDUFS4</i>  | X        | Variable. Nonsense, intronic, duplication and deletion causing frameshift. Unclear or undetected protein levels. | Variable presentations with predominant Leigh syndrome phenotype but also brain atrophy, cardiac hypertrophy, hypotonia, may present lactic acidosis and decreased CI activity.   | [108–111]        | 602694 |
| <i>NDUFS6</i>  | X        | Intronic, missense and deletion leading to uncertain or decreased protein level.                                 | Severe presentation of fatal lactic acidosis, Leigh syndrome.   | [112–114]        | 603848 |
| <i>NDUFV1</i>  | X        | Missense, nonsense and intronic leading to unclear protein impact.   | Leigh syndrome and may present progressive muscular hypotonia, myoclonic epilepsy, elevated plasma and cerebrospinal fluid lactate. CI deficiency.  | [95]             | 161015 |
| <i>NDUFV2</i>  | X        | Intronic deletion and insertion causing protein frameshift leading to decreased protein levels.                  | Hypertrophic cardiomyopathy and encephalopathy, Leigh syndrome, hypotonia.  | [100–102]        | 600532 |
| <i>NDUFAF2</i> | X        | Predominantly nonsense but also deletion causing frameshift leading to undetectable protein levels.              | Severe progressive brain abnormalities distinct from Leigh syndrome, hypotonia, may present apnoea, normal or mildly elevated plasma lactate.   | [77,116,118,119] | 609653 |
| <i>NUBPL</i>   | X        | Predominantly missense but also frameshift leading to decreased protein levels.                                  | Leukoencephalopathy, may present elevated serum and cerebrospinal fluid lactate.  | [77,104]         | 613621 |

S., subunit; A.F., assembly factor.

assembly factor NUBPL has a CXXC motif that binds iron–sulfur clusters and is thought to aid their delivery to **NDUFS1** and **NDUFV1**. Depletion of NUBPL in model systems leads to turnover of some N-module subunits and accumulation of intermediates [103]. NUBPL was identified in the same high-throughput sequencing study as FOXRED1 [77] and since then a few other patients have been identified [72,77,104]. Patients have milder symptoms than those harbouring mutations in the two known NUBPL substrates, typically presenting with leukoencephalopathy and childhood ataxia followed by other variable but progressive symptoms suggesting that biogenesis of **NDUFS1** and **NDUFV1** remains partially functional.

The subunits **NDUFA6**, **NDUFA7**, **NDUFA11**, **NDUFA12**, **NDUFS4**, **NDUFS6**, **NDUFV3** and the other copy of **NDUFAB1**, associated with LYR motif containing **NDUFA6**, seems to be incorporated at later

assembly stages of the complex I [32]. Some of these subunits appear to be able to be dynamically exchanged from the assembled complex, possibly to prevent accumulation of oxidatively damaged subunits [105]. Patients have been described for *NDUFA6* [106], *NDUFA12* [107], *NDUFS4* [108–111], and *NDUFS6* [112–114]. Patients with mutations in the latter three generally present with severe childhood Leigh syndrome (Table 4). Interestingly, loss of these proteins in the HEK293T model system leads to only mild defects in complex I assembly, turnover of only selected N-module subunits, and mild impacts on complex I function [22]. In line with this, mutations found in these patients generally lead to complete absence of the proteins (Table 4). In contrast, the known patients for *NDUFA6*, which is required for N-module assembly in model systems [22], present with variable symptoms ranging from severe infantile disease to survival into childhood, presumably due to differing effects on protein stability and function [106]. These examples highlight the delicate balance between the role of the protein in complex assembly and the severity of the variant and presentation of phenotypes in patients.

Finally, the N-module requires the assembly factor *NDUFAF2* for its biogenesis (Figure 2). *NDUFAF2* is found associated with a complex I intermediate that lacks the N-module, but contains a near complete Q–ND1–ND2–ND4–ND5 subcomplex [22,32,115]. HEK293T cell lines lacking some N-module subunits as well as patients with mutations in *NDUFAF2* contain this partially built assembly as a stable intermediate [22,105]. *NDUFAF2* is thought to have evolved through duplication of the *NDUFA12* gene [116], and the structure of the intermediate isolated from a fungal *NDUFS6* knockout model (lacking the N-module) shows *NDUFAF2* occupying the site usually taken by *NDUFA12* in the mature complex [117], giving rise to the suggestion that the assembly factor caps the near final Q–ND1–ND2–ND4–ND5 intermediate priming it for addition of the N-module. *NDUFAF2* patients present with complex I deficiency and severe progressive childhood disease. Most patients present with no detectable *NDUFAF2* protein [77,116,118,119], which given the loss of associated N-module in patients is reflected in the similar complex I defect upon loss of *NDUFAF2*, *NDUFA12*, *NDUFS4* and *NDUFS6* in gene-edited model systems [22], as well as the similarity of phenotypes observed in patients for these genes (Table 4).

## Complex II

Mitochondrial complex II (or succinate dehydrogenase) is an important enzyme that participates in both central metabolic processes relevant to mitochondrial energy generation: the MRC and the tricarboxylic acid cycle (TCA). This enzyme is responsible for the oxidation of succinate to fumarate with the extracted electrons used to reduce Coenzyme Q [120]. Complex II is the smallest complex in the OXPHOS system and the only one where all subunits are of nuclear origin (*SDHA*, *SDHB*, *SDHC*, *SDHD*). While all except for *SDHC* have been linked to mitochondrial disease (Table 5), genes associated with complex II are more commonly known for their association with tumorigenesis, particularly heritable paragangliomas (discussed below). *SDHA* is the most frequently associated with mitochondrial disease, with multiple variants and presentations leading to complex II deficiency having been described [121–129]. In general, patients carrying mutations in the *SDHA* locus predicted or shown to lead to reduced protein levels present with classical mitochondrial disease phenotypes including Leigh syndrome (Table 5). There are few examples of *SDHB* and *SDHD* patients with phenotypes in line with classical mitochondrial dysfunction. For the former, there are only three known patients [130,131] all harbouring the same homozygous transversion leading to an aspartate to valine substitution, reduced levels of *SDHB* protein and assembled complex II. Two of these patients presented neurologic impairment, developmental regression and leukoencephalopathy [130,131], but the third, a sibling of one affected individual, was asymptomatic [130] suggesting incomplete penetrance. As such this variant is still classified as a VUS (see OMIM 185470.0020). For *SDHD* there are only two known patients, both variants leading to reduced levels of protein and impaired complex II assembly, complex II deficiency, however, both presenting with fatal hypertrophic cardiomyopathy or encephalomyopathy [131,132] suggesting this is a genuine mitochondrial disease gene.

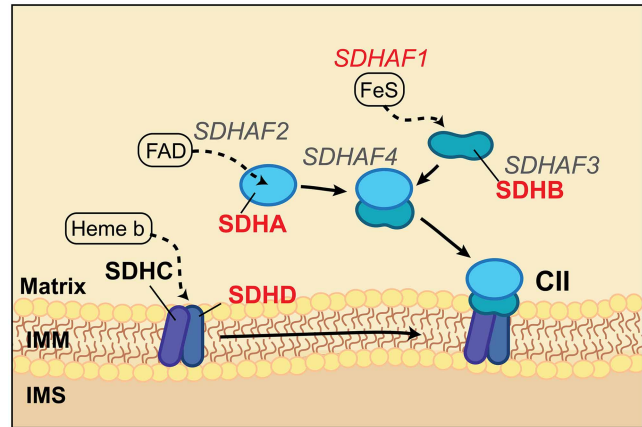
The assembly of this complex is not well characterised, with the known steps dominated by the insertion of two cofactors, thought to be catalysed by the assembly factors *SDHAF1–4* (Figure 3). The first step is likely the flavination of the *SDHA* subunit by the assembly factor *SDHAF2* [133,134]. Although the requirement for *SDHAF2* in the flavination of *SDHA* has been disputed in some cancer cell lines [135,136], evidence exists to the contrary, including both the absence of *SDHA* flavination in tumours harbouring heterozygous *SDHAF2* mutations [133] and a structure of the bacterial homologue of *SDHAF2* bound to *SDHA* [137]. *SDHA* also interacts with the assembly factor *SDHAF4*, which is proposed to prevent the generation of reactive oxygen species (ROS) from the oxidation of succinate by unassembled *SDHA* but has also been suggested to facilitate

Table 5. Defects affecting biogenesis of complex II

| Gene                     | S. | A. F. | Types of genetic variants and protein impact  | Clinical presentations and relevant information  | Ref.      | OMIM   |
|--------------------------|----|-------|---|--|-----------|--------|
| <i>SDHA</i>              | X  |       | Predominantly missense but also intronic and nonsense leading to unclear or decreased protein levels.                       | Majority presenting Leigh syndrome and may also present leukodystrophy, dystonia, ataxia, optic atrophy, lactic acidosis, cardiomyopathy.  | [121–129] | 600857 |
| <i>SDHB</i> <sup>1</sup> | X  |       | Single known homozygous missense variant with possible reduced penetrance but leading to decreased protein levels.          | Leukoencephalopathy, hypotonia or virtually asymptomatic, minor brain lesions.   | [127,130] | 185470 |
| <i>SDHD</i>              | X  |       | Predominantly missense but also disruption of stop codon and protein extension leading unclear or decreased protein levels. | Fatal hypertrophic cardiomyopathy or encephalomyopathy, developmental delay and lactic acidosis. Symptoms developed after viral infection. | [131,132] | 602690 |
| <i>SDHAF1</i>            |    | X     | Missense with unclear protein impact.   | Infantile leukodystrophy, spastic quadriparesis, lactate and succinate accumulation in the brain. Decreased CII activity/assembly.         | [143]     | 612848 |

<sup>1</sup>Possible VUS, see text for detail. S., subunit; A.F., assembly factor.

the interaction of **SDHA** with **SDHB** [138]. The other notable step is the insertion of an Fe–S prosthetic group into **SDHB**, which is thought to be incorporated prior to the formation of an **SDHA–SDHB** intermediate and be promoted by *SDHAF1* and *SDHAF3* [139–142]. *SDHAF1* is the only assembly factor known to be a mitochondrial disease gene, the two known homozygous missense variants lead to reduced complex II activity [143]. Patients present with infantile leukodystrophy and developmental regression, therefore having similarities



**Figure 3. Schematic depicting the complex II assembly pathway showing known mitochondrial disease genes in red and assembly factors in italics.**  
Heme b binds SDHC and SDHD. SDHA is flavinated (FAD) in the presence of *SDHAF2*. SDHB receives iron–sulfur (Fe–S) clusters through the assistance of *SDHAF1* and *SDHAF3* and binds SDHA to finalise the assembly of the complex. IMS, intermembrane space; IMM, inner mitochondrial membrane; Matrix, mitochondrial matrix.

to those harbouring the VUS in the associated subunit SDHB, discussed above (Table 5) [131,143]. Little is known about the assembly of the membrane anchored CII subunits, SDHC and SDHD. The heme b group situated at their interface is incorporated is required for their stability [144], but appears to play no catalytic role in the enzymatic function of the complex [145]. Interestingly the presence of both matrix exposed subunits, SDHA and SDHB is required for the stability of the SDHC/SDHD [134,141].

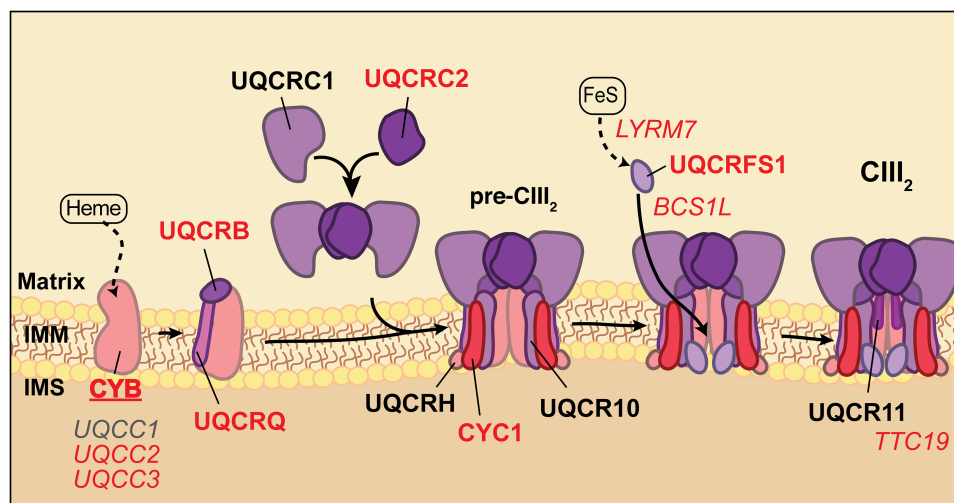
Unlike for the other OXPHOS complexes, the genes encoding succinate dehydrogenase subunits and assembly factors are better known as being implicated in tumorigenesis over mitochondrial disease. Mutations in SDHA [146], SDHB [147], SDHC [148], SDHD [147] and SDHAF2 [133,149] are linked to heritable paragangliomas, gastrointestinal stromal tumours, renal carcinomas and pituitary adenomas [150]. This is likely due to metabolic and epigenetic alterations [151] as unlike the mutations leading to complex II deficiency discussed above, those leading to tumorigenesis are typically heterozygous with modest effects on complex function. The impact of these mutations on complex II assembly and function are discussed in excellent recent reviews from Dalla Pozza *et al.* [151] and Bezawork-Geleta *et al.* [152].

## Complex III assembly

Complex III or the cytochrome bc<sub>1</sub> complex sits at the centre of the MRC, using electrons from complexes I and II via Coenzyme Q to reduce cytochrome c, while also pumping protons into the intermembrane space from the matrix [153]. Complex III forms an obligate homodimer (CIII<sub>2</sub>), with each monomer being composed of one mtDNA-encoded subunit (CYB) and nine nuclear-encoded subunits, CYC1, UQCRC1, UQCRC2, UQCRFS1, UQCRH, UQCRB, UQCRQ, UQCR10, UQCR11 [154,155]. CYB and the nuclear-encoded cytochrome c1 (CYC1) and UQCRFS1 are the three subunits with electron transfer capabilities [153,156]. Much of the human complex III assembly pathway has been extrapolated from studies of the yeast complex, which presents a similar structure and subunit composition [157–159]. Assembly can be broken down into many discreet steps (Figure 4), which are summarised below.

## Translation of CYB and initial steps of complex III assembly

The assembly of complex III begins with the translation of mtDNA-encoded cytochrome B (CYB) on mitribosomes and its co-translational insertion into the IMM (Figure 4) [158]. For efficient translation, the



**Figure 4. Schematic depicting the complex III assembly pathway showing known mitochondrial disease genes in red, mtDNA-encoded subunits underlined and assembly factors in italics.**

CYB is joined by UQCRB and UQCRQ. The subcomplex joins with a tetramer of UQCRC1 and UQCRC2, CYC1, UQCRH and UQCR10, resulting in the first dimeric intermediate, pre-CIII<sub>2</sub>. UQCRFS1 receives the iron–sulfur cluster from the assembly factor LYRM7 and is assembled into the pre-CIII<sub>2</sub> structure with the aid of the translocase BCS1L. The incorporation of UQCR11 subunit occurs at later stages of complex III assembly. The assembly factor TTC19 clears proteolytic fragments arising from UQCRFS1 maturation. IMS, intermembrane space; IMM, inner mitochondrial membrane; Matrix, mitochondrial matrix.

mitoribosome must be bound by a dimer of the assembly factors UQCC1 and UQCC2 [160,161]. This dimer interacts with the newly translated polypeptide at the mitoribosome exit tunnel and remains bound to **CYB** after its incorporation into the IMM [161], which is believed to be aided by the insertase OXA1L [162,163]. Like all other mtDNA protein coding genes, the phenotype and disease onset due to mutations in **CYB** depend on the variant and mutant load. Though many patients present with relatively mild symptoms (myopathy expressed as exercise intolerance; [164–169]), the classic LHON phenotype is also a common presentation [170–174] (Table 6). Interestingly, for many of these variants, complex III deficiency as well as high levels of mutant load appear to be restricted to muscle tissue. There are two known patients for the assembly factor UQCC2, both with mutations leading to near absence of the protein [160,175] (Table 6). In contrast with those with **CYB** mutations, the one UQCC2 patient harbouring complete loss of the protein due to defective mRNA splicing showed complex III deficiency in both the expected muscle tissue but also skin fibroblasts [160]. Another noteworthy point is that defects in UQCC2 also lead to a combined complex I defect [160,175], which is likely due to the reliance of complex I on the presence of complex III for its assembly [176].

**Table 6. Defects affecting translation of CYB and initial steps of complex III assembly**

| Gene          | S. | A.<br>F. | Types of genetic<br>variants and protein<br>impact  | Clinical presentations<br>and relevant information  | Ref.              | OMIM   |
|---------------|----|----------|---|---|-------------------|--------|
| <i>MT-CYB</i> | X  |          | Predominantly missense but also nonsense leading to reduced levels of protein. Effect is often restricted to muscle tissue. | Variable phenotypes and onset depending on variant, spanning from mild exercise intolerance and lactic acidosis, may develop encephalopathy and seizures in adulthood, may include multisystemic involvement (growth retardation, deafness, cognitive dysfunction) to LHON. A single severe case of fatal infantile cardiomyopathy has also been described. | [164–167,286,428] | 516020 |
| <i>UQCC2</i>  |    | X        | Missense or intronic splicing defect, leading to undetectable or very low protein levels.                                   | Lactic acidosis, growth retardation, neurological impairment.   | [160,175]         | 614461 |
| <i>UQCC3</i>  |    | X        | Single patient with missense leading to undetectable protein levels.  | Lactic acidosis, hypoglycaemia, hypotonia, delayed development.   | [178]             | 616097 |
| <i>UQCRB</i>  | X  |          | Single patient with deletion leading to C-terminal extension, but with unclear protein impact.                              | Episodic infantile lactic acidosis, hypoglycaemia, transient liver dysfunction, followed by normal psychomotor development in early childhood. Decreased CIII activity.   | [179]             | 191330 |
| <i>UQCRQ</i>  | X  |          | Missense leading to unclear protein impact.   | Severe non-lethal psychomotor retardation dystonia, athetosis, ataxia, mildly elevated blood lactate. Decreased CIII activity and variable CI deficiency.   | [180]             | 612080 |

S., subunit; A.F., assembly factor; LHON, Leber's hereditary optic neuropathy.

Following membrane integration of **CYB**, the first of two heme b molecules is incorporated at the  $b_L$  site [158,177], triggering the binding of the assembly factor UQCC3 which based on work on its fungal homologue is thought to promote the incorporation of a second heme b molecule at the  $b_H$  site [177]. This leads to dissociation of the UQCC1–UQCC2 dimer, which is free to initiate another round of **CYB** translation [161,177]. There is a single known patient for UQCC3, harbouring a missense variant leading to undetectable protein [178] (Table 6). The patient presented with isolated complex III deficiency (complex I was at the lower end of the control range in muscle), displayed lactic acidosis, hypotonia and delayed development, and fibroblasts exhibited defects in **CYB** and complex III assembly [178]. Fully hemylated **CYB** is then stabilised by the binding of the first nuclear-encoded subunits **UQCRB** and **UQCRQ** (Figure 4) [177]. Both of these subunits are mitochondrial disease genes, although there are only single variants known for each with limited cases (Table 6). For **UQCRB**, the single known case harbours a homozygous mutation leading to deletion of the last seven amino acids of the protein and inclusion of a new stretch of 14 amino acids derived from non-coding exons [179]. The protein impact of this mutation is not clear, although the patient, an infant who presented with episodic lactic acidosis, hypoglycaemia and liver dysfunction, had impaired complex III activity in liver, lymphocytes and fibroblasts. Interestingly, by the age of four the patient showed normal growth and psychomotor development. Without further cases or related functional studies, the mechanism underpinning the mild impact of this variant is unknown. For **UQCRQ** there is a single homozygous missense variant with multiple affected cases, however, these present with a more severe psychomotor retardation phenotype (Table 6). The impact of the mutation on protein function is unknown, although muscle from the patients has only a moderate complex III activity defect [180]. As for most other patients with defects affecting complex III, complex I activity was also moderately impaired in some, but not all of the reported cases.

### Assembly of the pre-complex III dimer

Parallel to the coalescence of hemylated **CYB** with **UQCRB** and **UQCRQ**, matrix facing subunits **UQCRC1** and **UQCRC2** form a separate tetrameric module (i.e. a dimer of each), which is likely incorporated into the **CYB**-containing module simultaneously with **CYC1** and **UQCRH** to yield the first dimeric intermediate of complex III, the pre-CIII<sub>2</sub> (Figure 4) [181]. This step coincides with the dissociation of UQCC3, which may prevent the dimerisation of earlier intermediates [181]. Following dimerisation, the subunit **UQCR10** is added to pre-CIII<sub>2</sub> [158]. **UQCRC2** and **CYC1** are mitochondrial disease genes (Table 7). Although there exist only a few patients for both, there is a consistent and similar phenotype and clinical progression to what is seen in the **UQCRB** patient [179]. Patients present with recurring episodes of metabolic acidosis that largely resolves in childhood or early adulthood [182–184]. Analysis of fibroblasts from the patients have combined complexes I and III defects in assembly, though the complex I enzyme defect is not significant for the **CYC1** patients [184]. The mechanism underpinning the similarity in phenotypes between these patients and those with mutations in **UQCRB** is not yet clear, though suggests a similar impact on complex III assembly and function.

**Table 7. Defects affecting assembly of the pre-complex III dimer**

| Gene          | S. | A.F. | Types of genetic variants and protein impact | Clinical presentations and relevant information  | Ref.      | OMIM   |
|---------------|----|------|--|--|-----------|--------|
| <i>CYC1</i>   | X  |      | Missense leading to near absence of protein  | Episodic severe infantile ketoacidosis, insulin-responsive hyperglycemia, hyperammonemia followed by normal development in childhood or early adulthood. Isolated CIII deficiency        | [184]     | 123980 |
| <i>UQCRC2</i> | X  |      | Missense leading to uncertain protein impact | Episodic infantile hypoglycaemia, lactic acidosis, ketonuria, variable liver failure followed by normal development in childhood. CIII deficiency and may present combined CI deficiency | [182,183] | 191329 |

S., subunit; A.F., assembly factor.

## Biogenesis of UQCRFS1 and final steps of assembly

The Rieske iron–sulfur protein, **UQCRFS1**, is first imported into the matrix where it is bound by the chaperone LYRM7 which stabilises it and mediates the insertion of a 2Fe–2S cluster (Figure 4) [185,186]. Now-folded and containing an iron–sulfur cluster, **UQCRFS1** is thought to be translocated into the IMM by the AAA+ ATPase BCS1L [187]. BCS1L is an inner membrane protein that forms a matrix-sided heptameric ring structure [188], allowing for the translocation of the hydrophilic folded C-terminus of **UQCRFS1** across the inner membrane [187–189]. The incorporation of **UQCRFS1** marks the important transition to a catalytically active complex III [158]. Both assembly factors and the **UQCRFS1** gene itself are linked to mitochondrial disease, with multiple variants described, most of which lead to severely reduced protein levels of the corresponding protein (Table 8). Functional studies in fungal models have indicated that absence of the LYRM7 homologue leads to destabilisation and degradation of **UQCRFS1** [185,190,191], although a small amount of **UQCRFS1** is still assembled into complex III, which is not seen in cells lacking the homologue of BCS1L [187]. In line with this, patients for all three genes have some similarity in clinical presentation, particularly LYRM7 and **UQCRFS1**, both of which present severe infantile conditions consistent with complex III deficiency (Table 8) [192–195]. While BCS1L patients can also present with these symptoms, there is considerable symptomatic variability depending on the variant, including hepatic iron overload and the absence of a complex III defect entirely (Table 8) [196–202]. This had led to suggestions that BCS1L may have another yet to be characterised role in mitochondrial function [196].

**Table 8. Defects affecting biogenesis of UQCRFS1 and final steps of CIII assembly**

| Gene           | S. | A. F. | Types of genetic variants and protein impact  | Clinical presentations and relevant information   | Ref.          | OMIM   |
|----------------|----|-------|---|---|---------------|--------|
| <i>UQCRFS1</i> | X  |       | Missense, intronic, nonsense leading to total absence or decreased protein levels.                      | Severe hypertrophic cardiomyopathy, lactic acidosis, alopecia. Isolated CIII deficiency. Depending on severity of variant survival into childhood with slightly lightly impaired gross and fine motor skills.   | [195]         | 191327 |
| <i>BCS1L</i>   |    | X     | Predominantly missense but also nonsense leading to decreased protein levels or unclear protein impact. | Variable phenotype including i) mild Björnstad syndrome (pili torti, neurosensory deafness), ii) severe Leigh syndrome, tubulopathy, hepatic dysfunction, metabolic acidosis, iii) GRACILE syndrome. May not present CIII deficiency.                                       | [196–201]     | 603647 |
| <i>LYRM7</i>   |    | X     | Multiple. Nonsense, intronic, deletion, duplication, missense leading to decreased protein levels.      | Lactic acidosis, early onset multifocal cavitating leukoencephalopathy, fatal neurologic decompensation. Isolated CIII deficiency.  | [192–194]     | 615831 |
| <i>TTC19</i>   |    | X     | Predominantly nonsense but also deletions likely leading to absence of protein.                         | Variable onset and neurological phenotypes including psychiatric symptoms, progressive neurodegenerative disorder, developmental delay, ataxia, as well as Leigh syndrome. Isolated CIII deficiency. Onset tends to late childhood with some cases presenting in adulthood. | [203,205–208] | 613814 |

S., subunit; A.F., assembly factor; GRACILE, growth retardation, amino aciduria, cholestasis, iron overload, lactic acidosis and early death.

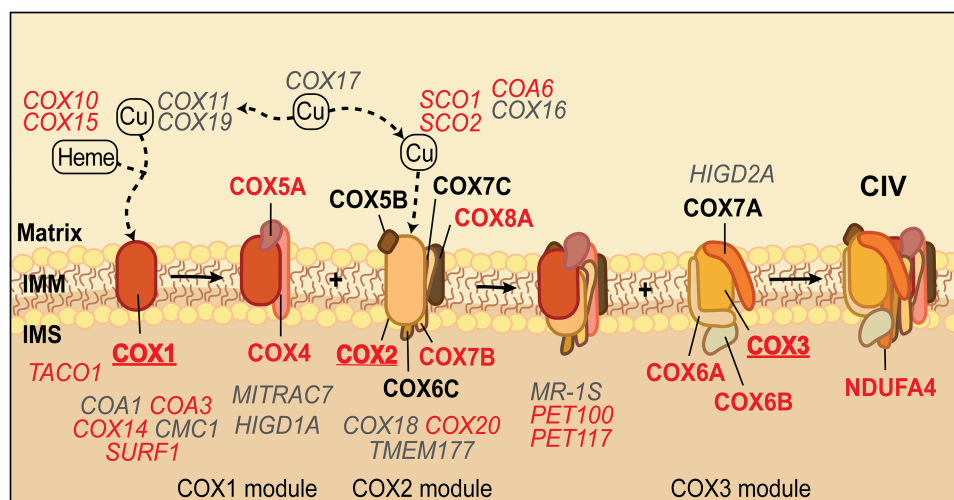
The final steps of complex III assembly include the incorporation of the last subunit, **UQCRI1** [158], followed by transient association of the assembly factor, **TTC19**. Although with unknown function at the time, **TTC19** was identified in patients with progressive neurological phenotypes and isolated complex III deficiency [203]. Functional studies using a mouse knockout model showed that **TTC19** is involved in proteolytic clearance of protein fragments derived from the N-terminus of **UQCRI1** that likely inhibit the enzyme [204]. Many other patients and variants have since been reported, generally leading to loss of detectable protein and similar late-onset phenotypes (Table 8) [192–201,205–208]).

## Complex IV assembly

Complex IV or cytochrome c oxidase (COX) is the last proton-pumping enzyme in the ETC and displays many interesting features compared with other OXPHOS complexes. For instance, complex IV has the highest ratio of known assembly factors per subunit as well as tissue and developmental specific isoforms which together adds complexity for the diagnosis of mitochondrial diseases [6,209,210]. Mammalian complex IV consists of 14 subunits of which three are core subunits encoded on mtDNA, **MT-CO1** (frequently referred to as **COX1**), **MT-CO2** (**COX2**) and **MT-CO3** (**COX3**) with the remainder encoded by nDNA, **COX4** (with 2 possible isoforms encoded on separate genes, **COX4I1** and **COX4I2**), **COX5A**, **COX5B**, **COX6A** (2 possible isoforms, **COX6A1-2**), **COX6B** (2 isoforms, **COX6B1-2**), **COX6C**, **COX7A** (3 isoforms, **COX7A1-3**), **COX7B**, **COX7C**, **COX8A** and **COX8C** [211–213]. It is generally thought that assembly of complex IV occurs in a modular fashion (Figure 5) [210,214,215], with the three mt-DNA encoded acting as platforms for assembly of nDNA encoded subunits into modules.

### Assembly of the COX1 module

The assembly of complex IV starts with translation of **COX1**, which requires a specific translational activator (**TACO1**) [216,217], and **COA3** and **COX14**, which appear to interact with **COX1** [218–220] and suggested to prevent its degradation [218,220]. Another assembly factor, **CMC1**, has also been implicated in stabilising **COX1** at this stage of assembly [221]. At this early step, mutations in **TACO1** have been linked to a range of phenotypes, commonly presenting as Leigh syndrome, optic atrophy and muscle involvement with variable



**Figure 5. Schematic depicting the complex IV assembly pathway showing known mitochondrial disease genes in red, mtDNA-encoded proteins underlined and assembly factors in italics.**

Complex IV assembly is driven by the coalescence of three distinct modules named after the mtDNA-encoded subunits they contain, COX1, COX2 and COX3. COX1 receives heme and copper (Cu) cofactors and joins COX4 and COX5A to form the COX1 module. COX2 receives copper and is assembled with COX5B, COX6C, COX7B, COX7C and COX8A to form the COX2 module. COX1 and COX2 module are integrated prior to addition of the COX3 module, which is composed of COX6A, COX6B and COX7A. NDUF4A is the last subunit to be added to form the mature complex IV. IMM, inner mitochondrial membrane; Matrix, mitochondrial matrix.

survival [216,222,223]. Importantly, TACO1 patients have overlapping phenotypes to those with disease caused by mutations in **COX1** (Table 9). Patients with mutations in **COX1** (*MT-CO1*) tend to survive into adulthood and present with phenotypes common to other mtDNA-encoded genes such as Leigh syndrome, lactic acidosis, hearing loss and myopathy, although other presentations including recurrent myoglobinuria and cerebellar ataxia have also been reported, and indeed overlap with patients with mutations in other mtDNA-encoded complex IV subunits [224–226]. Mutations in **COA3** have also been identified as the cause of a relatively mild phenotype of peripheral neuropathy, with exercise intolerance and short stature without clear involvement of heart, liver or brain [227], while a homozygous mutation in its assembly partner, **COX14**, leads to a severe phenotype of respiratory distress, lactic acidosis, hypertrophic cardiomyopathy, brain hypertrophy, microphthalmia and ketonuria [218]. The mild phenotype of the **COA3** patient compared with the severe phenotype of **COX14** patients might not be explained by residual protein levels as both patients showed almost or totally absent **COX14** protein [218,227] and reduced **COA3** levels [227]. This might suggest that although these two assembly factors are involved at the same step in complex IV assembly their precise function might differ.

The insertion of heme A is a two-step process performed by **COX10**, which converts heme B to heme O, and **COX15**, which converts heme O to heme A [228]. The heme A group is thought to be delivered to **COX1** by **SURF1** based on its ability to stoichiometrically bind heme A [229,230]. This step is thought to occur after dissociation of **CMC1**, which coincides with the binding of nuclear-encoded subunits **COX4** and **COX5A** [221] and the formation of the **COX1** module (Figure 5). Patients have been identified harbouring mutations in both **COX4** isoform encoding genes, with clinical phenotypes shown to differ depending on which isoform is affected. For example, variants in the ubiquitously expressed **COX4I1** [231] cause short stature, increase in chromosomal breaks and a phenotype similar to Fanconi anaemia [232], as well as more severe presentations of developmental delay, short stature and seizures, resembling Leigh syndrome in two siblings [233]. The two severely affected siblings also share compound heterozygous missense variants in **MDN1**, which is a AAA ATPase involved in cytosolic ribosomal biogenesis [234,235]. Despite the lack of functional studies to confirm pathogenicity of the **MDN1** variants, their involvement in the more severe phenotype cannot be discarded and might account for their combined OXPHOS deficiency, especially given that their unaffected sibling has inherited only one **MDN1** variant [233]. Likewise, **COX4I2** is highly expressed in lungs [231] and in the pancreas [236] with mutations in this gene leading to exocrine pancreatic insufficiency, dyserythropoietic anaemia, calvarial hyperostosis and failure to thrive, without a complex IV defect detected in fibroblasts [236] (Table 9). Taken together, the distinct expression patterns of **COX4I1** and **COX4I2** seem to correlate with their generalised or tissue-specific presentations. The third **COX1** module subunit, **COX5A**, has also been linked to disease, with mutations in **COX5A** leading to early onset pulmonary hypertension, brain abnormalities and lactic acidosis [237] (Table 9). Mutations in the assembly factors **COX10** and **COX15** have been reported to cause variable severe phenotypes including lactic acidosis, Leigh syndrome, hypertrophic cardiomyopathy and hypotonia [238–241], while mutations in **SURF1**, one of the most common causes of Leigh syndrome (reviewed in [242], has also been associated with other severe phenotypes such as Charcot–Marie–Tooth disease, rapidly progressive encephalopathy, ataxia, hypotonia, lactic acidosis and early death (Table 9). Analysis of patient-derived fibroblasts harbouring null mutation in **SURF1** revealed a rapidly degraded monomer and an accumulation of a complex IV subassembly, while a fully assembled complex IV was present in supercomplexes — higher order structures comprised of complexes I, III and IV [11]. Interestingly this phenomenon may be tissue specific, as analysis of different tissues from a patient harbouring truncating mutations in **SURF1** revealed decreased levels of fully assembled complex IV in heart, brain and muscle with increased accumulation of complex IV sub-assemblies in heart and muscle, but absent from brain [243].

The copper biosynthesis and insertion in mammalian cells has mostly been extrapolated from yeast studies. In this pathway, the  $\text{Cu}_B$  group is moved by **COX17** to **COX11**, which is a metallochaperone [244,245] containing a conserved copper-binding motif [246]. **COX11** also requires another assembly factor, **COX19**, to maintain its redox state [247] after copper binding. Finally, **MITRAC7** is a **COX1**-specific chaperone that prevents **COX1** degradation prior to the fusion of the **COX1** and **COX2** modules (Figure 5) [248]. Another protein thought to be involved in assembly of the **COX1** module is **HIGD1A**. Based mainly on studies primarily performed in yeast, **HIGD1A** is thought to bind and stabilise **COX4** and **COX5A** subunits prior to incorporation to **COX1** [215] and potentially stay bound to regulate complex IV activity [249]. However, while mammalian **HIGD1A** associates with **COX4** and **COX5A** [214,215], knockout of **HIGD1A** in mammalian cells had only a very minor effect on the stability of **COX4** and **COX5A** subunits, or complex IV assembly more broadly [214,250].

**Table 9. Defects affecting assembly of COX1 module**

Part 1 of 2

| Gene                             | S. | A.<br>F. | Types of genetic<br>variants and<br>protein impact   | Clinical presentations and<br>relevant information  | Ref.                   | OMIM   |
|----------------------------------|----|----------|--|---|------------------------|--------|
| <i>MT-CO1</i><br>( <i>COX1</i> ) | X  |          | Missense, nonsense<br>leading to unclear<br>protein impact.                                  | Majority of cases present<br>survival into adulthood and<br>phenotypes of late-onset<br>Leigh syndrome, recurrent<br>myoglobinuria, lactic acidosis,<br>cerebellar ataxia, optic<br>atrophy, hearing loss,<br>myopathy.                     | [224–226]              | 516030 |
| <i>COX4I1</i>                    | X  |          | Missense or<br>deletion/insertion<br>leading to unclear or<br>decreased protein<br>levels.   | Short stature, increased<br>chromosomal breaks,<br>resembling Fanconi anaemia<br>or severe cases of<br>developmental delay, short<br>stature and seizures,<br>resembling Leigh syndrome.  | [232,233]              | 123864 |
| <i>COX4I2</i>                    | X  |          | Missense leading to<br>unclear protein<br>impact.  | Exocrine pancreatic<br>insufficiency, dyserythropoietic<br>anaemia, calvarial<br>hyperostosis, failure to thrive.   | [236]                  | 607976 |
| <i>COX5A</i>                     | X  |          | Missense leading to<br>decreased protein<br>levels.  | Early-onset pulmonary<br>hypertension, lactic acidosis,<br>heart abnormalities, failure to<br>thrive.   | [237]                  | 603773 |
| <i>COA3</i>                      |    | X        | Missense and<br>duplication causing<br>frameshift leading to<br>decreased protein<br>levels. | Peripheral neuropathy,<br>exercise intolerance, short<br>stature. Survival to adulthood.  | [227]                  | 614775 |
| <i>COX10</i>                     |    | X        | Missense leading to<br>unclear protein<br>impact.  | Variable severe phenotypes<br>mostly presenting metabolic<br>acidosis and anaemia<br>combined with Leigh<br>syndrome or hypertrophic<br>cardiomyopathy but also<br>tubulopathy, ataxia, hypotonia<br>and early death.                       | [238,239]              | 602125 |
| <i>COX14</i>                     |    | X        | Missense leading to<br>undetectable protein<br>levels.                                       | Severe lactic acidosis,<br>microphthalmia, ketonuria,<br>hypertrophic cardiomyopathy,<br>respiratory distress, brain<br>hypertrophy.  | [218]                  | 614478 |
| <i>COX15</i>                     |    | X        | Missense, intronic<br>and nonsense<br>leading to unclear<br>protein impact.                  | Variable severe phenotypes.<br>Fatal infantile hypertrophic<br>cardiomyopathy, lactic<br>acidosis, seizures, hypotonia<br>or Leigh syndrome, failure to<br>thrive, psychomotor delay,<br>hypotonia, elevated plasma<br>lactate and pyruvate | [240,241]              | 603646 |
| <i>SURF1</i>                     |    | X        | Over 80 mutations<br>from variable<br>genetic nature.<br>Missense,<br>nonsense,              | Most common cause of Leigh<br>syndrome associated with CIV<br>deficiency but variable severe<br>phenotypes including<br>Charcot-Marie-Tooth disease,  | (Reviewed in<br>[242]) | 185620 |

Continued

Table 9. Defects affecting assembly of COX1 module

Part 2 of 2

| Gene  | S. | A. F. | Types of genetic variants and protein impact                                  | Clinical presentations and relevant information   | Ref.          | OMIM   |
|-------|----|-------|---|---|---------------|--------|
|       |    |       | insertions, deletions, intronic.  | rapidly progressive encephalopathy, ataxia, hypotonia, lactic acidosis and early death.   |               |        |
| TACO1 |    | X     | Duplication causing frameshift or missense leading to unclear protein impact. | Variable phenotype from mild mental retardation and survival to early adulthood to severe slowly progressive childhood-onset of Leigh syndrome, may present optic atrophy, dystonia, spastic tetraparesis, renal tubulopathy. | [216,222,223] | 612958 |

S., subunit; A.F., assembly factor.

## Assembly of the COX2 module

The assembly of the COX2 module starts with the translation and membrane insertion of the first transmembrane domain of COX2, which is stabilised by COX20 [Figure 5] [251]. The insertion of the second transmembrane domain also leads to translocation of the globular copper-binding domain which occurs with the assistance of assembly factor COX18 [251]. Patients harbouring mutations in the mtDNA-encoded COX2 (MT-CO2) have been reported to present with phenotypes ranging from mild exercise intolerance and recurrent myoglobinuria or late-onset cerebellar ataxia to severe fatal lactic acidosis, depending on variant and mutant load [252–255]. There are some overlap in phenotypes seen in cases caused by mutations in the assembly factor COX20 [256–259], particularly ataxia and hypotonia with survival into adulthood reported (Table 10).

The insertion of the copper centre (Cu<sub>A</sub>) in COX2 requires five known assembly factors: COX17, SCO1, SCO2, COA6 and COX16 [Figure 5]. COX17 donates copper to SCO1 [260], a metallochaperone that delivers the copper to COX2. Copper delivery to COX2 also requires SCO2, another metallochaperone that reduces the disulfide bonds in COX2 to allow the copper insertion [261]. COA6 and COX16 also interact with COX2, probably at the time of copper insertion, and promote the function of SCO1 and SCO2 [262–264]. COX16 has also been shown to act in the recruitment of the COX1 module to the COX2 module in the next step of assembly, in a COX2-dependent manner [264]. Another putative assembly factor, TMEM177, was shown to associate with the COX2/COX20/SCO1/SCO2 intermediate [265], and while TMEM177 is thought to regulate COX20 levels, loss of TMEM177 does not impair complex IV assembly in mammalian HEK293T cells [265]. Of the proteins involved in copper assembly, mutations have been identified in SCO1, SCO2 and COA6, all leading to severe phenotypes and commonly linked to fatal infantile cardiomyopathy, with shared clinical presentations of encephalopathy, liver failure, respiratory distress and metabolic acidosis [88,266–272] (Table 10).

The timing of the incorporation of the mammalian subunits COX5B, COX6C, COX7B, COX7C and COX8A to the COX2 module remain unclear as well as the precise functions of associated assembly factors PET100, PET117 and MR-1S [215,273–275]. Despite their unclear roles, functional studies suggest the interaction between PET117, MR-1S and complex IV subunits are mediated by PET100 [215]. At this step, mutations in COX7B and COX8A and the genes encoding assembly factors PET100 and PET117 have been linked to diseases with variable presentations [274,276–280] (Table 10). COX7B is an X-linked gene, and while many variants have been described, all known cases are in females who present with facial dysmorphism, linear skin defects, short stature with variable presentation of tetralogy of Fallot and ventricular hypertrophy [276,277]. For COX8A, a homozygous intronic mutation has been shown to cause a more severe phenotype of Leigh-like syndrome, developmental delay, pulmonary hypertension, epilepsy and elevated lactate in blood and cerebrospinal fluid [278]. For PET100 and PET117 it is useful to consider the fungal studies where much more is known about their function. The yeast homologue pPet100 was found in an assembly intermediate containing pCox7, pCox7a and pCox8 (human COX7A, COX6C and COX7C) and loss of pPet100 leads to its

**Table 10. Defects affecting assembly of COX2 module**

Part 1 of 2

| Gene                             | S. | A.<br>F. | Types of genetic<br>variants and protein<br>impact  | Clinical presentations and<br>relevant information  | Ref.         | OMIM   |
|----------------------------------|----|----------|---|---|--------------|--------|
| <i>MT-CO2</i><br>( <i>COX2</i> ) | X  |          | Missense, nonsense,<br>deletion leading to<br>unclear or decreased<br>protein levels.                       | Variable phenotypes from<br>mild exercise intolerance,<br>recurrent myoglobinuria,<br>late-onset cerebellar ataxia<br>and survival into adulthood<br>to severe cases of lactic<br>acidosis and early death.                     | [252–255]    | 516040 |
| <i>COA6</i>                      |    | X        | Mostly missense but<br>also nonsense leading<br>to decreased protein<br>levels.                             | Hypertrophic<br>cardiomyopathy may present<br>lactic acidosis. Decreased CI<br>and CIV in heart but not<br>effect in fibroblasts.   | [88,271,272] | 614772 |
| <i>COX7B</i>                     | X  |          | Intronic and deletion<br>causing protein<br>frameshift or nonsense<br>leading to unclear<br>protein impact. | X-linked gene. Facial<br>dysmorphism, linear skin<br>lesions with survival into<br>childhood, may present<br>tetralogy of Fallot, ventricular<br>hypertrophy.   | [276,277]    | 300885 |
| <i>COX8A</i>                     | X  |          | Intronic leading to<br>unclear protein impact.  | Leigh-like syndrome,<br>developmental delay,<br>pulmonary hypertension,<br>epilepsy, elevated blood and<br>cerebrospinal fluid lactate.<br>Decreased CIV assembly.  | [278]        | 123870 |
| <i>COX20</i>                     |    | X        | Predominantly<br>missense but also<br>intronic leading to<br>decreased protein<br>levels.                   | Most presentations include<br>ataxia, hypotonia but can<br>also present mild elevation of<br>blood lactate, sensory<br>neuropathy and static<br>encephalopathy with<br>reported survival into<br>adulthood.                     | [256–259]    | 614698 |
| <i>PET100</i>                    |    | X        | Nonsense or missense<br>abolishing first<br>methionine leading to<br>unclear protein impact.                | Variable severe phenotypes.<br>Fatal infantile lactic acidosis,<br>brain abnormalities, severe<br>coagulopathy or Leigh<br>syndrome, elevated blood<br>lactate, seizures, hypotonia.<br>but Decreased CIV<br>assembly/activity. | [274,279]    | 614770 |
| <i>PET117</i>                    |    | X        | Nonsense leading to<br>unclear protein impact.  | Brain and motor<br>development regression with<br>survival into adulthood.<br>Decreased CIV activity/<br>assembly.  | [280]        | 614771 |
| <i>SCO1</i>                      |    | X        | Predominantly<br>missense or frameshift<br>may lead to decreased<br>protein levels.                         | Liver failure, encephalopathy,<br>hypotonia, metabolic<br>acidosis, may present<br>cardiac hypertrophy or<br>respiratory distress.  | [266,267]    | 603644 |

Continued

**Table 10. Defects affecting assembly of COX2 module**

Part 2 of 2

| Gene | S. | A. F. | Types of genetic variants and protein impact  | Clinical presentations and relevant information  | Ref.      | OMIM   |
|------|----|-------|---|--|-----------|--------|
| SCO2 |    | X     | Mostly missense but also nonsense and duplication leading to unclear or reduced protein levels. | Fatal infantile hypertrophic cardiomyopathy, encephalopathy, elevated blood lactate, respiratory distress. | [268–270] | 604272 |

S., subunit; A.F., assembly factor.

accumulation together with another subcomplex composed of **Cox5a** and **Cox6** (human **COX4I1** and **COX5A**), thus preventing the assembly of the mature complex IV [273]. Consistently, the patients reported to harbour **PET100** mutations show undetectable [279] or residual mature complex IV [274], with the former displaying a more severe phenotype and neonatal death [279]. In the case of pPet117, yeast studies have shown that it stabilises Cox15 oligomers and might function in heme A synthesis and/or transfer to **pCox1**, and is also required for complex IV assembly [275]. In contrast with this, mammalian studies have shown that **PET117** interacts with **PET100**, **MR-1S** and other subunits of the **COX1** and **COX2** modules [215], suggesting that mammalian **PET117** could have a distinct function to its yeast counterpart. Despite unknown effect of **PET117** mutation on protein levels, the patients reported with a homozygous nonsense mutation display decreased complex IV assembly and a milder phenotype [280] when compared with **PET100** patients [274,279].

## Assembly of the COX3 module

The **COX3** module is the last module to be added to the now complete **COX1** and **COX2** modules in the nascent complex IV assembly (Figure 5). This module consists of the core subunit **COX3** and the nuclear-encoded subunits **COX6A**, **COX6B** and **COX7A**. Even though complex IV displays the highest assembly factor to subunit ratio amongst other OXPHOS complexes, it was surprising that no assembly factors have been found to be required for building the **COX3** module. Although **COX3** is not directly involved in electron transport, it is thought to play a regulatory role in enzyme function [211,281]. **HIGD2A** was previously thought to act as an assembly factor for the complexes I, III, IV supercomplex [282] but recently shown to be required for the assembly of the **COX3** module [214]. While **HIGD2A** appears to be needed for the early steps of **COX3** biogenesis, it is unclear if it is involved in **COX3** translation, membrane integration or its integration with the nascent complex IV assembly. Following coalescence of the **COX1**, **COX2** and **COX3** modules, **NDUFA4**, formerly thought to be a complex I subunit [212], is likely the last subunit to be incorporated to form the mature complex IV [210].

Similar to other mitochondrially encoded genes, mutations in **MT-CO3 (COX3)** cause a variety of phenotypes and onsets including Leigh-like syndrome, myopathy, lactic acidosis, MELAS, LHON and recurrent myoglobinuria with survival into adulthood reported [172,283–286] (Table 11). Interestingly, the phenotype of recurrent myoglobinuria is also found in patients harbouring mutations in **COX1 (MT-CO1)** [225] and **COX2 (MT-CO2)** [252]. In the case of **COX6A1** and **COX6A2**, mutations have been reported to cause different clinical presentations. While a homozygous deletion in **COX6A1** was shown to cause a neuromuscular disease called Charcot–Marie–Tooth disease [287], mutations in **COX6A2** lead to a muscle-specific presentation of myopathy and cardiomyopathy [288]. As **COX6A2** is exclusively expressed in heart and skeletal muscle [289], the mutations reported in **COX6A2** were shown to only affect complex IV activity in differentiated muscle and not in undifferentiated myoblasts [288]. For the only hydrophilic extramembrane subunit of complex IV facing the IMS, **COX6B1** [290], pathogenic variants have been shown to cause severe infantile encephalomyopathy, cardiomyopathy and lactic acidosis. The last subunit to be assembled into complex IV, **NDUFA4**, has been reported to cause Leigh syndrome, congenital lactic acidosis and variable presentation of dystonia due to a homozygous intronic variant. The intronic variant leads to undetectable **NDUFA4** protein levels via western blot analysis and was shown to not impair the assembly of the other 13 complex IV subunits [291].

**Table 11. Defects affecting assembly of COX3 module**

| Gene                             | S. | A.<br>F. | Types of genetic<br>variants and protein<br>impact   | Clinical presentations and<br>relevant information   | Ref.              | OMIM   |
|----------------------------------|----|----------|--|--|-------------------|--------|
| <i>MT-CO3</i><br>( <i>COX3</i> ) | X  |          | Missense, nonsense,<br>insertion, deletion<br>leading to unclear or<br>decreased protein<br>levels depending on<br>variant and mutation<br>load. | Variable phenotype, including<br>Leigh-like syndrome,<br>myopathy, lactic acidosis,<br>MELAS, LHON, recurrent<br>myoglobinuria with cases of<br>survival into adulthood. | [172,283–<br>286] | 516050 |
| <i>COX6A1</i>                    | X  |          | Deletion leading to<br>decreased protein<br>levels.  | Recessive axonal type of<br>Charcot-Marie-Tooth with<br>survival into adulthood.   | [287]             | 602072 |
| <i>COX6A2</i>                    | X  |          | Missense leading to<br>decreased protein<br>levels.  | Congenital myopathy,<br>cardiomyopathy, isolated CIV<br>deficiency in muscle and<br>absent from undifferentiated<br>myoblast.  | [288]             | 602009 |
| <i>COX6B1</i>                    | X  |          | Missense leading to<br>decreased protein<br>levels.  | Severe infantile<br>encephalomyopathy,<br>cardiomyopathy, lactic<br>acidosis. CIV deficiency<br>detected in muscle and mildly<br>in fibroblasts.                         | [429,430]         | 124089 |
| <i>NDUFA4</i>                    | X  |          | Intronic leading to<br>undetectable protein<br>levels.   | Leigh syndrome, congenital<br>lactic acidosis, may present<br>dystonia with survival into<br>adulthood.  | [291]             | 603833 |

S., subunit; A.F., assembly factor; LHON, Leber's hereditary optic neuropathy; MELAS, mitochondrial encephalopathy, lactic acidosis and stroke-like episodes.

Although the exact mechanism in which COA7 is involved in complex IV assembly remains unclear, mutations in COA7 have been shown to cause leukoencephalopathy or spinocerebellar ataxia and axonal neuropathy with variable onset and survival into late adulthood is reported (Table 12). A patient reported with undetectable COA7 levels via western blot analysis also showed decreased levels of COX2 and COX3 subunits, and decreased assembly of complex IV [292], providing insights into the stage of assembly of which COA7 may be involved.

## Complex V assembly

Complex V, also known as F<sub>0</sub>F<sub>1</sub>-ATPase, is the last enzyme in the OXPHOS system, utilising the proton gradient generated by complexes I, III and IV to power ATP synthesis. Complex V is composed of a membrane-embedded F<sub>0</sub> section connected by an external stalk to a matrix soluble F<sub>1</sub> section containing the ATPase domains [293]. Like complexes I, III and IV, complex V is under dual genetic control, with two of the membrane subunits encoded on mtDNA (*ATP6*, *MT-ATP6*; *ATP8*, *MT-ATP8*) and the remaining 16 encoded on nDNA [294]. Much of what is known for complex V is extrapolated from studies in fungal models, though in recent years there is an increasing amount of literature documenting the assembly in mammals. The nomenclature for nuclear complex V genes has also undergone a recent overhaul [23], now all being prefixed *ATP5* (e.g. *ATP5F1A*). Typically, the corresponding proteins are represented by single Greek and Latin letters (e.g. *α* or *α*-subunit for **ATP synthase subunit alpha**, which is the recommended name for the protein product of *ATP5F1A*) though there are some inconsistencies. As for other complexes, we have chosen to refer to the subunits and assembly factors by their gene name using the aforementioned formatting (**bold** typeface for subunit, regular typeface for assembly factor) though have included the commonly used protein symbol on the first instance.

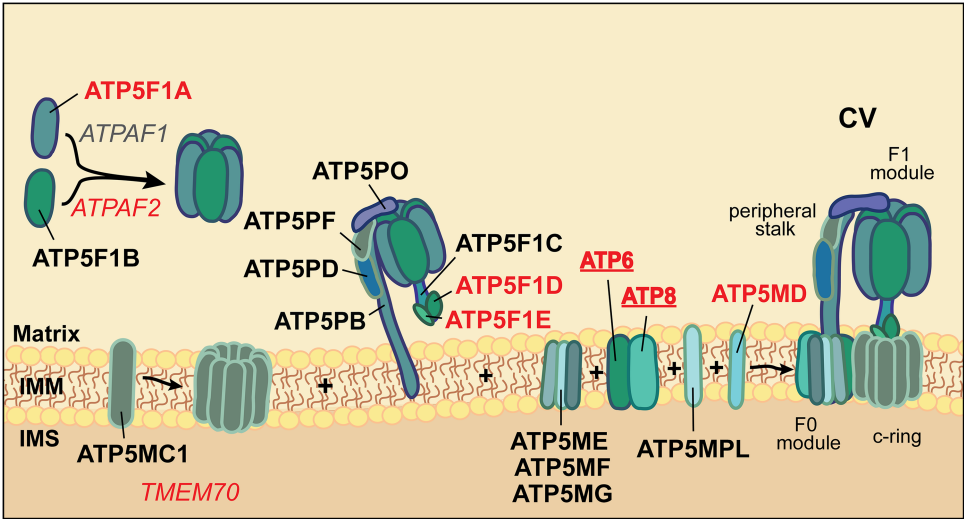
Table 12. Defects affecting unknown steps of complex IV assembly or function

| Gene (alias) | S. | A. F. | Types of genetic variants and protein impact   | Clinical presentations and relevant information   | Ref.      | OMIM   |
|--------------|----|-------|--|---|-----------|--------|
| COA7         |    | X     | Predominantly missense but also intronic and deletion causing protein frameshift and undetectable protein levels | Variable phenotypes and onset with survival into late adulthood presenting leukoencephalopathy or spinocerebellar ataxia with axonal neuropathy | [292,431] | 615623 |

S., subunit; A.F., assembly factor.

Assembly of the F<sub>1</sub> catalytic module and central stalk

The assembly of complex V starts with the oligomerisation of three **ATP5F1A** ( $\alpha$ -subunit) and three **ATP5F1B** ( $\beta$ -subunit) subunits into an alternating hexamer, in a series of assembly steps mediated by the assembly factors **ATPAF1** and **ATPAF2** respectively (Figure 6) [295]. These proteins likely act as placeholders to prevent the formation of homomeric complexes [296,297] and their loss in fungal models leads to aggregation of the subunits into large insoluble complexes [298]. In general, there are few reported cases for patients harbouring mutations affecting assembly or function of this module (Table 13). For **ATP5F1A** there are two known variants [299,300] with cases for both presenting with severe encephalopathy or microcephaly, followed by early infantile death. Tissue and fibroblast material from affected patients had reduced but not absent levels of **ATP5F1A** protein as well as lower levels of other subunits, suggesting stability of the F<sub>1</sub> module is



**Figure 6. Schematic depicting the complex V (F<sub>1</sub>F<sub>0</sub>-ATPase) assembly pathway showing known mitochondrial disease genes in red, mtDNA-encoded proteins underlined and assembly factors in italics.** To aid correlation with disease genes, subunits have been labelled according to their gene name. The commonly used protein names are in parenthesis as follows: three copies of the **ATP5F1A** ( $\alpha$ -subunit) and **ATP5F1B** ( $\beta$ -subunit) are assembled with the aid of the chaperones **ATPAF1** and **ATPAF2** with later binding of the subunits **ATP5F1C1** ( $\gamma$ -subunit), **ATP5F1D** ( $\delta$ -subunit) and **ATP5F1E** ( $\epsilon$ -subunit). The membrane ring composed of **ATP5MC1** (c-subunits; also encoded by **ATP5MC2** and **ATP5MC2**) subunits is assembled and joins the pre-complex V prior to the addition of the subunits **ATP5PB** (b-subunit), **ATP5PD** (d-subunit), **ATP5PF** (F6-subunit) and **ATP5PO** (OSCP). The assembly pathway is followed by integration of **ATP5ME** (e-subunit), **ATP5MF** (f-subunit) and **ATP5MG** (g-subunit) and then by the mtDNA-encoded **ATP6** (subunit 6) and **ATP8** (subunit 8). The last subunits **ATP5MPL** (MP68) and **ATP5MD** (DAPIT) are added to complete the assembly of complex V. IMM, inner mitochondrial membrane; Matrix, mitochondrial matrix.

**Table 13. Defects in the F<sub>1</sub> catalytic module and central stalk**

| Gene<br>(protein <sup>1</sup> )          | S. | A.<br>F. | Types of<br>genetic<br>variants and<br>protein impact                | Clinical presentations and<br>relevant information  | Ref.      | OMIM   |
|--|----|----------|--|---|-----------|--------|
| <i>ATP5F1A</i><br>( $\alpha$ -subunit)   | X  |          | Missense<br>leading to<br>unclear protein<br>impact.                 | Fatal neonatal encephalopathy, lung<br>hypoplasia or hypertension, may<br>present seizures, heart failure.<br>Decreased CV assembly/activity.                         | [299,300] | 164360 |
| <i>ATPAF2</i><br>( <i>Atp12</i> )        |    | X        | Single known<br>missense<br>leading to<br>unclear protein<br>impact. | Degenerative encephalopathy,<br>severe developmental delay, death<br>in early childhood. Decreased CV<br>assembly/activity.   | [301]     | 608918 |
| <i>ATP5F1D</i><br>( $\delta$ -subunit)   | X  |          | Single known<br>missense<br>leading to<br>normal protein<br>levels.  | Hyperammonemia, lactic acidosis<br>or ketoacidosis, may present<br>cardiomyopathy, delayed speech<br>with survival into childhood.<br>Decreased CV assembly/activity. | [305]     | 603150 |
| <i>ATP5F1E</i><br>( $\epsilon$ -subunit) | X  |          | Single known<br>missense<br>leading to<br>reduced protein<br>levels. | Neonatal onset lactic acidosis, mild<br>mental retardation, severe peripheral<br>neuropathy with survival into<br>adulthood. Reduced levels of fully<br>assembled CV. | [306]     | 606153 |

<sup>1</sup>Commonly used protein name; S., subunit; A.F., assembly factor.

compromised [299]. For *ATPAF2*, there is a single known example [301]. In line with the requirement for this protein in assembly of the F<sub>1</sub> module, the patient presented with similar symptoms to those with mutations in *ATP5F1A*, degenerative encephalopathy, severe developmental delay and death in early childhood (Table 13). The next step in the assembly pathway is the incorporation of the subunits belonging to the central stalk, *ATP5F1C* ( $\gamma$ -subunit), *ATP5F1D* ( $\delta$ -subunit) and *ATP5F1E* ( $\epsilon$ -subunit). Functional studies have shown that loss of any of these subunits leads to similar defects in complex V assembly, lower levels of mature complex and turnover of subunits for the F<sub>1</sub> module [302]. *ATP5F1C* is the key structural molecule connecting the F<sub>1</sub> catalytic module to the F<sub>0</sub> module and through structural similarities of its coiled-coiled tail with the C-terminal regions of *ATPAF1* and *ATPAF2*, likely displaces the assembly factors during module biogenesis [303,304]. There are no known patients for *ATP5F1C*, although a few cases have been reported for *ATP5F1D* and *ATP5F1E* [305,306]. Although patients present with similar, relatively mild phenotypes in line with expectations based on functional studies (Table 13), the molecular underpinnings are different. In the case of *ATP5F1D*, the patient had normal levels of the mutant protein, whereas other subunits of the F<sub>1</sub> module were destabilised [305]. In the case of *ATP5F1E*, the patient had reduced levels of protein, though retained a fully assembled complex (including the mutant protein) albeit at lower levels than controls [306]. Both patients had similar net effects on total levels of assembled Complex V and function (Table 13).

## Assembly of the c-ring

The membrane-embedded c-ring and the central stalk are components of the rotor part of the Complex V and therefore essential for ATP synthesis. In humans, the c-ring consists of eight **c-subunits** encoded by *ATP5MC1*, *ATP5MC2* and *ATP5MC3* (Figure 6). Interestingly, all three genes encode the same mature protein, the proteins only differing in the sequence of their cleaved mitochondrial targeting signals [307,308]. There are no reported cases of mitochondrial disease linked to mutations in these genes, however, many cases and >5 different variants have been linked to defects in the assembly factor *TMEM70* (Table 14) which has been linked with its biogenesis. *TMEM70* has been suggested to be required for assembly of the **c-subunits** into the c-ring [80]. Although this has been argued in the literature [75], it is clear from these studies that *TMEM70* is required for the joining of the c-ring to the F<sub>1</sub> module, and its absence leads to a severe assembly

**Table 14. Defects affecting the c-ring**

| Gene          | S. A.F. | Types of genetic variants and protein impact  | Clinical presentations and relevant information  | Ref.           | OMIM   |
|---------------|---------|---|--|----------------|--------|
| <i>TMEM70</i> | X       | Multiple. Intronic, insertion, deletion, duplication, nonsense, missense leading to unclear or absent protein levels. | Variable phenotype including hypertrophic cardiomyopathy, lactic acidosis, hyperammonemia, persistent pulmonary hypertension, encephalocardiomyopathy, neonatal hypotonia or hypertonia, facial dysmorphism, bilateral cataracts, leukoencephalopathy. Presentations vary from normal to defective isolated (CI or CIII) or combined (CI + CIII) OXPHOS activities in muscle or fibroblasts. | [81,82,84,432] | 612418 |

S., subunit; A.F., assembly factor.

defect. As a disease gene, *TMEM70* is further complicated by variable presentations, depending on the variant patients either present with isolated or combined Complex V or Complex I defects, as well as severe cardiac and neuronal phenotypes (Table 14) [81–84]. Although the precise involvement in Complex I assembly is even less clear, recent studies in gene-edited cells completely lacking *TMEM70* protein [75] are consistent with suggesting it has a dual role in assembly of both complexes.

## Assembly of the peripheral stalk and F<sub>0</sub> module

The peripheral stalk assembles with the F<sub>1</sub> subcomplex in a two-step manner. The first four subunits to be incorporated are **ATP5PB (b-subunit)**, **ATP5PD (d-subunit)**, **ATP5PF (F6-subunit)**, **ATP5PO (OSCP)** followed by the membrane-associated subunits **ATP5ME (e-subunit)**, **ATP5MF (f-subunit)** and **ATP5MG (g-subunit)** [294,309,310] (Figure 6). In the absence of the c-ring, an intermediate complex V is assembled containing the F<sub>1</sub> catalytic module, the peripheral stalk and the membrane subunits **ATP5ME**, **ATP5MF** and **ATP5MG** [311].

Once the previously formed subcomplex containing the F<sub>1</sub> module and peripheral stalk join with the c-ring, this provides the scaffold necessary for the incorporation of the two mitochondrially-encoded subunits **ATP6 (subunit 6)** and **ATP8 (subunit 8)** (Figure 6) [309]. Mutations in both lead to disease (Table 15). More than

**Table 15. Defects in the Fo module**

| Gene (protein <sup>1</sup> ) | S. A.F. | Types of genetic variants and protein impact  | Clinical presentations and relevant information  | Ref.              | OMIM   |
|------------------------------|---------|---|--|-------------------|--------|
| <i>MT-ATP6</i> (subunit 6)   | X       | Several missense variants with protein levels dependant on variant and mutant mtDNA load. | Most commonly presented as Leigh syndrome, NARP, spinocerebellar ataxia but also Charcot-Marie-Tooth, hypertrophic cardiomyopathy, lactic acidosis. Higher heteroplasmic levels correlate with earlier-onset phenotypes. Often but not always show ATP synthesis rate. | Reviewed in [312] | 516060 |
| <i>MT-ATP8</i> (subunit 8)   | X       | Missense or nonsense leading to unclear protein impact.                                   | Hypertrophic cardiomyopathy and neuropathy with survival into adolescence. Decreased CV assembly/activity. Alternately has presented with reversible cognitive dysfunctions with seizures and brain pseudoatrophy.   | [313–316,433]     | 516070 |
| <i>ATP5MD (DAPIT)</i>        | X       | Single known intronic variant leading to undetectable protein levels.                     | Leigh syndrome, developmental regression after febrile illness. Survival into childhood. Decreased proportion of CV dimer.   | [317]             | 615204 |

<sup>1</sup>Commonly used protein name; S., subunit; A.F., assembly factor; NARP, neuropathy, ataxia and retinitis pigmentosa.

200 *ATP6* cases have been reported with 19 different underlying mutations and a large variability in mutant mtDNA load (reviewed in [312]). Phenotypes are highly variable and both severe infantile Leigh syndrome as well as adult onset disease has been reported (Table 15), and as such there is no clear assembly phenotype underpinning mutations in this gene. There are comparatively few reported patients for *ATP8*, however, like for *ATP8* these cases report with a different phenotype likely underpinned by differences in the variant and mutant load. Phenotypes include cardiomyopathy and neuropathy [313] as well as seizures and neuropsychologic decline [314] as well as defects in complex V activity (Table 15). In the case of the former, the fibroblasts from the patient accumulated subcomplexes of unassembled complex V, including a free catalytically active F<sub>1</sub> domain, suggesting the protein may be structurally important [313]. Interestingly, *ATP6* and *ATP8* are transcribed as a polycistronic mRNA and there are a few cases known to likely affect both proteins [315,316]. Patients present with severe early onset cardiomyopathy or neurological symptoms, although there have been one case of a kindred with adult onset cerebellar ataxia and peripheral neuropathy (Table 15). Aside from defects in complex V function, the molecular impacts in these cases are unknown.

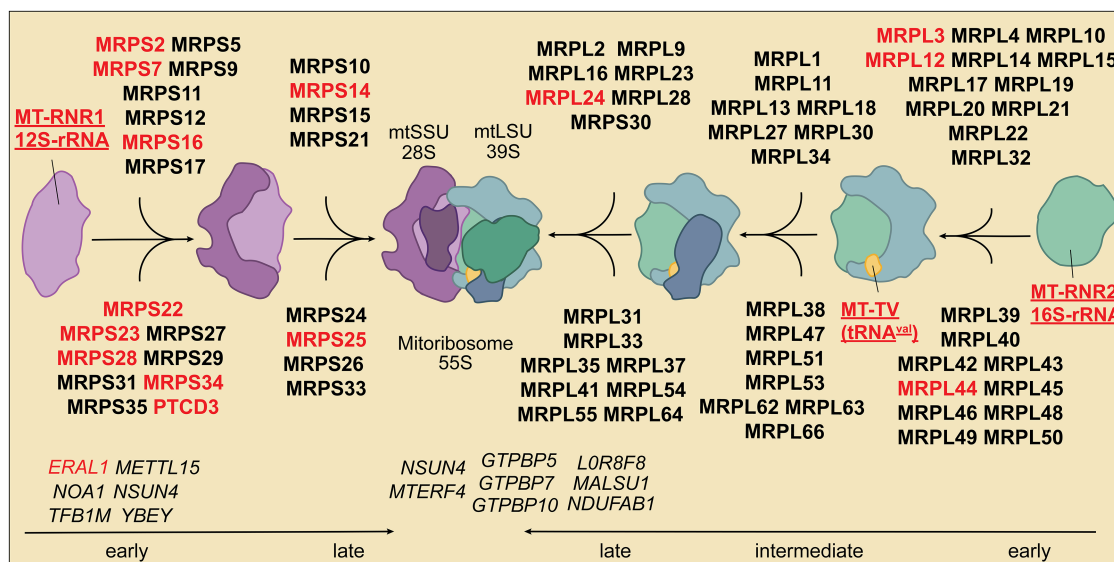
Finally, the subunit *ATP5MPL* (**MP68**), which is required for *ATP6* and *ATP8* stability is incorporated, followed by *ATP5MD* (**DAPIT**) [309]. A single variant of the latter is known to cause Leigh syndrome with childhood onset [317]. Fibroblasts from patients had no detectable protein and reduced ATP synthesis. Fully assembled complex V has been shown to assemble into dimers [318,319] and, more recently, in tetramers [293]. Interestingly, fibroblasts from *ATP5MD* patients had markedly reduced dimerisation [317], suggesting the protein is involved in this process.

## Mitochondrial translation and the mitoribosome

Mitochondrial protein synthesis is a complex process that has its own components such as a mitochondria-specific genetic code, an exclusive set of tRNA and tRNA synthetases and its own ribosome known as the mitoribosome [320]. The composition of ribosomes has considerably changed over the course of evolution, specially the RNA to protein ratio which has reversed from 1 : 2 protein : RNA in bacteria and cytosolic ribosomes to 2 : 1 protein : RNA in the mammalian mitoribosome. This explains why almost half of the mitoribosome proteins are mitochondrion-specific and absent from the bacterial ancestor [4]. Like other ribosomes, the mammalian mitoribosome is composed of two subunits, the small (mtSSU) and the large (mtLSU). The mRNA engages with the mtSSU while the mtLSU can anchor itself to the IMM and catalyse translation [321]. The mitoribosome has a total sedimentation coefficient of 55S, composed of the 28S for the isolated mtSSU and the 39S for the mtLSU [322]. The mtSSU consists of the 12S rRNA encoded by the mtDNA, and 30 MRP mitoribosomal proteins encoded by the nDNA [323]. The mtLSU consists of a tRNA valine (tRNA<sup>val</sup>) [2] and a 16S rRNA, both encoded by the mtDNA, and 52 nuclear-encoded mitoribosomal proteins. The peptidyl transferase centre (PTC) catalyses the formation of the peptide bonds of nascent polypeptides [324] and is located in the internal part of the mitoribosome formed exclusively by the 12S and 16S rRNAs [325,326]. The polypeptide exit tunnel (PET) starts at the PTC and ends at the polypeptide exit site (PES) where the translated polypeptide leaves the mitoribosome [327]. The polypeptide tunnel is an important region that is targeted in bacteria as a binding site for antibiotics [321]. The PET is surrounded by ring composed of bacterial conserved proteins **bl17m** (encoded by the gene *MRPL17*), **uL22m** (*MRPL22*), **uL23m** (*MRPL23*), **uL24m** (*MRPL24*), **uL29m** (*MRPL47*) and the mitochondrial specific **mL45** (*MRPL45*) which promotes the tethering of the mitoribosome to the IMM [321]. The translocation of mitochondrial proteins is mediated by OXA1, which binds newly synthesised polypeptides [162,163] and the mitoribosome [328,329]. The nomenclature for mitoribosomal proteins has recently been overhauled for consistency between cytosolic and mitochondrial ribosomes found across different organisms [330]. To assist readers linking phenotype to function we have chosen to refer to the protein using their HUGO assigned gene name and have listed the revised protein name in parenthesis at first mention.

## Assembly of the mtSSU

Even though the mitoribosome has unarguable importance, its assembly pathway has not been studied in as much detail as the assembly of OXPHOS complexes [331]. Recent research has begun to solve the timing of incorporation of mitoribosome protein using pulse and pulse-chase stable isotope labelling with amino acids in cell culture (SILAC) proteomics approaches [331,332], suggesting that the mtSSU displays an early and a late class of incorporation of mitoribosomal proteins, while the mtLSU has an additional intermediate class (Figure 7).



**Figure 7. Schematic model depicting the mitoribosome assembly pathway based on data from [331].** To aid correlation with disease genes, subunits have been labelled according to their gene name (outlined in 332). The incorporation stage of MRPS6 (bS6m), MRPS18C (bS18m), MRPS37 (mS37), MRPS38 (mS38), MRPL36 (bL36m) and MRPL52 (mL52) remains unclear. Known mitochondrial disease genes in red, mtDNA-encoded rRNAs and tRNA underlined, assembly factors in italics. The mtSSU proteins are incorporated at early or late stage in the assembly pathway while the mtLSU proteins are incorporated at early, intermediate or late stages. Mitoribosome small subunit, mtSSU; mitoribosome large subunit, mtLSU.

The mitoribosome biogenesis has been suggested to start at or near the mitochondrial nucleoid and present subclusters of assembly proteins for each incorporation class or ‘module’ in the terminology used for the OXPHOS complexes [331]. The 12S rRNA is stabilised by the RNA chaperone ERAL1 [333,334] and metal-dependent endoribonuclease YBEY [335], and methylated by two proteins, the adenine dimethyltransferase TFB1M [336] and the cytosine methyltransferase NSUN4 [337]. The early mitochondrial proteins participating in mtSSU assembly are mainly localised at the top and bottom of the 12S rRNA core and seem to be grouped into three different clusters [331]. One cluster contains MRPS5 (uS5m), MRPS16 (bS16m), MRPS22 (mS22), MRPS27 (mS27), MRPS34 (mS34) and MRPS18B (mS40). A second assembly cluster contains MRPS7 (uS7m), MRPS9 (uS9m), MRPS29 (mS29), MRPS31 (mS31), MRPS35 (mS35) and MRPS39 (mS39). A third cluster of SSU early binding proteins consists of MRPS2 (uS2m), MRPS23 (mS23) and MRPS28 (bS1m). The remaining proteins involved in the early assembly of the mtSSU are MRPS11 (uS11m), MRPS12 (uS12m) and MRPS17 (uS17m) appear to incorporate in an independent manner, with recent studies suggesting that YBEY may incorporate MRPS11 (uS11m) to the nascent mtSSU [335]. The early assembly of mitoribosomal proteins seem to be facilitated by the GTPase NOA1/C4orf14, which was shown to interact with several early assembled mtSSU proteins and the mitochondrial nucleoid [338]. In addition to that, METTL15 was shown to methylate the 12S rRNA at position C839 and be required for the biogenesis of mtSSU with its absence affecting both early assembled proteins MRPS12 (uS12m), MRPS17 (uS17m) and late assembled protein MRPS15 (uS15m), as well as MRPS38 (mS38) [339] which has not yet been assigned an incorporation class [331].

For the later incorporated mtSSU proteins, two assembly clusters seem to be present. One cluster consists of MRPS10 (uS10m), MRPS14 (uS14m), MRPS24 (uS3m) and MRPS33 (mS33) located at the top of the 12S rRNA, and another assembly cluster comprised of MRPS15 (uS15m), MRPS25 (mS25) and MRPS26 (mS26) locating at the bottom of the 12S rRNA with the remaining proteins incorporating in an independent manner [331].

To date, mutations in eleven components and in one assembly factor of the mtSSU have been identified, causing a range of phenotypes, onset and variable survival (Table 16). Interestingly, majority of the disease genes participate in the early stages of the mtSSU assembly (Figure 7) and patients tend to have a combined OXPHOS deficiency depending on tissue type and mutation [340–345]. Several cases of mutations in

*MT-RNR1* (12S rRNA) have been reported to cause hearing loss induced by aminoglycoside exposure (Table 16) with one family also presenting cardiomyopathy [346]. While mutations in *ERAL1*, the RNA chaperone shown to stabilise 12S rRNA [333,334], have also been shown to cause sensorineural deafness, it has not been linked to aminoglycoside exposure as have mutations in *MT-RNR1* [347].

Both *MRPS2* and *MRPS7* encode proteins assembled early in the mtSSU (**uS2m** and **uS7m**, respectively) and mutations in these genes have been reported to cause sensorineural hearing impairment and hypoglycaemia but with survival into adulthood [344,345,348] (Table 16). On the other hand, mutations in *MRPS16* (**bS16m**) and *MRPS22* (**mS22**) commonly lead to clinical presentations of fatal lactic acidosis and tubulopathy, with *MRPS22* mutations also leading to hypertrophic cardiomyopathy, Leigh-like brain lesions and delayed sexual development [342,349–351]. Interestingly, a homozygous missense mutation in *MRPS23* leads to hypoglycaemia and hepatic disease [66], sharing partial phenotypic similarities with other genes encoding early assembled proteins, *MRPS2*, *MRPS7*, *MRPS16* and *MRPS22* (Table 16) (Figure 7). Comparably, mutations in *MRPS28* (**bS1m**) were shown to present before birth as intrauterine growth retardation (IUGR) and progress with multisystemic involvement including sensorineural deafness, brain abnormalities, hyperlactatemia and failure to thrive with survival into adulthood [352,353]. The last two genes linked to mitochondrial disease participating in the early assembly of the mtSSU are *MRPS34* and *PTCD3*. Mutations in both genes have been shown to cause Leigh or Leigh-like syndrome with variable presentations of hyperlactatemia, optic atrophy, hearing loss, microcephaly and variable OXPHOS defects and survival depending on tissue type and mutation [341,354] (Table 16).

While the majority of mutations in the mtSSU lead to decreased protein levels (Table 16), a missense homozygous mutation was identified in *MRPS14* (**uS14m**) leading to increased levels of corresponding protein, and clinical presentation of lactic acidosis, hypertrophic cardiomyopathy and hypotonia [340]. The unaffected assembly of the mitoribosome suggests that the incorporation of the mutant protein causes impaired mitochondrial translation which was predicted to disrupt the mitoribosome mRNA channel [340]. The other late assembled mtSSU protein linked to disease, *MRPS25* (**mS25**), assembles in a different cluster to *MRPS14* (**uS14m**) [331] and has been linked to a different phenotype including encephalopathy, short stature, muscle fatigue, dystonia, mild elevation of plasma lactate [343].

## Assembly of the mtLSU

The assembly of the mtLSU seems to present an early, an intermediate and a late class of incorporation of proteins (Figure 7) with several subclusters of proteins present at each stage [331]. Assembly factors involved in the biogenesis of the mtLSU have been identified mostly at late stages. *MALSU1* is a member of the DUF143 family of proteins of conserved ribosomal silencing factors and is required for mtLSU biogenesis and translation [355–357] (Figure 7). Recently, the structure of an mtLSU intermediate containing *MALSU1* was solved [358] revealing the involvement of two other proteins, *NDUFAB1*, a subunit of complex I with an essential secondary role in mitochondrial function (discussed above) [22], and *LOR8F8*, the product of a bicistronic transcript that also encodes *MID51*, a mitochondrial protein involved in morphology [85]. While the function of these new assembly factors is not yet known, they have been suggested to act as caps to prevent premature association of the nascent mtLSU with the mtSSU [358]. At least five other assembly factors have been characterised to be involved in late mtLSU assembly and the formation of the 55S monosome. Three of these are quality control GTPases proposed to prevent premature monosome formation: *GTPBP5* (*MTG2*) [359], *GTPBP7/MTG1* [360] and *GTPBP10* [361,362], and two others form a heterodimeric complex comprised of *NSUN4* and *MTERF4* assembly factors which facilitates the monosome formation and enables mtDNA translation [337,363,364].

Once the 55S monosome is assembled, the tRNA<sup>val</sup> is nestled between two groups of proteins: the early assembled *MRPL40* (**mL40**), *MRPL46* (**mL46**) and *MRPL48* (**mL48**) proteins on one side and the intermediate assembled proteins *MRPL18* (**mL18**), *MRPL38* (**mL38**) and *MRPL27* (**bL27m**) on the other side [4,331]. Although several components required for the assembly of the human mitoribosome have been identified over the years, the lack of assembly factors characterised for the different incorporation clusters and independently incorporated proteins for both mtSSU and mtLSU might suggest that some assembly factors are yet to be discovered.

Even though the mtLSU contains more structural proteins than the mtSSU, there are only four nuclear disease genes associated with the mtLSU, as well as 2 mtDNA-encoded rRNAs, with majority of these participating in the early assembly steps (Figure 7). The majority of mutations associated with defects in the mtLSU are missense mutations leading to a variable phenotype and OXPHOS defect, depending on mutation and

**Table 16. Defects in the assembly of the mtSSU**

| Gene (RNA/protein name <sup>1</sup> ) | A. S. F. | Types of genetic variants and protein impact  | Clinical presentations and relevant information   | Ref.                                 | OMIM   |
|---------------------------------------|----------|---|---|--------------------------------------|--------|
| <i>MT-RNR1</i> (12S rRNA)             | X        | Transition, transversion and deletion/insertion.  | Aminoglycoside-induced hearing loss, incomplete penetrance reported, may include cardiomyopathy.  | [346,434,435], and reviewed in [436] | 561000 |
| <i>ERAL1</i>                          | X        | Missense leading to decreased protein levels.   | Perrault syndrome expressed as sensorineural hearing loss and ovarian dysgenesis with survival into late adulthood.   | [347]                                | 607435 |
| <i>MRPS2</i> (uS2m)                   | X        | Missense leading to decreased protein levels.   | Sensorineural hearing impairment, mild developmental delay and hypoglycaemia. Variable OXPHOS deficiency depending on the tissue type. Survival into childhood.   | [344]                                | 611971 |
| <i>MRPS7</i> (uS7m)                   | X        | Missense leading to decreased protein levels.   | Congenital sensorineural hearing impairment, lactic acidosis, hypoglycaemia. Variable OXPHOS deficiency depending on the tissue type. Survival into childhood.  | [345,348]                            | 611974 |
| <i>MRPS14</i> (uS14m)                 | X        | Missense leading to increased protein levels.   | Lactic acidosis, hypertrophic cardiomyopathy, hypotonia with survival into childhood. Unaffected mitoribosome assembly but decreased assembly of CI, CIII, CIV and CV suggesting incorporation of the mutant protein causes impaired mitochondrial translation. | [340]                                | 611978 |
| <i>MRPS16</i> (bS16m)                 | X        | Nonsense leading to unclear protein impact but decreased mitochondrial translation.                 | Fatal lactic acidosis, tubulopathy, hypotonia. Variable OXPHOS deficiency depending on the tissue type.   | [342]                                | 609204 |
| <i>MRPS22</i> (mS22)                  | X        | Missense or duplication causing frameshift leading to unclear or decreased protein levels.          | Variable phenotypes including fatal lactic acidosis, hypertrophic cardiomyopathy, tubulopathy, dysmorphic features, hypotonia, Leigh-like lesions, hypergonadotropic hypogonadism. Variable OXPHOS deficiency depending on the tissue type.                     | [349–351]                            | 605810 |
| <i>MRPS23</i> (mS23)                  | X        | Missense leading to unclear protein impact.   | Hepatic disease and hypoglycaemia. Combined CI and CIV deficiencies in fibroblasts.   | [66]                                 | 611985 |
| <i>MRPS25</i> (mS25)                  | X        | Missense leading to decreased protein levels.   | Multiple presentations including encephalopathy, short stature, muscle fatigue, dystonia, mild elevation of plasma lactate with survival into adulthood.  | [343]                                | 611987 |
| <i>MRPS28</i> (bS1m)                  | X        | Missense and deletion causing frameshift and early termination leading to decreased protein levels. | IUGR, cerebellar atrophy, microcephaly, hyperlactatemia, developmental delay, sensorineural deafness, failure to thrive with survival into adulthood. CIV deficiency in muscle, fibroblasts and liver.  | [352,353]                            | 611990 |
| <i>MRPS34</i> (mS34)                  | X        | Intronic, nonsense, missense leading to decreased protein levels.                                   | Leigh or Leigh-like syndrome, mild or hyperlactatemia, may present microcephaly, optic atrophy. Variable OXPHOS defect and survival depending on tissue type and mutation.  | [341]                                | 611994 |
| <i>PTCD3/MRPS39</i> (mS39)            | X        | Intronic and insertion causing frameshift leading to decreased protein levels.                      | Severe Leigh syndrome, optic atrophy, hearing loss. Decreased CI and CIV in fibroblasts.  | [354]                                | 614918 |

<sup>1</sup> Commonly used protein name; S., subunit; A.F., assembly factor; IUGR, intrauterine growth restriction.

**Table 17. Defects in the assembly of the mtLSU**

| Gene (RNA/<br>protein<br>name <sup>1</sup> ) | S. | A.<br>F. | Types of genetic<br>variants and<br>protein impact                               | Clinical presentations and<br>relevant information   | Ref.          | OMIM   |
|--|----|----------|--|--|---------------|--------|
| <i>MT-RNR2</i><br>(16S rRNA)                 | X  |          | Transition leading<br>to unclear RNA<br>impact.                                  | Myopathy, hypertrophic<br>cardiomyopathy. Survival into<br>adulthood reported. Decreased<br>ATP production.  | [365,366]     | 561010 |
| <i>MT-TV</i><br>(tRNA <sup>val</sup> )       | X  |          | Transition leading<br>to unclear or<br>decreased tRNA <sup>val</sup><br>levels.  | Variable phenotype and onset<br>depending on mutation and<br>mutant load with survival into<br>adulthood reported.<br>Predominantly presenting ataxia,<br>hearing and/or visual loss,<br>bilateral cataracts or migraines<br>and muscle weakness, MELAS<br>severe Leigh or Leigh-like<br>syndrome, hypertrophic<br>cardiomyopathy. | [367–<br>372] | 590105 |
| <i>MRPL3</i><br>(uL3m)                       | X  |          | Missense or<br>deletion leading to<br>unclear or<br>decreased protein<br>levels. | Early onset severe<br>cardiomyopathy, psychomotor<br>retardation, failure to thrive, lactic<br>acidosis. Variable OXPHOS<br>defect depending on tissue type<br>and mutation.   | [437,438]     | 607118 |
| <i>MRPL12</i><br>(bL12m)                     | X  |          | Missense leading<br>to decreased<br>protein levels.                              | Antenatal hypotrophy, tonic<br>seizures, ataxia, hyperlactatemia,<br>failure to thrive. Variable<br>OXPHOS defect depending on<br>tissue type and mutation.  | [439]         | 602375 |
| <i>MRPL24</i><br>(uL24m)                     | X  |          | Missense leading<br>to decreased<br>protein levels.                              | Cerebellar atrophy,<br>choreoathetosis of limbs and<br>face, Wolff–Parkinson–White<br>syndrome, increased lactate in<br>blood with survival into<br>adolescence. Combined CI and<br>CIV defect in muscle.  | [377]         | 611836 |
| <i>MRPL44</i><br>(mL44)                      | X  |          | Missense leading<br>to unclear or<br>decreased protein<br>levels.                | Predominantly hypertrophic<br>cardiomyopathy, hepatopathy,<br>but also muscle weakness,<br>granular pigmentation of retina,<br>metabolic acidosis. Survival into<br>early adulthood reported.<br>Variable OXPHOS defect<br>depending on tissue type and<br>mutation.   | [378,379]     | 611849 |

<sup>1</sup>Commonly used protein name; S., subunit; A.F., assembly factor; MELAS, mitochondrial encephalopathy, lactic acidosis and stroke-like episodes.

tissue type analysed (Table 17). Mutations identified in the 16SrRNA (*MT-RNR2*) have been reported to cause hypertrophic cardiomyopathy and myopathy with survival into adulthood [365,366], while mutations in the tRNA<sup>val</sup> (*MT-TV*) appear to cause a broader spectrum of phenotypes including muscle weakness, hearing and visual loss, MELAS, Leigh syndrome and hypertrophic cardiomyopathy [367–372] (Table 17). It is not clear if mutations in tRNA<sup>val</sup> lead to combined defects in mitoribosome assembly and defective polypeptide elongation, although it should be noted that mitoribosomes are able to incorporate tRNA<sup>phe</sup> when the levels of tRNA<sup>val</sup> are severely decreased [373]. Despite this implying that the phenotypes are due to defective elongation, the adaptive switch in tRNA composition still leads to impaired mitochondrial translation [374] hinting at a combined defect. Severe phenotypes leading to early death have been associated with *MRPL3* (uL3m) and *MRPL12*

(bL12m) suggesting these proteins play an essential for mitochondrial translation. In fact, uL3m, encoded by *MRPL3* displays extensive binding contact with the 16S rRNA and is one of the earliest assembled proteins, providing an anchor to *MRPL39* (mL39) followed by *MRPL45* (mL45) [331], the latter known to tether the mitoribosome at the IMM for translation [321]. Recent structural studies also revealed that *MRPL12* (bL12m) plays an important role in interacting with mitochondrial elongation factor (EF-G1<sub>mt</sub>) [375] and promoting tRNA translocation on the mitoribosome during translation [376]. On the other hand, mutations in *MRPL24* (uL24m) and *MRPL44* (mL44) have been associated with severe to mild phenotypes with survival into adolescence and early adulthood [377–379] (Table 17). *MRPL24* is a late assembled protein that lacks extensive contact with early assembled proteins and is involved in the formation of the PET [331]. Despite its involvement with the PET, a teenager was recently reported harbouring a homozygous missense variant leading to cerebellar atrophy, choreoathetosis, increased lactate in blood and tachycardia (Wolff–Parkinson–White syndrome) [377]. Finally, four patients have been reported harbouring missense mutations in the gene encoding early assembled protein *MRPL44* (mL44). The patients commonly presented with hypertrophic cardiomyopathy with majority presenting stabilisation of their phenotype over the years [378,379].

## Conclusion

In this review, we have attempted to catalogue the breadth of genetic and clinical phenotypes associated with impaired assembly of mitochondrial OXPHOS complexes and the mitoribosome. Along with highlighting the intricacy of this system, we hope to have demonstrated the high heterogeneity in clinical presentations that challenges the diagnosis of new patients and the validation of novel disease genes linked to dysfunction in this critical process.

## Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

## Funding

We acknowledge funding from the National Health and Medical Research Council (NHMRC Fellowship 1140851 to DAS). DHH is supported by a Melbourne International Research Scholarship and the Mito Foundation PhD Top-up Scholarship.

## Open Access

Open access for this article was enabled by the participation of The University of Melbourne in an all-inclusive *Read & Publish* pilot with Portland Press and the Biochemical Society under a transformative agreement with CAUL.

## Acknowledgements

We apologise to those colleagues whose work we could not cite due to space restrictions. We thank Alison Compton, David Thorburn and all members of the Stroud lab for their input.

## Abbreviations

COX, cytochrome c oxidase; IMM, inner mitochondrial membrane; LHON, Leber hereditary optic neuropathy; MCIA, mitochondrial complex I intermediate assembly; MRC, mitochondrial respiratory chain; mt, mitochondrial; nDNA, nuclear DNA; OXPHOS, oxidative phosphorylation; PTC, peptidyl transferase centre; VUS, variants of uncertain significance; WES, whole exome; WGS, whole genome.

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