# The ins and outs of the flavin mononucleotide cofactor of respiratory complex I

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**Abstract**

The flavin mononucleotide (FMN) cofactor of respiratory complex I occupy a key position in the electron transport chain. Here, the electrons coming from NADH start the sequence of oxidoreduction reactions which drives the generation of the proton-motive force necessary for ATP synthesis. The overall architecture and the general catalytic proprieties of the FMN site are mostly well established. However, several aspects regarding the complex I flavin cofactor are still unknown. For example, the flavin binding to the N-module, the NADH-oxidizing portion of complex I, lacks a molecular description. The dissociation of FMN from the enzyme is beginning to emerge as an important regulatory mechanism of complex I activity and ROS production. Finally, how mitochondria import and metabolize FMN is still uncertain. This review summarizes the current knowledge on complex I flavin cofactor and discusses the open questions for future research.

## Introduction

Heterotrophic organisms live by the oxidation of high energy bonds of organic substrates derived from foodstuffs. The breakdown of carbohydrates, proteins and fats provides the free energy that maintains the [ATP]/[ADP] ratio far from equilibrium, allowing endergonic processes coupled with ATP hydrolysis to proceed at a physiologically useful rate (1).

The main pattern of ATP synthesis in aerobic organisms is oxidative phosphorylation, in which the transfer of electrons from a reduced substrate to molecular oxygen is coupled to the phosphorylation of ADP. During oxidative phosphorylation a few selected reduced soluble molecules (e.g. NADH, succinate and fatty acids) are oxidized by the electron transport chain, a sequence of distinct polypeptides and cofactors localized in the mitochondrial inner membrane.

The first event that inputs electrons into the chain is a redox reaction between a reduced substrate and a coenzyme form of riboflavin, either FMN or flavin adenine dinucleotide (FAD) (2). These cofactors are redox-active compounds placed within respiratory complexes in clefts accessible to their respective substrate. The FMN of respiratory complex I reacts specifically with NADH. From here, high-energy electrons enter the chain and move forward to complex III and complex IV and, ultimately, to molecular oxygen. Besides its fundamental role in oxidative phosphorylation, the flavin group of complex I is also a major contributor to production of reactive oxygen species (ROS) by mitochondria (3-5). For these reasons, the structural and functional proprieties of the FMN site of complex I have been an uninterrupted area of research since its discovery.

In this review we discuss several aspects of complex I biology from the point of view of its cofactor, FMN. We begin by describing the molecular structure within complex I that binds FMN. We then review the evolution, the assembly pathway and the turnover mechanism of the N-module, the functional unit that harbors the flavin cofactor. Next, we comment upon the open questions regarding the complex I flavin association pathway and underscore the role of FMN in both catalysis and ROS production by complex I. Finally, we discuss how complex I FMN cofactor is integrated in the context of mitochondrial flavin homeostasis. We focus mainly on human complex I. When a specific reference to a gene of another species is made, we will explicitly state both names, the human homologue and the other.

**1. Complex I FMN binding site**

NADH:Ubiquinone oxidoreductase, or respiratory complex I, is an energy transducing enzyme that couples the vectorial proton translocation across the internal mitochondrial membrane to the scalar reaction of electron transport. The catalytically active components of complex I are fourteen highly conserved subunits which contain FMN and eight iron-sulfur clusters (6, 7). In the human enzyme, 31 accessory subunits are added to form a shell around the core (6-8).

The structure of this multisubunit membrane-bound enzyme has been solved by cryo-electron microscopy at 3.9 Å resolution (6, 7) and substantial progress has been made towards the understanding of the coupling mechanism (9).

These remarkable achievements are the results of a series of uninterrupted investigations that began when complex I was first isolated from beef heart mitochondria almost 60 years ago (10). Subsequent attempts were made to identify the flavin component of the preparation. It was shown that upon heat and acid treatment

68 neither FAD nor Riboflavin but only FMN was released from the beef heart
69 NADH:ubiquinone oxidoreductase (11, 12). Furthermore, it was possible to dissociate
70 the bound cofactor from its enzyme by mild procedures and to restore the activity of
71 the resulting apoenzyme by the addition of FMN (12), thereby proving once and for all
72 the nature of the flavin. Later, the refinement of the isolation and purification
73 techniques enabled the identification of the coenzyme binding site.

74 Taking advantage of the experimental resolution of complex I into three discrete
75 fragments by chaotropic agents, it was possible to determine the one where FMN is
76 bound to (10, 13, 14). It is a soluble flavo-iron sulfur subcomplex that retains the
77 capability to oxidize NADH in the presence of various electron acceptors (10, 13, 14). For
78 this reason, it was named "Flavoprotein" (Fp). The Fp fragment can be further separated
79 into three polypeptides of 51, 24 and 10 kDa called NDUFV1, NDUFV2 and NDUFV3
80 respectively (the latter may be present or not depending on the resolution method) (15,
81 16). Nowadays it is known that the 51 kDa subunit (NDUFV1) is a highly conserved
82 protein (17-19) that contains the NADH binding site (20) and a tetranuclear Fe-S center
83 known as N3 (14). Structural (17, 18, 21) and functional (20) data indicate that the FMN
84 binding site is located in this subunit (Figure 1). Alignment of the NDUFV1 aminoacidic
85 sequence with Nubm and Nqo1, its homologues from *Yarrowia lipolitica* and *Thermus*
86 *thermophilus* respectively, reveals a Rossmann fold-like domain (residues 96 to 264) that
87 appears to bind the flavin cofactor (18, 19, 22). This unique domain differs from a
88 classical Rossmann fold, the common nucleotide binding structure of dehydrogenases
89 (23), for it has only four stranded parallel ß-sheets instead of six (18). This unique feature
90 allows to bind both FMN and NADH. Further details on the complex I FMN binding
91 domain were provided by a study which employed *Y.lipolitica* as a model organism to

investigate the molecular consequences of single amino acid substitutions in conserved residues of Nubm (NDUFV1) (19). Mutations in the amino acids directly involved in the formation of non-covalent bonds with the flavin cofactor, like the conserved $Tyr^{204}$ (Figure 1), are sufficient to provoke the complete loss of mature complex I (19). On the other hand, mutations in nearby residues, like $Cys^{206}$ and $Glu^{377}$ of the human NDUFV1, result in loss of the flavin from the complex, that, nevertheless, can normally assemble. In this case, incubation of the mutated enzyme with FMN is able to restore some activity, but never to the wild type level, indicating that the enzyme is still able to bind the cofactor but not tightly (19). While $Cys^{206}$ is located in the Rossmann fold-like domain, $Glu^{377}$ is in the 4Fe-4S binding domain, showing that derangement of the iron sulfur cluster is, in turn, able to destabilize FMN binding (19).

Collectively, these experiments show that proper arrangement of the FMN site can impact on the correct maturation of respiratory complex I. Unlike complex II, for which the overall flavinylation pathway has been described (for a recent review see (24)), very little is known on when and how FMN binds to complex I. However, the available information about the assembly pathway and the evolution of complex I still allows us to draw some conclusions.

## 2. Evolution and assembly of complex I N-module

### *2.1 Evolution of complex I N-module*

The organization of the structural subunits and the sequence of their association into the mature holoenzyme support the notion that human complex I is formed by three defined functional modules: the NADH-oxidizing N-module, the ubiquinone-reducing Q-module and the proton-pumping P-module (25, 26). Phylogenetic analyses reveal that these building blocks evolved separately from distinct ancient bacterial

dehydrogenases (27-29) that progressively combined to form the contemporary members of complex I superfamily. It seems that the core structure of the ancestor of complex I contained just the Q and the P-module and that the electron-input N-module was added only later during evolution (27-29). The core subunits of the N-module (NDUFV1, NDUFV2 and the N-terminus of NDUFS1) are closely related to components of a soluble hydrogen:$NAD^+$ oxidoreductase from the Gram-negative bacterium *Cupriavidus metallidurans* (17, 21). Thus, the N-module of mitochondrial complex I probably evolved from a hydrogen-oxidizing enzyme of an aerobic bacterium. Complex I kept the cofactor and the substrate of the ancestor enzyme, FMN and $NAD^+$ respectively, for a completely new purpose, i.e. aerobic respiration. Nevertheless, the study of complex I assembly reveals that the N-module, despite the loss of its original hydrogenase function, still behaves as a functional unit.

*2.2 Assembly of complex I N-module*

NDUFV1, which contains the FMN binding site, is a nuclear encoded protein that is first translated inside the cytoplasm and is then imported into the mitochondrial matrix through the TIM23 complex (30). In the matrix, NDUFV1 is found in three main states: as a free soluble monomer, as part of soluble assembly intermediates or bound to the mature holo-Complex I (31-34). NDUFV1 association with others N-module subunits precedes its incorporation into complex I, as showed by experiments of expression of GFP-tagged subunits and by complexome analysis of recovery from mitochondrial translation inhibition (32, 33). The gradual maturation of the N-module proceeds through two subsequent assembly intermediates: a low molecular weight one that contains only NDUFV1 and NDUFV2 (that we name "$N_1$"), and a high molecular weight one (that we name "$N_2$") in which NDUFS1 and the accessory

NDUFA2 join as well (Figure 2). Eventually, the incorporation of this hydrogenase-like building block into the Q/P intermediate (33, 35) activates the NADH:ubiquinone oxidoreductase activity. The remainder of the N-module accessory subunits joins lastly: NDUFA12, NDUFS6 and NDUFS4 first (36) and then NDUFA6, NDUFA7 and NDUFV3 (Figure 2). Therefore, during complex I maturation, the N-module is incorporated only at the last stage, when most of the enzyme is already assembled into a Q/P subcomplex (Figure 2) (33). This is a remarkable analogy with the modular evolution proposal, showing how ontogeny recapitulates phylogeny at the molecular level as well.

Interestingly, $\rho^0$ 143B cells, which lack mitochondrial DNA and therefore are not able to assemble the holo-enzyme, accumulate the lower molecular weight NDUFV1/NDUFV2 assembly intermediate $N_1$ (33). However, pre-association in subassembly seems not to be obligatory for NDUFV1 to be loaded on complex I: studies *of in vitro* importing of radio-labeled peptides showed that NDUFV1 can be loaded on already-existing complex I through exchange of preexisting subunits (34). In any case, it is still uncertain at which stage NDUFV1 binds its cofactor FMN and if the N-module assembly intermediates present NADH dehydrogenase activity *in vivo*.

**3. Flavin binding to respiratory complex I**

It should be clear at this point that the "$N_1$" module, observed during complex I assembly, and the "Fp" fragment, which may be separated from complex I by chaotropic agents, represent the same entity (14, 15, 33, 37) (Figure 2). They are constituted of two subunits: NDUFV1, which contains the flavin and a 4Fe-4S cluster, and NDUFV2, which contains a 2Fe-2S cluster (14). The Fp fragment, i.e. the "$N_1$" module, is therefore generally considered, the minimal catalytic competent unit of complex I with NADH

dehydrogenase activity, at least *in vitro*. NDUFV3, which is never found in the "$N_1$" module, sometimes co-purifies with the "Fp" fragment depending on the isolation procedure, but its presence is not required for catalytic activity (15).

Interestingly, when the Fp fragment is incubated with NADH in the absence of any electron acceptor, it loses irreversibly its activity due to the dissociation of the FMN from the enzyme. The inactivation is probably due to the denaturation of the protein because, once it happens, it cannot be reversed by FMN addition (37). Importantly, the same does not occur when isolated complex I is incubated with NADH, presumably because, in the native enzyme, the flavin binding site is sheathed by other subunits that confer further structural stability (12, 37, 38).

A similar phenomenon is observed when the Fp fragment is resolved into NDUFV1 and NDUFV2 by sodium tri-chloroacetate treatment (16). In this case, it is the physical separation of the two subunits that triggers the loss of FMN and, in turn, of the NADH dehydrogenase activity. Of note, this treatment does not affect the iron sulfur clusters, which are kept in place. This line of evidence suggests that: 1) the reduced Fp fragment without the FMN is highly unstable (37) and 2) NDUFV1 cannot bind the FMN by itself, but it requires the presence of NDUFV2 to stabilize the interaction (16). Since the resolution process maintains intact the iron sulfur clusters it is then probable that, during complex I assembly, their insertion precedes the binding of the flavin (16). Therefore, the FMN cofactor would bind to NDUFV1 only when it joins NDUFV2 to form the "$N_1$" module and not before.

It has been shown that insertion of flavin cofactors into the catalytic domain of apoenzymes might or might not depend on specific molecular partners (39). For example, in the SDHA subunit of complex II FAD is covalently linked via the 8-α-methyl

group to the N-3 atom of a conserved histidine (24). In this case, flavinylation occurs after the peptide has entered the mitochondrial matrix and its targeting sequence has been removed (24, 40). It has been shown that, in order to bind FAD, complex II requires a dedicated assembly factor, SDHAF2, whose germline loss-of-function mutations are found to be associated with hereditary paraganglioma (41). In the case of NDUFV1, no such assembly factor has been described and it is unlikely that one is needed, since FMN binds to complex I non-covalently and it can dissociate and re-associate from the holo-enzyme without the requirement of any specific chaperone (12, 42). Nevertheless, it is still possible that the insertion of the flavin during NUDFV1 folding is aided by the association to other subunits during the assembly process and/or by the delivery of the iron sulfur clusters. The human protein NUBPL, homologous to the yeast Ind1, is required for the incorporation of the Fe-S centers in complex I core subunits and is found mutated in human complex I deficiencies (43, 44). Knockdown of NUBPL causes a decrease in the total amount of the NDUFV1 protein, but not of the NDUFV1 transcript, further highlighting the importance of the cofactors for the stability of core complex I subunits (44).

When *E. coli* NDUFV1, NDUFV2 and NDUFS1 analogues (NuoF, NuoE and NuoG respectively) are over expressed in a bacterial system, no production of an active Fp fragment is observed (45). Still, the co-expression of NuoC and NuoD (analogues of the human NDUFS3 and NDUFS2) allows the formation of an active NADH dehydrogenase *in vivo.* By adding riboflavin, ferric ammonium citrate, and sodium sulfide to the culture medium the production of the Fp fragment can be stimulated even more (45). The same group reported later that deletion of the *E. coli* complex I genes encoding for Q- and P-module subunits, results in accumulation of an active NADH dehydrogenase fragment

(46). The fragment is very similar to the human $N_2$ module (Figure 2), it contains the NuoF, NuoE and NuoG proteins, the iron sulfur clusters and the FMN cofactor. Collectively, it seems that the insertion of the flavin cofactor already happens during the maturation of low molecular weight N-module subassemblies. It is therefore possible that, during human complex I assembly, the N-module intermediates are already catalytically active before the incorporation into the holo-enzyme.

On the ground of the previous considerations, we can now assume that the assembly of the human N-module may proceed through the following steps (Figure 2): (i) Import of the nuclear encoded peptides into the mitochondria. (ii) Insertion of at least one iron sulfur cluster by NUBPL. (iii) Formation of the NDUFV1/NDUFV2 heterodimer ($N_1$ module). (iv) Binding of the FMN cofactor (at this point NADH dehydrogenase activity would start), (v) Association with NDUFS1 and NDUFA2 ($N_2$ module). (vi) Incorporation into the Q/P intermediate to form the holo-enzyme

The presence of active assembly intermediates during complex I assembly still lacks a molecular confirmation. If true, it poses a problem: to avoid the accumulation of unassembled fragments that would result in futile NADH oxidation and ROS production (51). In fact, the degradation of the N-module subunits in mitochondria is regulated by a dedicated molecular machinery independently of the rest of complex I.

## 4. Stability and turnover of the N-module

### *4.1 N-module turnover and maintenance*

To maintain a proper mitochondrial function, the synthesis and import of oxidative phosphorylation proteins must be fine-tuned with their degradation rate (for a recent review, see (47)). The average lifespan of different peptides varies greatly even between subunits that are part of the same respiratory complex (48, 49). In particular,

236 the N-module of complex I has a relatively short lifetime, and it is the portion of the
237 oxidative phosphorylation system whose components' turnover is the fastest (48-50).
238 There may be several reasons behind the need for a faster turnover: in the case of the
239 FMN containing N-module, the most straightforward explanation is that being one of
240 the major sites of ROS production in mitochondria results into extensive oxidative
241 damage and loss of function (51, 52). Whatever the purpose, N-module subunits are
242 targets of two ATP-dependent proteases inside the mitochondria: LonP and ClpXP (50,
243 53). LonP-mediated NDUFV1 removal seems to be responsive to mitochondrial inner
244 membrane depolarization (54) and to the increase in intramitochondrial cAMP levels
245 with consequent activation of PKA kinase (55). Interestingly, this pathway is activated in
246 astrocytes in response to cannabinoids signaling and causes a decrease in complex I
247 activity through N-module destabilization (56), demonstrating that regulation of the
248 FMN binding domain of complex I can be used *in vivo* for metabolic adaptation. On the
249 other hand, ClpXP is the protease that regulates the levels of N-module subunits in the
250 steady state. Upon ClpXP deletion, in mammalian cells (50), murine tissues (57) and
251 plants (58) the protein abundance of NDUFV1, NDUFV2 and NDUFS1 increases, and blue
252 native gel analysis reveals the accumulation of N-module subcomplexes. Some of these
253 subassemblies correspond to the intermediates of complex I assembly pathway and at
254 least one of them, probably the $N_1$ module, retains NADH dehydrogenase activity as
255 shown by in-gel activity experiments (50, 57, 58) (Figure 2). Thus, ClpXP is a protease
256 that promptly degrades complex I N-module subunits avoiding the accumulation of
257 subassembly intermediates regulating the fast turnover of this domain of the respiratory
258 chain. Recently, thanks to studies on patients suffering from Leber's hereditary optic
259 neuropathy, DNACJ30 was described to be an additional important protein for N-

module repair (59). It seems that DNACJ30 is a chaperone that controls the turnover of the accessory N-module subunits NDUFV3, NDUFS4, NDUS6, NDUFA6 and NDUFA7 (59) (Figure 2).The joint action of ClpXP (which regulates the core) and DNACJ30 (which regulates the accessory) would then assure the proper maintenance of complex I N-module.

*4.2 N-module stability in disease*

Next, we comment upon one last instance in which the N-module reveals its uniqueness, i.e. complex I deficiencies. Several mutations in nuclear and mitochondrial genes are cause of complex I dysfunction (60) and the study of these pathologies help us to understand many aspects of Complex I biology. Blue native gel electrophoresis is an important tool that allows to study the structural organization of the electron transport chain components and it is used to characterize the underlying biochemical defects of disorders. Thanks to the increased usage of this technique throughout the years, it is now clear that some patients exhibit a similar blue native migration profile despite bearing mutations in different genes. This is the case for complex I deficiencies caused by N-module genes defects (61). Accumulation of the stalled P/Q assembly intermediate with the associated assembly factor NDUFAF2 seems to be a common finding in mitochondria from patients carrying mutations in the *NDUFS4* (34, 62-64), *NDUFV1* (64, 65), *NDUFS1* (66), *NDUFS6* (34, 67) and *NDUFA2* (68) genes. Moreover, the same blue native migration pattern is observed in mitochondria from *NDUFS1*, *NDUFA2*, *NDUFS6*, *NDUFA6* knockout cell lines (8, 36) and in a *Ndufs4* knockout mouse model (69). These evidences suggest that a single defect in one of the N-module subunits is sufficient to impair the incorporation of the others all-at-once, while the rest of the complex is assembled normally.

A crucial advance in the knowledge of complex I dysfunction was provided by an elegant study in which knockout cell lines of each one of the nuclear encoded 31 accessory subunits were generated and characterized by proteomics, transcriptomics, and functional analysis (8). The huge amount of data generated by this study provides several insightful hints to elucidate the biology of the N-module. Firstly, this study clarified that absence of NDUFS6, NDUFS4, NDUFA2 and NDUFA6 proteins impairs the stability of the N-module on complex I by different mechanisms. Indeed, in the *NDUFS4* knockout cell line, the N-module is not degraded but it is only dislodged from the holo-enzyme, probably during the isolation procedure (70) , as it can be seen as a faster migrating subcomplex in the blue native gel. In fact, the proteomic analysis shows that, in this cell line, the overall level of N-module subunits does not change. Complex I is still able to provide at least partial activity, as revealed by the functional studies and by the viability of the cells in galactose. On the other hand, *NDUFA2* and *NDUFA6* knockout cell lines lose completely the N-module and, consequently, they are unviable in galactose. *NDUFS6* knockout cells situate somehow in between these two extremes. Secondly, this study showed that in each knockout cell line which is unviable in galactose (25 out 31) the whole N-module is always absent no matter which subunit is missing. This specific regulatory mechanism operates at the protein level because the lack of the subunits was not parallel by a decrease in the correspondent messenger RNA. This observation further corroborates that whenever the N-module is not able to be assembled on complex I it is rapidly degraded, probably by the ClpXP machinery (50). The same regulatory mechanism is activated upon inhibition of mitochondrial ribosome by antibiotics such as chloramphenicol, where absence of mitochondrial encoded subunits induces a marked decrease in the overall abundance of nuclear encoded subunits (33).

When the protease component of ClpXP is deleted, the decrease in the N-module subunits does not happen, proving that ClpXP is directly involved in the degradation process (50). As a matter of fact, bacteria, which lack a dedicated protease system such as ClpXP, do accumulate the N-module when another portion of complex I is deleted (46).

Thus, proper function of most of the N-module components seem to be a prerequisite for the stability of the whole FMN binding portion of complex I and, on the other hand, a defect either in the Q or the P-module triggers a rapid degradation of the N-module as a whole. However, the opposite is not true, i.e. a defect in the N-module do not impact on the stability of the Q/P-module. Then, it naturally arises to question why such a fine tuning of the NADH dehydrogenase fragment of complex I evolved, to which we will attempt to propose some answer in the next paragraph.

## 5. The mode of action and ROS production of FMN cofactor

### *5.1 Reactivity of the flavin cofactor of complex I*

Several chemical compounds are used to dissect the catalytic proprieties of the FMN site of complex I *in vitro*. Hexacyanoferrate (III), hydrophilic quinones, hexamine ruthenium (III), $APAD^+$ and paraquat are the molecules that react with the reduced flavin of complex I, although through different mechanisms (71, 72). Furthermore, NADH oxidation by FMN can be inhibited by ADP-ribose (73), NADH-OH (74) and diphenyleneiodoium (75).

The reason why FMN is such an extremely versatile redox center is revealed by the same chemical structure of flavin cofactors. Their characteristic isoalloxazine ring system is able to accept either one or two electrons at a time and therefore it is a suitable intermediate between the obligate two electron donors (e.g. NADH) and

obligate one electron acceptors (e.g. the iron-sulfur centers of complex I) (2). Furthermore, in contrast to the freely diffusing NADH, FMN is tightly bound to complex I. This arrangement achieves two important outcomes. Firstly, the midpoint potential of the two-electron reduction of the cofactor is modified by the protein environment; as a result, the reduction potential of the complex I bound FMN (-340 mV at physiological pH, 133 mV more negative than the one for free FMN) is optimized to match that of NADH (-320 mV) for more efficient energy conservation (15, 76). Secondly, the interaction between free flavins and dissolved oxygen is minimized. Nevertheless, despite the conformation of the flavin binding site favors the reaction with the NADH/$NAD^+$ couple, the FMN moiety of complex I remains an important source of ROS by mitochondria (3). In complex I, various sites can generate ROS and their relative contribution is still debated, (4, 51, 77, 78) however, FMN seems to be one of the most important. In the presence of NADH, isolated complex I generates $O_2^{\cdot-}$, and to a lesser extent $H_2O_2$, depending on the NADH/$NAD^+$ ratio (3-5). In respiring mitochondria, when the NADH/$NAD^+$ ratio is low, little amounts of superoxide are constantly generated by this modality (3-5). It has been proposed that $NAD^+$ blunts ROS generation by complex I by occupying the substrate binding site and precluding molecular oxygen access (51).

On the other hand, complex I can generate large amounts of ROS through a mechanism called reverse electron transport (RET). RET happens when most of the ubiquinone pool is in the reduced state and the proton motive force across the inner mitochondrial membrane is high. If such conditions are met, electrons flow back to the complex I to form superoxide (52, 79-81). During ischemia reperfusion injury, the specific conditions that allows ROS production through RET are met: the accumulation

of succinate in hypoxia constitutes an electrons pool that, upon reoxygenation, drives the superoxide formation (82) .

*5.2 Reversible FMN dissociation*

Since FMN was demonstrated to play such an important role in ROS production by complex I, the binding of the flavin cofactor to the enzyme has been recognized as a potential regulatory mechanism for mitochondrial superoxide generation (83). From the first functional studies it was noted that the affinity of the reduced $FMNH_2$ is orders of magnitude lower than that of the oxidized FMN (15, 42, 76). As we mention in the previous sections, FMN probably binds to NDUFV1 during the maturation of the $N_1$ module and it is required for the proper folding of the protein (37). Until complex I is fully assembled, dissociation of the cofactor results in the irreversible destabilization of the $N_1$ intermediate (37). In the mature human enzyme however, the dissociation of the FMN is reversible, probably thanks to the stabilizing action of the other subunits (16, 37). Similarly, when *Escherichia coli* complex I is reduced by NADH in the absence of acceptors, the flavin cofactor is released, abolishing the enzyme NADH dehydrogenase activity and ROS production (38). Here, the decreased affinity of $FMNH_2$ for its binding pocket is probably due to the reduction of the nearby iron sulfur clusters, which, in turn, induce a structural modification of the flavin-binding site (38). Nevertheless, it is unlikely that FMN loss from human complex I happens during fast turnover conditions because the acceptor (ubiquinone) is constantly regenerated by complex III (38, 42). However, during hypoxia, it is possible that the interruption of the respiratory chain, together with the increase of the $NADH/NAD^+$ ratio, would recreate the conditions necessary for FMN dissociation from complex I (38); flavin loss would then result in a drop of complex I activity. In fact, it has been recently suggested that the loss of flavin by complex I might

play a critical role both in the short- and long-term effects of ischemia-reperfusion injury (38). Murine models of ischemia reperfusion injury in brain (84, 85) and in liver transplant (86) seems to support the proposed role of FMN loss in the post-ischemic dysfunction of complex I.

It should be added that, during ischemia (87-89) and hypoxia (90, 91), complex I undergoes a dramatic structural change known as "active/deactive transition" (A/D). ROS production by RET markedly decreases when complex I is in the deactive form and, for this reason, modulation of the A/D transition is a promising therapeutic strategy for ischemia/reperfusion injury (92, 93). It remains to be elucidated whether loss of flavin by complex I and active/deactive transition during hypoxia are structurally correlated; further research is needed to clarify their relative contribution to the pathogenesis of ischemia/reperfusion injury (94).

*5.3 Redox state of cysteines and the FMN binding site*

The experiments on *E. coli* complex I suggest that changes in the overall redox state of active groups in proximity of the FMN binding pocket are sufficient to induce the dissociation of the flavin cofactor from the enzyme (84). Therefore, the affinity of FMN for its binding site might change in response to oxidative modifications that introduce negative charges and/or alter the structure of the protein (84). Human NDUFV1 contains a total of twelve cysteine residues; four of them participates in the 4Fe-4S cluster ($Cys^{379}$, $Cys^{382}$, $Cys^{385}$, $Cys^{425}$), five form part of the Rossmann fold-like domain of the nucleotide binding pocket ($Cys^{125}$, $Cys^{142}$, $Cys^{187}$, $Cys^{206}$, $Cys^{255}$) and the remaining three ($Cys^{238}$, $Cys^{286}$, $Cys^{332}$) form part of the peptide backbone. A single oxidative modification in NUDFV1 would have a different effect depending on the type of modification and on the localization of the residue affected.

For example, the redox-active thiols of NDUFV1 have been shown to be sensitive to superoxide-induced intramolecular disulfide formation (95), glutathionylation by GSSG (96), sulfonation (97) and S-nitrosation (98). Protein S-glutathionylation is a reversible cysteine modification that is sensitive to the redox state of the mitochondrial glutathione pool and has important regulatory functions (99). Glutathione disulfide (GSSG) can oxidize NDUFV1 in $Cys^{206}$, $Cys^{187}$ (both in the nucleotide binding domain) and $Cys^{425}$ (in the iron sulfur cluster) (100). The formation of GSSG mixed disulfide bonds with NDUFV1 (and NDUFS1) correlates with an increase in superoxide formation by mitochondria (96). Interestingly, incubation with GSSG causes a gradual decrease in the activity of complex I over time (96), but the underlying mechanism is still under debate.

As we previously discussed, high amounts of ROS can be produced at the FMN site of complex I. ROS are, for their chemical nature, extremely reactive, and cysteines are the most sensitive amino acid residues to ROS-mediated oxidation (99). Studies on the isolated Fp fragment showed that, in the presence of NADH, the generation of $O_2^{\cdot-}$ leads to irreversible inactivation of the enzyme (101). The reduction of the activity correlates with the oxidative modification of $Cys^{206}$ and $Tyr^{177}$, both localized in the FMN binding site of NDUFV1 (101). Complex I degradation in cellular models of electron transport chain deficiency is regulated by RET (80). Quantitative redox proteomics analyses showed that the oxidation $Cys^{125}$, $Cys^{142}$ and $Cys^{187}$ of NDUFV1 correlates with the reoxygenation-induced degradation of this subunit (80). Therefore, RET regulates the oxidation of critical cysteine residues of NDUFV1. Interestingly, when cysteines of NDUFV1 are oxidized, the mitochondrial protease ClpXP promptly degrades the subunit (50), providing a molecular explanation for the RET-induced NDUFV1 degradation.

The effect of the oxidative modifications described above on the affinity of FMN for its binding pocket is still unknown. However, if a cysteine located close to the flavin is affected, a change in the activity and in the association/dissociation properties of the cofactor is likely. As a matter of fact, in the model organism *L. lipolytica*, a single point mutation in the conserved $Cys^{206}$ (C206G) causes complete loss of flavin from complex I (19). Thus, oxidative modification of cysteine residues in the FMN binding pocket might be an important level of regulation of complex I activity and ROS production in response of the overall redox state of mitochondria.

## 6. Complex I and the mitochondrial flavin cofactor homeostasis

### *6.1 Mitochondrial flavin cofactor homeostasis*

In the previous section we reviewed how and when complex I binds its cofactor FMN and some aspects of its catalytic properties. In this last section we will discuss how complex I FMN cofactor integrates in the context of mitochondrial flavin homeostasis. Various molecular aspects of the overall human flavin metabolism were recently reviewed in (102) and here we will focus mainly on the most relevant aspects to complex I biology.

In humans, flavin homeostasis is regulated by a set of genes that controls riboflavin transport and metabolism assuring proper delivery of cofactors to nascent apoenzymes (103). Plasma membrane transport of riboflavin in the whole body, from the intestinal absorption to the urinary secretion, is jointly orchestrated by the three members of SLC52 family is (RFVT1, RFVT2 and RFVT3) (104). Once inside the cell, riboflavin is then converted to its catalytically active cofactor forms by the sequential action of two enzymes: Riboflavin kinase (RFK) and FAD synthase (FADS) (102). FAD and FMN are cofactors of the at least 90 flavoproteins that compose the “human

450 flavoproteome" (105). Of all these 90 genes, according to the updated version of
451 Mitocarta 3.0 (106), almost half (43 out of 90) encodes for a mitochondrial flavoproteins
452 (105).

453 How human mitochondria manage to uptake flavin cofactors from the cytosol is
454 still uncertain. It is not clear whether FMN can be transported across the inner
455 mitochondrial membrane directly by a specific carrier or if it is generated inside the
456 mitochondrial matrix (Figure 3). In the latter case, a mitochondrial targeted isoform of
457 the cytosolic RFK or FADS enzymes would be required to form FMN from the imported
458 riboflavin or FMN, respectively. While the existence of a mitochondrial FAD synthase
459 isoform has been extensively demonstrated (107, 108), the presence of a mitochondrial
460 RFK isoform and of a putative mitochondrial riboflavin transporter still require further
461 molecular confirmation (107). The passage of flavin cofactors through the
462 mitochondrial outer membrane probably happens through VDAC channels (109) as it is
463 the case for many small metabolites. On the other hand, it seems that FAD can be
464 transported across the mitochondrial inner membrane thanks to a carrier called MFT
465 (mitochondrial folate carrier) which transports folate but also FAD (110). The gene
466 encoding MFT, *SLC25A32*, is closely related to the yeast mitochondrial FAD transporter
467 *FLX1* gene (110, 111) and it was found to be mutated in patients with a riboflavin-
468 responsive neuromuscular disease (112). In line with its proposed role, patient's
469 *SLC25A32*-deficient fibroblasts present deficient activities in components of the electron
470 transport chain such as complex II (112). If FAD is the form in which riboflavin is
471 transported inside the mitochondria, FMN must be formed not by the phosphorylation
472 of riboflavin, but through hydrolysis of FAD into FMN and AMP. Two types of enzymes
473 were proposed to carry out this reaction in humans and, surprisingly, one of them is the

very same FADS. In fact, beside the FAD synthase domain located at the C-term of the protein, some isoforms of FADS present a N-term molybdo-pterin-binding domain capable of FAD hydrolysis (113). The reason behind the localization of two distinct domains that catalyze the functional opposite reactions on the same enzyme and how the regulation of the two activities may happen is still not known; however, it has been proposed that redox modifications of the cysteine residues of the protein may ensure a potential regulatory mechanism capable of tuning the overall direction of catalysis (102, 113). Another putative class of FAD hydrolyzing enzymes that could convert FAD into FMN inside the mitochondria is the NUDIX family (114), yet a formal demonstration is still lacking. On this account, summarizing the information we have at our disposal, we can hypothesize that FMN delivery to the nascent complex I would occur through the following steps (Figure 3): (i) Uptake of extracellular riboflavin by a plasma membrane carrier of the *SLC52* family. (ii) Conversion of riboflavin into FMN first by cytosolic RFK. (iii) Conversion of FMN into FAD by cytosolic FADS. (iv) Transport into the mitochondrial inter-membrane space through VDAC. (v) Mitochondrial FAD uptake by MFT. (vi) Conversion of FAD into FMN (probably by the FAD hydrolase activity of the mitochondrial FADS isoform). (vii) Binding of FMN to NDUFV1 subunit

A defect in one of these steps should then result, among other clinical findings, in a deficient complex I activity, as it is the case for patients bearing mutations in the genes encoding RFVT1/2 (115) and FADS (116, 117). To our knowledge, mutations in the RFK gene were never observed to be the cause of a clinical syndrome and this is not surprising because it reflects the unconditioned essentiality of this gene, as shown by the embryonic lethality of the mouse knockout model (118). Apart of the scheme

proposed above, we expect that upcoming investigations will soon help to clarify the doubtful aspects of mitochondrial flavin homeostasis.

*6.2 Mitochondrial FMN pool*

As soon as the coenzyme is finally produced and transported, the likelihood of FMN binding to complex I NDUFV1 subunit would be regulated, among other factors, by the availability of FMN in the mitochondrial matrix and by the competition with other FMN binding enzymes. An exact quantification of matrix flavin coenzymes is challenging for the tendency that these compounds have to be degraded during sample handling (119, 120), nevertheless, various estimations of FMN matrix concentration can be found in the literature, albeit often expressed in different units of measurement (mol/g of protein, g/g, molarity). Based upon the quantifications made in a variety of biological samples, FAD seems to be the main flavin compound present in the cell, meanwhile FMN occurs to be less abundant; riboflavin is often found only in traces or completely absent (111, 121-123). The preference towards FAD could be explained by the fact that most it is the most common coenzyme of the flavoproteome (84 % of the human flavoenzyme uses FAD and only 16 % uses FMN) (105) and by its lower reactivity in solution when compared with FMN and riboflavin (111). The ratio between FAD and FMN concentrations appears to fit between 3 and 8 (i.e. FAD being 3-10 times more abundant than FMN) both in total cell lysates and in mitochondrial matrix preparations (111, 121-123). Moreover, a recent study (124) reported an absolute matrix concentration value for FAD of 26.21 ± 7 µM. By combining the two measurements, the estimation of the mitochondrial matrix FMN concentration would be situated between 8.3 and 2.62 µM. According to Mitocarta 3.0 (106), in human mitochondria, there are only 3 enzymes which employ FMN as a cofactor and one of them, Dihydroorotate dehydrogenase

(DHODH), is anchored to the internal mitochondrial membrane facing the intermembrane space, therefore, it has no access to the matrix FMN pool (125). The remaining two, complex I NDUFV1 and pyridoxamine 5'-phosphate oxidase (PNPO), are thus the only enzymes located in the mitochondrial matrix that bind FMN. A quantitative study of protein abundance in mouse (126) showed that, in most tissues, the amount of NDUFV1 is 5 to 20-fold higher than the amount of PNPO, with liver being the exception because here the two proteins have comparable levels. It follows that, apart from liver, most of the protein-bound fraction of mitochondrial FMN belongs to complex I. Whether FMN abundance could be a limiting factor for the assembly and the activity of complex I or not is still not known and it would require a dedicated set of studies to be resolved. If the intramitochondrial NDUFV1 concentration is in the same range, or even below, the FMN one, then, availability of the cofactor would be an important point of control of complex I activity; this consideration would further strengthen the rationale behind the clinical usage of riboflavin supplementation therapy in complex I deficient patients.

The link between complex I and its cofactor is not solely related to its activity but it extends to its structural stability as well. Indeed, the binding of the flavin cofactors to the respective apoenzyme not only provides catalytic activity but it is often crucial for the correct folding of the protein (127, 128). The decreased abundance of N-module subunits observed upon riboflavin withdrawal in cultured cells (127, 129) may be explained by a post translational mechanism that recognizes and degrades the flavin-deficient peptides and the ClpXP machinery seems to be a likely candidate for such a task (50).

*6.2 Further connections between complex I and flavin coenzymes*

While the connection between complex I structure and function and flavin cofactor homeostasis seems to be unidirectional at first (i.e. the cofactor regulates the enzyme), there are some studies in which the opposite is observed, in other words, where the function of the electron transport chain influences the cofactor levels. For example, complex I inhibition by rotenone in HeLa cells results in decreased levels of FAD and FMN (130). Moreover, in knockouts cell lines for $NAD^+$ and $P_i$ mitochondrial transporters (*SLC25A51* and *SLC25A3* respectively) where oxidative phosphorylation is blunted, riboflavin is one of the most significatively depleted mitochondrial metabolites (131). Finally, oligomycin a or piericidin A treatment induce a depletion in the mitochondrial FAD pool (124). These results highlight the mutual interdependence between complex I and its coenzyme, and that regulatory mechanisms may exist at many levels.

We will comment upon one last case in which this intimate relationship is unveiled. Complex I assembly is an intricate process which requires the coordinate action of at least 18 dedicated assembly factors (8, 132-134) whose list keeps expanding every year. Of the several proteins that were proposed to play a role in complex I assembly, three of them are flavoproteins and play a role in the maturation of the P-module, namely FOXRED1, ACAD9 and AIFM1. FOXRED1 is a FAD-dependent oxidoreductase involved in the maturation of the ND4 membrane subcomplex (8, 33, 135) and it was found to be mutated in patients with defects in complex I (43). ACAD9 is a FAD-dependent acyl-CoA dehydrogenase specific for long chain unsaturated fatty acids with a secondary role in beta-oxidation but a primary role in complex I biogenesis (133). ACAD9 is crucial for the formation of the MCIA complex, an inner membrane machinery composed of various assembly factors, that assists the formation of the ND2

subcomplex (133, 136). ACAD9 mutations are known cause of complex I deficiency and are often riboflavin-responsive (137). Interestingly, the switch between the two functions of ACAD9 seems to be regulated by the de-flavinylation of the enzyme induced by other components of the MCIA complex (138). Finally, AIFM1 is an inner membrane protein that binds FAD and NADH and cooperate with CHCHD4 to assist the proper deliver of nuclear encoded subunits to the nascent ND2/ND6 portion of complex I (8, 139). At this point, it should be clear that mitochondrial flavin cofactor homeostasis and respiratory complex I regulate each other at multiple levels and that the elucidation of their mutual influence is a promising research field for the years to come.

**Future directions and Perspectives**

Since its first description, our knowledge about the NADH dehydrogenase component of the mitochondrial respiratory chain has kept expanding. Thanks to the impressive resolution of single-particle electron cryomicroscopy (6, 7), the structural features of mammalian complex I are now being employed to decipher its coupling mechanism (9). These studies provide an important framework for the rationale behind the development of therapeutic strategies (92, 93) and new drugs (140) for human diseases. Nevertheless, whereas the general architecture of the FMN binding site is known, several functional aspects regarding the flavin cofactor remain unknown. In this review, we discussed the main topics regarding the complex I FMN that we think still need a molecular description. For example, the regulatory mechanisms beneath the flavin binding of the N-module are still unknown, and they might be an important point of control of the assembly of complex I. The association/dissociation of the FMN cofactor during ischemia/reperfusion injury and the mitochondrial metabolism of flavins are other crucial processes that are starting just now to be recognized.

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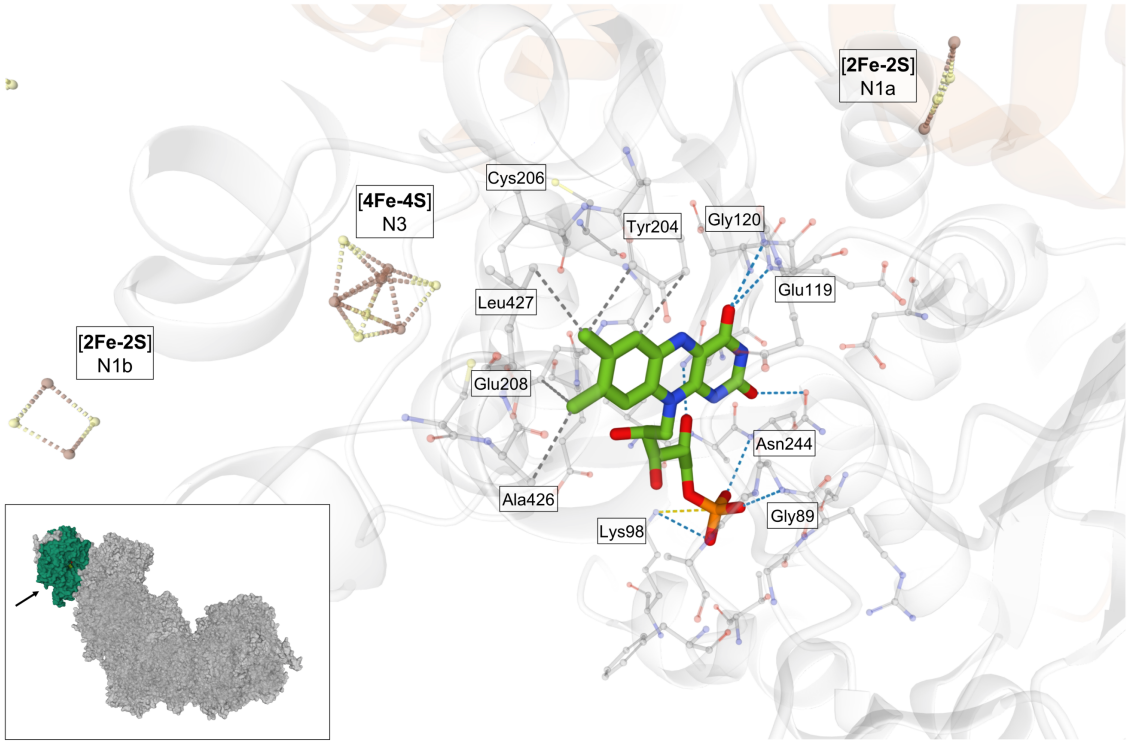



**Figure 1.-** ***3D structure of the FMN binding site*****.** The non-covalent interactions between FMN and the amino acidic residues of the binding site are colored to show hydrogen bonds (blue), hydrophobic interactions (gray) and ionic interactions (yellow). The cofactor and the main amino acids are shown as sticks, the rest of the NDUFV1 and NDUFV2 subunits are shown as white and orange cartoons, respectively. The relative position of the N1a, N1b and N3 iron sulfur clusters and of $Cys^{206}$ is shown. In the insert, the NDUFV1 subunit is colored in dark green to show its position in the assembled mammalian complex I. The arrow indicates the direction of the view, the orientation of the main picture was chose to obtain the best clarity of the FMN binding site. (PDB accession number utilized 6ZKQ, all the amino acids shown are conserved in the human protein).

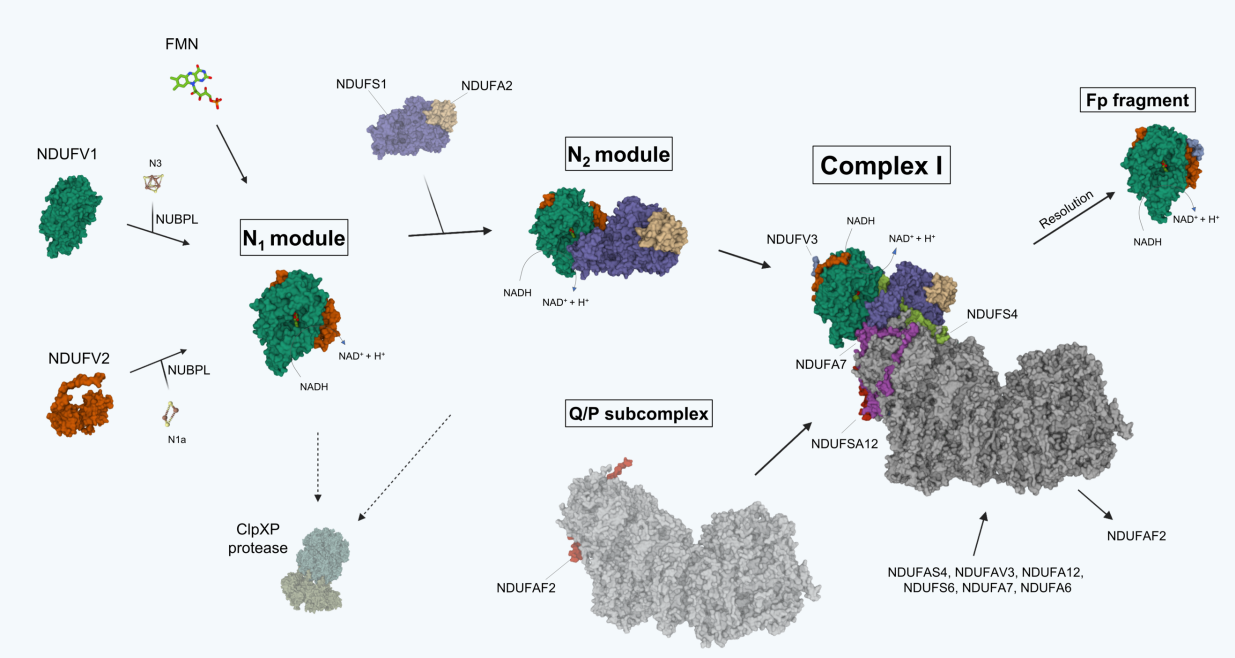
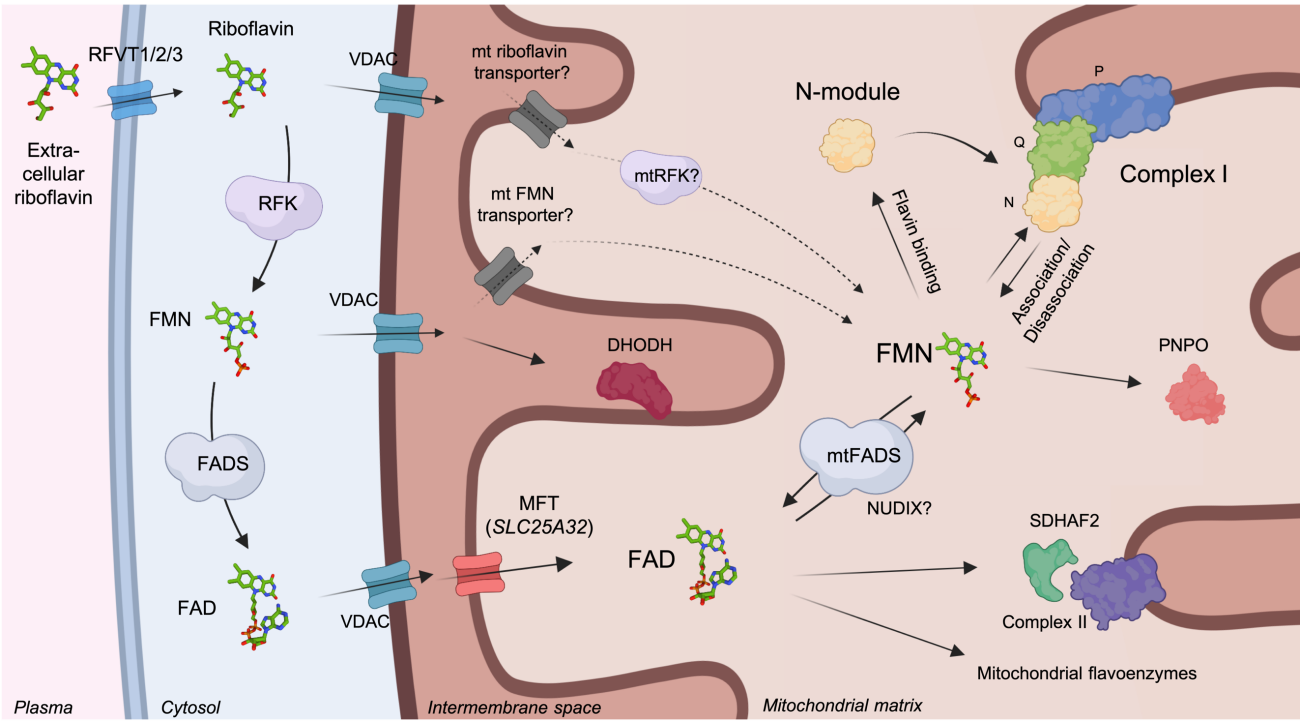



**Figure 2.-** ***Assembly pathway and flavin binding model of the N-module*****.** The proposed assembly pathway and flavin binding model of the N-module is shown. The insertion of the iron sulfur cluster of NDUFV1 (dark green) and NDUFV2 (orange) is mediated by NUBPL. Flavin binding occurs together with or just after the formation of the low molecular weight $N_1$ module. The insertion of the flavin marks the start of the NADH dehydrogenase activity. Upon addition of the NDUFS1 and NDUFA2 subunits the high molecular weight $N_2$ module is formed. In the last step of complex I assembly the $N_2$ module binds the Q/P subcomplex, the NDUFAF2 cofactor is substituted by NDUFA12 and the other accessory subunits are finally connected. Upon chaotropic agent treatment, the Fp fragment is resolved from complex I, retaining the NADH dehydrogenase activity. The action of ClpXP protease controls the abundance of the N-module. (PDB accession number 6ZKQ)




999

1000 **Figure 3.-** ***Integration of mitochondrial FAD and FMN homeostasis and complex I***
1001 ***maturation.*** The cytosolic and mitochondrial metabolism of flavin cofactors are shown.
1002 The uptake of extra-cellular riboflavin is mediated by one of the RFVT (*SLC52*) family
1003 members. The conversion of riboflavin into FMN and FAD occurs in the cytosol thanks
1004 to the enzymes RFK and FADS. The three cofactors pass through the outer mitochondrial
1005 membrane through VDAC channels. In the inter membrane space DHODH is the only
1006 FMN-containing enzyme. The uptake of FAD by mitochondria is mediated by the MFT
1007 transporter. The existence and the identity of putative mitochondrial riboflavin/FMN
1008 transporters and RFK isoform need further molecular confirmations. Once in the matrix,
1009 FAD either binds to apoenzymes (such as complex II, which is shown) or is converted to
1010 FMN by the mitochondrial FADS and/NUDIX enzymes. FMN binds to one of the only two
1011 matrix FMN-containing enzymes: NUDFV1, of the N-module, and PNPO. The FMN pool
1012 is also in equilibrium with the complex I FMN through the process of
1013 association/dissociation.