

# **NDUFAF3 is involved in the assembly of the Q/P modules of respiratory complex I in the green microalga *Chlamydomonas reinhardtii***

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## Abstract

The mitochondrial NADH:ubiquinone oxidoreductase, or complex I, is composed of a hydrophobic arm comprising the P module and a hydrophilic arm comprising the N and Q modules. The assembly of complex I is well characterized in humans and catalyzed by a series of assembly factors that join the Q, P, and N modules sequentially. The complex I of protists and plants, however, contain additional ancestral features namely a ferredoxin bridge that connects the matrix and the membrane arms and a  $\gamma$  carbonic anhydrase domain, whose mechanisms of assembly are unknown. In this work, a strain where the complex I assembly factor NDUFAF3 has been tagged with a 3xFLAG at the C-terminus is investigated in the green microalga *Chlamydomonas reinhardtii*. Like its human homolog, NDUFAF3 interacts strongly with the classical subunits of the Q and P modules, but also with the  $\gamma$  carbonic anhydrase domain and C1-FDX, a subunit of the ferredoxin bridge. The predicted structural positioning of NDUFAF3 within the Q module suggests a role in the formation of this bridge. In contrast, subunits of the N module are only loosely associated with NDUFAF3. We further demonstrate that the N module is attached at a later stage of assembly, suggesting that *Chlamydomonas* complex I assembles in a human-like sequence. This contrasts with what is documented in Angiosperms where the N and the Q modules are attached together before anchoring to the P module. Altogether, these results highlight a conserved and ancestral role of NDUFAF3 in complex I manufacture.

## 1. Introduction

Mitochondrial oxidative phosphorylation drives the synthesis of ATP within eukaryotic cells. This process involves five multisubunit complexes: four oxidoreductases (complexes I-IV) and ATP synthase (complex V). NADH:ubiquinone oxidoreductase (complex I) is the main entry point of electrons into the respiratory chain. It catalyzes NADH oxidation and connects ubiquinone reduction to proton pumping from the matrix side to the intermembrane space of the mitochondrion. In eukaryotes, such as the green microalga *Chlamydomonas reinhardtii* (henceforth *Chlamydomonas*) (Cardol *et al.*, 2004; Waltz *et al.*, 2025) complex I is approximately 1 MDa and consists of about 50 subunits, of which 14 core subunits are of bacterial origin. X-ray and cryo-EM analyses revealed that complex I is composed of two arms, structured into an L-shape in all organisms examined so far (reviewed in (Galemou Yoga *et al.*, 2020)) (**Fig. 1A**). The hydrophilic arm protrudes into the mitochondrial matrix, and it is composed of the N module (NADH dehydrogenase) and the Q module (ubiquinone reduction), both of which together harbor critical prosthetic groups (8 Fe–S clusters, 1 flavin mononucleotide). The membrane arm is involved in proton translocation (P) and is divided into two modules, one proximal (P<sub>p</sub>) and one distal (P<sub>d</sub>) with respect to the location of the peripheral arm (**Fig. 1A**). The hydrophobic arm contributes to proton pumping by a mechanism hypothesized to rely on a long-range propagation of conformational rearrangements driven by ubiquinone reduction (Kampjut and Sazanov, 2020). In many protists (Zhou *et al.*, 2022; Mühleip *et al.*, 2023) and photosynthetic eukaryotes, including *Chlamydomonas*, an additional matrix-exposed domain attached to the P<sub>p</sub> module of the membrane arm is composed of  $\gamma$  carbonic anhydrase subunits (Dudkina *et al.*, 2008; Perales *et al.*, 2004; Sunderhaus *et al.*, 2006; Fromm *et al.*, 2016; Soufari *et al.*, 2020; Klusch *et al.*, 2021; Waltz *et al.*, 2025). In addition, a bridge connects the two arms of plant complex I between the Q module and the  $\gamma$  carbonic anhydrase domain (**Fig. 1A**). The bridge contains a ferredoxin-like protein (C1-FDX), which plays a critical role in the establishment of the connection (Klusch *et al.*, 2021). Together with the  $\gamma$  carbonic anhydrase domain, this bridge is considered a conserved and ancestral feature of complex I in protists, green algae, and land plants, lost in fungi and mammal complex I (Braun and Klusch, 2024).

Complex I formation begins with the independent assembly of the four modules (N, Q, P<sub>d</sub> and P<sub>p</sub>) described above. These modules are then further combined in a stepwise manner to yield the active holoenzyme. The modular assembly has been extensively investigated in humans (Guerrero-Castillo *et al.*, 2017; Stroud *et al.*, 2016) and to a lesser extent in plants where *Arabidopsis thaliana* has remained the model of investigation (Ligas *et al.*, 2019). Complex I assembly, is *grosso modo* conserved in the two model organisms, but some differences do exist, e.g., at the matrix arm level. The matrix arm is fully assembled before connecting to the P<sub>p</sub> module in *Arabidopsis*. In humans, the Q module is first connected to the P<sub>p</sub> module. Then, the membrane arm is formed by integrating the P<sub>d</sub> module before the N module is connected last to the structure. Two assembly intermediates, corresponding to 200 and 700 kDa, have been identified in the green microalga *Chlamydomonas*. The 200 kDa module is soluble and exhibits NADH dehydrogenase activity (Cardol *et al.*, 2002). The 700 kDa subcomplex contains the matrix arm, the P<sub>p</sub> module, and the  $\gamma$  carbonic anhydrase module and is accumulated when subunits of the P<sub>d</sub> module are absent (Barbieri *et al.*, 2011; Cardol *et al.*, 2008; Cardol *et al.*, 2002; Cardol *et al.*, 2006).

Numerous assembly factors participate in complex I assembly in mammals (Guerrero-Castillo *et al.*, 2017; Formosa *et al.*, 2018). In land plants, four assembly factors are

functionally characterized. The L-galactono-1,4-lactone dehydrogenase (GLDH) (Pineau *et al.*, 2008; Schertl *et al.*, 2012; Schimmeyer *et al.*, 2016; Soufari *et al.*, 2020), and CRK1 (Wang *et al.*, 2022) participate in the assembly of the membrane arm. The protein INDH/NUBPL (Wydro *et al.*, 2013) and the LYR protein CIAF1 (Ivanova *et al.*, 2019) deliver Fe-S clusters to subunits of the matrix arm. INDH/NUBPL is also involved in mitochondrial translation (Wydro *et al.*, 2013). Both INDH and CRK1 are conserved and have been functionally examined for their role in complex I assembly in mammals (Sheftel *et al.*, 2009; Dunning *et al.*, 2007), while GLDH and CIAF1 are plant-specific (Ivanova *et al.*, 2019; Ligas *et al.*, 2019).

Only two assembly factors have been functionally analyzed in *Chlamydomonas*. AMC1 is only found in *Chlamydomonas* and plays a role in the expression of the mitochondrial *nd4* gene (Subrahmanian *et al.*, 2020), which encodes a core subunit of the P<sub>d</sub> module. The absence of the assembly factor NDUFAF3 results in a lack of fully-formed complex I in *Chlamydomonas* and a decrease in the abundance of some subunits of the Q module (Massoz *et al.*, 2017). NDUFAF3 is conserved in plants and mammals. In humans, defects in NDUFAF3 are responsible for neonatal death and neurodevelopmental disorders and are characterized by a decreased abundance of Q module subunits. NDUFAF3 is required to join the Q and P modules (Saada *et al.*, 2009; Ishiyama *et al.*, 2018; Baertling *et al.*, 2017; van der Ven *et al.*, 2023; Guerrero-Castillo *et al.*, 2017) and is proposed to work in association with NDUFAF4 (Guerrero-Castillo *et al.*, 2017), an assembly factor present in land plants (Ligas *et al.*, 2019), but absent in *Chlamydomonas* (Subrahmanian *et al.*, 2016). Considering that NDUFAF3 is only functionally characterized in mammals and that NDUFAF4 is missing in *Chlamydomonas*, we decided to examine the role of NDUFAF3 in complex I assembly in *Chlamydomonas*.

Our analyses demonstrate the association of NDUFAF3 with an assembly intermediate containing subunits of the Q/P modules, along with additional complex I components, not found in mammals and fungi, such as the  $\gamma$  carbonic anhydrase domain and C1-FDX of the ferredoxin bridge. Structural predictions position NDUFAF3 at a stage preceding the consolidation of this bridge. Further analyses indicate that the N module is the last to be assembled, demonstrating that the mitochondrial complex I of *Chlamydomonas* follows a human-like construction pathway.

## 2. Material and methods

### 2.1. Strains and culture media

All strains were derived from the 137C strain from the *Chlamydomonas* collection. The *ndufaf3* mutant (Massoz *et al.*, 2017), wild-type (Ct), and complemented strains isolated in this work were grown at 25°C under mixotrophic conditions in Tris-acetate phosphate (TAP) medium (Harris, 1989) under continuous low light (50  $\mu\text{mol photons.m}^{-2}.\text{s}^{-1}$ ) or in heterotrophic conditions. Division rates in exponential phase were measured according to (Pulich and Ward, 1973).

### 2.2. Transformation

Complementation experiments were performed as described in (Massoz *et al.*, 2017), using the *ndufaf3* mutant as the recipient strain. The mutant was transformed with 5  $\mu\text{g}$  of the pGH-NDUFAF3-3xFLAG plasmid (Fig. S1) and 3  $\mu\text{g}$  of the pSL18 plasmid containing the *aphVIII* gene encoding resistance to paromomycin (Depège *et al.*, 2003). Transformants were selected on paromomycin (10  $\mu\text{g ml}^{-1}$ ) containing TAP medium. The pGH-NDUFAF3-3xFLAG construct was obtained by replacing a *PstI-XbaI* fragment of the untagged synthetic NDUFAF3 gene

described in (Massoz *et al.*, 2017) with a fragment containing a 3xFLAG encoding sequence (GAC TAC AAG GAC CAC GAC GGT GAC TAC AAG GAC CAC GAC ATC GAC TAC AAG GAC GAC GAC GAC AAG) before the STOP codon (**Fig. S1**). This construct was synthesized by IDT (<https://eu.idtdna.com/>).

### 2.3. Biochemical analyses

As described in (Remacle *et al.*, 2001), membrane fractions were recovered by sonication and differential centrifugations. Soluble fractions were obtained by recovery of the supernatant of the membrane fractions. Purified mitochondria fractions were isolated from a cell wall-less strain, as described in (Cardol *et al.*, 2002). Protein amounts were determined using the Bradford assay method (Bradford, 1976). Immunoblotting was performed using standard protocols. Antibodies against TYKY (NUO8, (Barbieri *et al.*, 2011)) and NAD1 (kind gift of E Meyer, Germany) were used at 1/3500 dilution, antibodies against NAD7 (NUO7, (Barbieri *et al.*, 2011)) were used at 1/2000 dilution. Antibodies against the FLAG tag (Sigma-Aldrich) were used at 1/5000 dilution. Solubilization of crude membrane and mitochondria-enriched fractions using *n*-dodecyl- $\beta$ -D-maltoside (1.7% w/v), BN-PAGE (4-15% pre-cast gradient gels, Bio-Rad), and the subsequent staining of the gel by NADH/NBT (nitroblue tetrazolium) were done as previously published (Cardol *et al.*, 2002).

For 2D-BN/SDS PAGE, BN gels were sliced in between each well (150-200  $\mu$ g of solubilized mitochondria per well) and incubated in 2x Laemmli buffer for 45 minutes before being transferred to a 15% polyacrylamide (with 4% stacking gel) SDS gel. The second dimension SDS gels were run at 180 V for  $\sim$ 1 hour at 4°C in buffer (0.25 M Tris-HCl pH 8.3, 0.192 M glycine, 0.1% SDS). Immunoblotting was performed using standard protocols and the same antibody dilutions listed above. Images were aligned based on complex I and complex I subunit signaling location and gel/blot size.

The molecular masses of the two species detected by the FLAG antibodies in the 2D BN-SDS PAGE were estimated based on their migration distances, using the respiratory complexes as size references (the linear regression between the logarithm of the molecular mass (kDa) and the migration distance of the respiration complexes has a  $R^2$  of 0.9993).

### 2.4. Co-immunoprecipitations

Immunoprecipitations were conducted using purified mitochondria corresponding to 0.5-1 mg of protein with the  $\mu$ MACS™ DYKDDDDK (FLAG) or Protein G Isolation Kits (Milenyi Biotec), following the methodology of (Waltz *et al.*, 2019). The immunoprecipitations with DYKDDDDK (FLAG) beads involved purified mitochondria from the NDUFAF3-3xFLAG-tagged strain compared to those from the wild-type strain. Immunoprecipitations using Protein G beads were carried out in the presence or absence (as control) of 2.5  $\mu$ g of TYKY antibodies with purified mitochondria from the NDUFAF3-3xFLAG-tagged strain. Purified mitochondria were lysed using a lysis buffer composed of 20 mM HEPES-KOH (pH 7.6), 100 mM KCl, 30 mM MgCl<sub>2</sub>, 1 mM DTT, 0.3% *n*-dodecyl- $\beta$ -D-maltoside (v/v), and 1% Triton X-100 (v/v), supplemented with protease inhibitors (cOmplete™, EDTA-free protease inhibitor cocktail, Roche). Washing steps were performed with a wash buffer similar to the lysis buffer described above, but containing 0.01% *n*-dodecyl- $\beta$ -D-maltoside and 0.1% Triton X-100 (v/v).

Mass spectrometric analyses were conducted at the Strasbourg-Esplanade Proteomics Facility (<https://ibmc.cnrs.fr/en/laboratoire/mass-spectrometry/equipes/strasbourg-esplanade-proteomics-facility/>). Eluted proteins from the immunoprecipitated samples were reduced (5 mM DTT), alkylated (10 mM iodoacetamide) and digested with 300 ng trypsin.

Generated peptides were analyzed by nanoLC-MS/MS on a reversed phase nanoElute 2 coupled to a TIMS-TOF Pro 2 mass spectrometer (Bruker Daltonik GmbH) using a data-dependent acquisition (DDA). Peptides were separated on an IonOpticks Aurora Elite column (25 cm × 75µM, 1.7µm particle size and 120 Å pore size; AUR3–15075C18-CSI) with 70 min gradients. Data were searched against the *Chlamydomonas reinhardtii* CC-4532 v6.1 annotated genome (Phytozome v13) using Mascot (Matrix Science, v2.8). Proteins were then validated in Proline (ProFI) with an FDR < 1% (false discovery rate) at both Peptide Spectrum Mass (PSM) and protein levels, and a PSM score > 25. Statistical analysis of the immunoprecipitation data from three biological replicates was conducted using Extract Ion Chromatogram (peptides intensities) (XIC) abundance using the R software package (<https://github.com/hzuber67/IPinquiry4>). The data have been deposited on the PRIDE platform.

## 2.5. Confocal microscopy

Fixed *Chlamydomonas* cell imaging was performed on a Leica TCS SP5 inverted confocal laser microscope (Leica Microsystems). Images were collected using a water-immersion objective at 512x512 pixel resolution. Alexa Fluor 488 conjugated FLAG-tag antibodies (ThermoFisher Scientific) were visualized using the 488 nm line of the argon laser, and the emission light was dispersed and recorded at 495 to 532 nm (for an optimized detection at 525 nm). MitoTracker™ Orange (ThermoFisher Scientific) was visualized using the HeNe 543 nm laser, and the emission light was dispersed and recorded at 564 to 602 nm (for an optimized detection at 576 nm).

## 2.6. Structural modeling

Complex I subunits and NDUFAF3 structures and interactions were predicted using version/release 2.2.0 of the software AlphaFold2. Predictions were performed and run locally using our in-house computer cluster (Jumper *et al.*, 2021). For the prediction, the hypothesized mitochondrial targeting sequences (MTS) were trimmed from the sequences. Figures featuring atomic models were visualized with UCSF ChimeraX (Pettersen *et al.*, 2021). The complex I subunits were colored according to  $-\text{Log}_{10}$  (adjusted *p*-value) by creating a custom attribute assignment file in ChimeraX.

## 2.7. Accession numbers

Raw proteomic data have been deposited on the PRIDE platform (Project PXD048077, Username: [reviewer\\_pxd048077@ebi.ac.uk](mailto:reviewer_pxd048077@ebi.ac.uk) Password: BvX2SMXN).

## 3. Results

### 3.1. NDUFAF3-3xFLAG restores complex I assembly in complemented *ndufaf3* strains

To examine the role of NDUFAF3 in complex I assembly, we generated an algal strain bearing a tagged version of NDUFAF3. To achieve this, we transformed a previously characterized *ndufaf3* mutant (Massoz *et al.*, 2017) with a resistance cassette to paromomycin and a construct encoding a 3xFLAG-tag at the C-terminus of NDUFAF3 (see experimental procedures for details) (**Fig. 1B**, **Fig. S1**). The non-tagged version of this construct has been successfully used to complement the *ndufaf3* mutant (Massoz *et al.*, 2017). Complex I was detected in eight paromomycin-resistant transformants (A-H) by NADH/NBT staining after separating native protein complexes from membrane extracts via BN-PAGE. A wild-type strain

(Ct) and the *ndufaf3* mutant were used as positive and negative controls, respectively. The transformants showed restored NADH dehydrogenase activity and assembly of complex I and cannot be distinguished from the wild type except for clone G (**Fig. 1C**). Among them, four transformants (A, B, C, H) were chosen for further analysis. The presence of the FLAG epitope of NDUFAF3 was verified by immunoblotting after separation of membrane extracts by SDS-PAGE (**Fig. 1D**). **Fig. 1D** also shows that the NAD7 subunit of the Q module is detected in all these transformants like in Ct, but not in the *ndufaf3* mutant, as already reported in *Chlamydomonas* and humans (Massoz *et al.*, 2017; Baertling *et al.*, 2017; Ishiyama *et al.*, 2018). The H transformant was chosen for further analyses. Since the Q module is part of the hydrophilic arm, we asked if NDUFAF3 could be detected in a soluble extract. As shown in **Fig. 1E**, the signal related to NDUFAF3 is detected in the membrane fraction of the H transformant and not in the soluble fraction. It can thus be hypothesized that NDUFAF3 is associated with membrane-anchored intermediate modules in complex I assembly. The same result is obtained for the TYKY subunit of the Q module. Immunofluorescence staining of type H transformant cells with a probe to detect mitochondria (MitoTracker) and the FLAG epitope of NDUFAF3 (with Alexa Fluor 488) is shown in **Fig. 1F**. The yellow color in the merged image illustrates the colocalization of the fluorescent signals identifying mitochondria and NDUFAF3 (**Fig. 1F**). We concluded that the tagged subunit is indeed localized in mitochondria. At last, the growth rate of the H transformant is the same as that of the Ct strain in light (light + acetate, mixotrophy) and dark (dark + acetate, heterotrophy) conditions (**Fig. 1G**). In contrast, growth of the *ndufaf3* mutant is null in heterotrophic conditions (not presented on the graph). Together these results indicate that the *NDUFAF3-3xFLAG* gene copy present in the H transformant restores the respiratory capacity when expressed in the *ndufaf3* mutant.

### 3.2. NDUFAF3-3xFLAG-tagged is detected in two assembly intermediates by immunodetection analysis

To detect complex I assembly intermediates associated with NDUFAF3-3xFLAG, native protein complexes from membrane extracts of the Ct strain and the H transformant were separated by BN-PAGE, followed by immunoblotting analysis. When the anti-FLAG antibodies were used, two subcomplexes were detected in the H transformant. The size of these subcomplexes is smaller than the whole complex I, revealed by NADH/NBT staining (**Fig. 2A**). As a control, anti-TYKY antibodies were also used since this subunit belongs to the Q module, which is known to interact with NDUFAF3 (Guerrero-Castillo *et al.*, 2017). A signal corresponding to whole complex I is detected with the anti-TYKY antibodies for the Ct strain and the H transformant, confirming the integrity of complex I in the complemented strain (**Fig. 2B**). No signal is detected in membrane extracts of the *ndufaf3* mutant since this subunit of the Q module is not accumulated in this mutant (Massoz *et al.*, 2017), like NAD7 (**Fig. 1D**).

Since the signals detected using the anti-FLAG antibodies were noisy on membrane fractions, mitochondria were isolated from the strain expressing the *NDUFAF3-3xFLAG* construct (H transformant) and two-dimensional BN-SDS-PAGE (2D BN/SDS-PAGE) followed by immunoblotting analysis using specific antibodies were performed on mitochondrial extracts (**Fig. 2C**). Two protein complexes are recognized by the FLAG antibodies, with an apparent molecular mass of approximately 700 and 500 kDa, based on the migration pattern of the respiratory complexes (see the experimental procedure for details). These findings are in agreement with the results obtained in **Fig. 2A**. In addition, a smear is found both below and above these two main spots. Distinct signals revealing the presence of NAD7 and TYKY align with in-gel staining for complex I activity, indicating the presence of these subunits in



the holoenzyme, as expected from results obtained on membrane fraction (**Fig. 2B**). NAD7 and TYKY are also immunodetected below and above complex I, at a higher molecular weight, also stained in the NBT gel, which corresponds to the documented supercomplex I+III<sub>2</sub> (Cardol *et al.*, 2006; Cardol *et al.*, 2008; Waltz *et al.*, 2025). Considering the two main spots detected using the anti-FLAG antibodies, these results suggest that NDUFAF3 is probably transiently associated with two intermediates of complex I, although we do not have confirmation that they are *bona fide* complex I assembly submodules.

### 3.3. Physical interaction of NDUFAF3-3xFLAG-tagged with subunits specific to the Q, P<sub>p</sub> and P<sub>d</sub> modules

Co-immunoprecipitation analysis was performed to confirm the physical interactions of NDUFAF3 with complex I subunits. Purified mitochondrial solubilized extracts from the H transformant expressing NDUFAF3-3xFLAG-tagged were immunoprecipitated with anti-FLAG antibodies, and subsequent elutions were analyzed by nanoLC-MS/MS. We also conducted the co-immunoprecipitation experiment with a wild-type strain (Ct) as a negative control. Three biological replicates were prepared for both strains (NDUFAF3-3xFLAG-tagged and Ct). Analysis showed that 119 proteins were statistically relevant with an adjusted *p*-value < 0.05. Among them, 35 subunits of complex I were found: 27 presented a Log<sub>2</sub>FC>3, while 8 were below this threshold. Since two subunits presenting a Log<sub>2</sub>FC<3 (the 75 kDa subunit log<sub>2</sub>FC=2.39 and Nad1 log<sub>2</sub>FC=2.43) were detected on membrane extracts of the *ndufaf3* mutant [(Massoz *et al.*, 2017) and **Fig. S2**], this suggested that interactions with NDUFAF3 were weak below Log<sub>2</sub>FC=3. For the 52 proteins with a Log<sub>2</sub>FC>3, a functional enrichment analysis confirmed that 'mitochondrial respiratory chain complex I' was by far the most enriched cellular component using anti-FLAG antibodies (**Fig. S3A**). This led us to conclude that NDUFAF3 mostly interacts with complex I subunits and to consider the 27 complex I subunits with Log<sub>2</sub>FC>3 as potential interactors of NDUFAF3.

NDUFAF3-3xFLAG-tagged is the fifth most enriched protein with a Log<sub>2</sub>FC = 5.84 (**Fig. 3A, Table S1**). This is expected since NDUFAF3 was the bait used for this analysis. The subunits identified belong mostly to the Q, P<sub>p</sub>, and P<sub>d</sub> modules (**Fig. 3B**). Included are several protist and plant subunits not found in mammals and fungi complex I: the three  $\gamma$  carbonic anhydrase subunits (CAG1-3), of which CAG3 is the strongest interactant with NDUFAF3, C1-FDX (NUOP3 in *Chlamydomonas*) of the ferredoxin bridge, P1 (NUOP5, in *Chlamydomonas*), NUOP7 and P9 (Cardol *et al.*, 2004; Klusch *et al.*, 2021) (**Fig. 3A, B, Table S1**). Only one subunit of the N module was detected with a Log<sub>2</sub>FC>3, the 18 kDa subunit, which forms contacts between the N and the Q modules (Waltz *et al.*, 2025). By summing the molecular masses of the detected proteins, the expected size of the module is 579 kDa (**Table S2**), which is in the range of the sizes of the two complexes identified by 2D BN/SDS-PAGE (500 and 700 kDa) (**Fig. 2C**). In conclusion, these findings demonstrate that NDUFAF3 is present in a large subcomplex, comprising subunits of both the hydrophilic and hydrophobic arms of complex I, with strong interactions with the Q module and the carbonic anhydrase domain.

### 3.4. The N module is the last to be assembled in *Chlamydomonas* complex I

Except the 18 kDa subunit, the other subunits of the N module co-immunoprecipitated with NDUFAF3 are only loosely attached to the complex I subassembly detected (log<sub>2</sub>FC =2.39 for the 75 kDa subunit, 1.75 for the 24 kDa subunit, 1.70 for the 51 kDa subunit, 1.53 for the 13 kDa subunit, **Table S1**). This suggests that the N module is the last one to join complex I, after the association between the Q and P modules. This contrasts with the situation in

Angiosperms where the N and Q modules join before their anchoring to the membrane. A co-immunoprecipitation with antibodies against a subunit of the Q module, the TYKY subunit, was thus performed on solubilized mitochondria of the H transformant to determine which type of subassembly (N/Q, or NQP) could be captured. As expected, immunoprecipitation data showed that TYKY was the most enriched protein ( $\log_2FC=7.68$ ) (**Fig. S4**). A functional enrichment on the 167 statistically relevant proteins with a  $\log_2FC>3$  showed that the cellular component 'mitochondrial respiratory chain complex I' was also the most enriched, like for the co-immunoprecipitation with anti-FLAG antibodies, demonstrating the specificity of the anti-TYKY antibodies towards complex I subunits (**Fig. S3B**). Results also showed that the subunits of the N module are indeed much more enriched compared to the co-immunoprecipitation with anti-FLAG antibodies (NDUFAF3), together with subunits of the Q, P<sub>p</sub> and P<sub>d</sub> modules (**Fig. 4, Fig. S4, Table S3**). Thirty-eight subunits (against 27 when co-immunoprecipitation is performed with anti-FLAG antibodies) were detected using the same threshold of  $\log_2FC>3$ , which suggests that a nearly mature form of complex I has been captured for a size of 858 kDa (**Table S2**), close to the 1050 kDa of the mature complex I. These findings agree with the hypothesis that the N module is attached at a late assembly stage, when the Q, P<sub>p</sub> and P<sub>d</sub> modules are already joined together.

#### 4. Discussion

NDUFAF3 is one of the seven assembly factors (NDUFAF2-7, INDH/NUBPL) responsible for assembling the matrix arm of complex I in humans and conserved in plants (Guerrero-Castillo *et al.*, 2017; Ligas *et al.*, 2019). However, NDUFAF3 was not found to be associated with complex I modules in Arabidopsis, which prevented the assessment of its role in the assembly process (Ligas *et al.*, 2019). In addition, NDUFAF3 is encoded by two isoforms in Arabidopsis (At3g60150/At2g44525), which suggests possible redundancy (Ligas *et al.*, 2019). To further elucidate the steps of complex I assembly, we constructed a strain with a functional flagged NDUFAF3 in Chlamydomonas. The physical association of NDUFAF3 with a complex I intermediate could be demonstrated by co-immunoprecipitation followed by nanoLC-MS/MS analysis of detergent-solubilized mitochondria from the *NDUFAF3-3xFLAG* expressing strain. According to the assembly pathway of human complex I proposed by Guerrero-Castillo *et al.*, NDUFAF3 is present at multiple stages of the assembly process (Guerrero-Castillo *et al.*, 2017). Its association to complex I subassemblies starts with a soluble form of the Q module, and remains until the Q module is attached to the P<sub>p</sub> and the P<sub>d</sub> module (Guerrero-Castillo *et al.*, 2017). The data presented here show that the co-immunoprecipitation using the anti-FLAG antibodies has captured a late stage of NDUFAF3-associated subassembly, where the Q, P<sub>p</sub> and P<sub>d</sub> modules are joined together. In addition, our results suggest that NDUFAF3 joins the assembly when the Q module is already attached to the inner mitochondrial membrane since NDUFAF3 is only recovered in membrane extracts by immunodetection. This is not surprising since it has recently been demonstrated (Sung *et al.*, 2024) that in humans the Q module attaches to the inner mitochondrial membrane earlier than was previously suggested in the model of Guerrero-Castillo *et al.*, 2017. Our findings also show that subunits present in plant, algal, and protist complex I but not in mammal and fungi complex I (Braun and Klusch, 2024) are present in the subassembly intermediate associated to NDUFAF3 in Chlamydomonas.

The superimposition of the subunits detected by co-immunoprecipitation with anti-FLAG antibodies within complex I highlights that NDUFAF3 interacts strongly with subunits of the Q and P modules (**Fig. 3B**), which is expected based on the role of NDUFAF3 (Guerrero-Castillo *et al.* 2017). Using AlphaFold2 multimer (Jumper *et al.*, 2021), a structural model of

NDUFAF3 in association with Q module subunits (NAD7, 9, TYKY, PSST, B13) was generated (Fig. S5). The structural prediction of association is strong (pLDDT score>90, Fig. S5C), confirming the essential role of NDUFAF3 in the assembly of key subunits of the Q module, as already proposed (Guerrero-Castillo *et al.*, 2017). Moreover, the predicted positioning of NDUFAF3 within the Q module reveals a potential steric hindrance with the B14 and SDAP subunits which belongs to the ferredoxin bridge, in addition to C1-FDX (Klusck *et al.*, 2021). As mentioned above, C1-FDX was identified as an interactor of NDUFAF3 via the co-immunoprecipitation experiments, while B14 was not detected and SDAP was below the threshold of  $\log_2FC=3$  (Table S1), which explains the partial enrichment of the ferredoxin bridge on Fig. 3B. Given the context of the position of NDUFAF3 at the level of the Q module, the structural prediction suggests that NDUFAF3 plays a role in finalizing the assembly of the ferredoxin bridge, likely present in the Last Eukaryotic Common ancestor (LECA) (Braun and Klusck, 2024).

Of note is the presence of the 18 kDa subunit of the N module in the subassembly complex detected with anti-FLAG antibodies. This subunit is proposed to stabilize the N module in mammal complex I (Yin *et al.*, 2024). The presence of this subunit suggests that the subassembly captured with the anti-FLAG antibodies is at a stage where the N module starts to be assembled. This would explain why the NDUFAF2 assembly factor, which is binding to to Q module and prevents the joining of the N module (Guerrero-Castillo *et al.*, 2017) is not found in the co-immunoprecipitation. The other subunits of the N module are below the threshold when probing for interactions with NDUFAF3-3xFLAG but are well detected together with subunits of the Q, P<sub>p</sub> and P<sub>d</sub> modules when co-immunoprecipitation is performed with TYKY antibodies. This demonstrates that the N module is attached at the last stage of complex I assembly, like in mammal and fungal complex I. In Angiosperms, the entire matrix arm (N and Q modules) is formed before the addition of the membrane arm (Ligas *et al.*, 2019; López-López *et al.*, 2022).

In the assembly of human complex I, NDUFAF3 is associated with NDUFAF4 (Guerrero-Castillo *et al.*, 2017). In fruit fly, overexpression of *NDUFAF4* can partially overcome some NDUFAF3 defects (Murari *et al.*, 2021), indicating functional redundancy between the two assembly factors. The absence of NDUFAF4 and the existence of only one isoform of *NDUFAF3* in *Chlamydomonas*, contrary to *Arabidopsis*, is unclear. One interpretation is that complex I assembly in *Chlamydomonas* is simple and this could reflect the basal phylogenetic position of this microalga in eukaryotes.

In conclusion, these results underscore the uniqueness of *Chlamydomonas* for the study of complex I manufacture in non-mammal systems.

### Author contributions

T S-G designed and performed the experiments; MT, FW, NC, SF, GH, JC, PH performed the experiments; T S-G, MT, FV, NC, SF, GH, DV, JC, PH, PPH, CR analyzed the data. CR designed and conducted the experiments and wrote the initial version of the manuscript. T S-G, MT, FW, and PPH reviewed the manuscript, and all authors approved the final version.

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### Data availability statement

Raw proteomic data have been deposited on the PRIDE platform (Project PXD048077, Username: [reviewer\\_pxd048077@ebi.ac.uk](mailto:reviewer_pxd048077@ebi.ac.uk) Password: BvX2SMXN).

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## Short legends for Supporting Information

### Figure S1. Synthetic *NDUFAF3* gene (Cre12.g496800) of *C. reinhardtii*.

**A.** Nucleotide sequence (<https://phytozome-next.jgi.doe.gov/> Chlamydomonas reinhardtii v5.6). Grey background: promoter region; yellow background: 5' and 3' UTR; blue background: coding sequence; orange letters: first intron of *RBCS2*; underlined in bold and black: ATG (start codon), TGA (stop codon), and putative polyadenylation site (TGTAA); in bold and italics: *PstI* (CTGCAG) and *XbaI* (TCTAGA) restriction sites. The 3xFLAG addition is underlined in blue. **B.** Amino acid sequence of the NDUFAF3 protein. The 3xFLAG sequence is in bold.

### Figure S2. Nad1 is detected in the *ndufaf3* mutant strain.

Immunoblot of membrane fractions (20 µg of proteins per lane) from the *ndufaf3* and H strains incubated with antibodies against Nad1 antibodies. A Coomassie blue staining of a gel loaded with the same extracts is shown as a control for loading. Note that this Coomassie blue staining is identical to the one presented in Fig. 1E, as the same membrane extracts are used.

### Figure S3. Functional analysis of proteins enriched by NDUFAF3 and TYKY co-immunoprecipitation.

To perform the functional analysis of proteins enriched by NDUFAF3 (A) or TYKY (B) co-immunoprecipitation (coIP), we used ShinyGO v0.80 (<https://bioinformatics.sdstate.edu/go80/>) (1). Since ShinyGO relies on the *C. reinhardtii* genome annotation version 5.5, we first converted the protein IDs obtained from our IP experiments, which were based on version 6.1, to version 5.5. This conversion was carried out using the g:Profiler ID conversion tool (<https://biit.cs.ut.ee/gprofiler/convert>) (2). The conversion settings were as follows: Organism – Chlamydomonas reinhardtii; Target namespace – ENSP; Numeric IDs interpreted as ENTREZGENE.

For both NDUFAF3 and TYKY IP datasets, we selected proteins with a log<sub>2</sub> fold change (log<sub>2</sub>FC) > 3. The resulting protein IDs were submitted to g:Profiler for conversion. In the case of the NDUFAF3 IP dataset, 50 out of 52 proteins were successfully converted to version 5.5 IDs. For the TYKY IP dataset, 164 out of 167 proteins were converted.

These converted IDs were subsequently analyzed in ShinyGO v0.80 using default parameters, with the “GO Cellular Component” ontology selected as the pathway database. Results were visualized based on Fold Enrichment and –log<sub>10</sub>(FDR) values.

Fold Enrichment quantifies the magnitude of enrichment with higher values indicating a greater overrepresentation of a category and serving as an indicator of effect size.

P-values assess the statistical significance of the enrichment, with lower values indicating a lower probability that the observed result is due to random chance under the null hypothesis.

FDR q-values represent the False Discovery Rate-adjusted p-values, accounting for multiple hypothesis testing and reducing the likelihood of Type I errors.

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**Figure S4. Volcano plot illustrating the global enrichment of proteins identified through co-immunoprecipitation performed with purified mitochondria from the NDUFAF3-3xFLAG-tagged strain, in the presence of TYKY antibodies or without antibodies as a control (n=3).** The x-axis displays the  $\text{Log}_2$  fold change ( $\text{LogFC}$ ), while the y-axis represents the  $-\text{Log}_{10}$  of the adjusted  $p$ -value ( $-\text{Log}_{10}(\text{padj})$ ), obtained using the IPInquiry4 R software package. Significantly enriched proteins are defined by a  $\text{Log}_2\text{FC} > 3$  and a  $-\text{Log}_{10}(\text{padj}) > 1.3$  (corresponding to an adjusted  $p$ -value  $\leq 0.05$ ), as indicated by the red axes. NDUFAF3 is marked as a red dot, with N module subunits shown in violet, Q module subunits in yellow,  $P_p$  module subunits in blue,  $P_d$  module subunits in pink, the subunits of the  $\gamma$  carbonic anhydrase domain in orange, and the ferredoxin bridge subunits in green (see **Table S2**). The complete datasets and analysis are presented in **Tables S1 and S3**.

**Figure S5. Structure prediction of the Q module of Chlamydomonas in complex with NDUFAF3.**

**A.** Overall view of the mature complex I (Chlamydomonas model PDB: 9FRY). The subunits of the Q module are colored, and the dashed square indicates the zoomed-in view shown in B. **B.** The Predicted model of the Chlamydomonas Q module subunits and NDUFAF3, generated by AlphaFold2 Multimer. **C.** pLDDT score for the AlphaFold2 Multimer prediction. Amino acid residues in the predicted model are colored according to their pLDDT score; the higher the score, the more confident the prediction is. Most of the model is predicted with high confidence, except for the C and N terminal parts of the proteins, which is expected. **D.** Chlamydomonas AlphaFold prediction overlaid with the Chlamydomonas model. NDUFAF3 clashes with subunits B14 and SDAP, which connect the matrix and membrane arms via the ferredoxin bridge.

**Table S1. Dataset of proteins identified on the NDUFAF3 co-immunoprecipitation.**

List of proteins identified by nanoLC-MS/MS following co-immunoprecipitation experiments with anti-FLAG antibodies on purified mitochondria from the NDUFAF3-3xFLAG-tagged transformant, compared to purified mitochondria from the wild-type strain (n=3).

**Table S2. Summary table of the subunits identified in NDUFAF3 and TYKY co-immunoprecipitations.**

Only proteins with a  $\text{log}_2\text{FC} > 3$  are listed. The first column corresponds to the names of complex I subunits as described by (1). The associated Chlamydomonas gene names and accession numbers are provided. The molecular weights (MW), highlighted in bold in column 6, represent those of the predicted mature forms of the proteins, calculated using the Predalgo tool (2) or the TargetP prediction tool (<https://services.healthtech.dtu.dk/services/TargetP-2.0/>) for the targeting sequences.

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**Table S3. Dataset of proteins identified on the TYKY co-immunoprecipitation.**

List of proteins identified by nanoLC-MS/MS following co-immunoprecipitation experiments conducted with purified mitochondria from an NDUFAF3-3xFLAG-tagged strain in the presence of TYKY antibodies or, as a control, in the absence of antibodies (n=3).

## Figures legends

### Figure 1. The *NDUFAF3-3xFLAG* gene restores complex I activity in a *ndufaf3* mutant and its corresponding gene product is addressed to mitochondria.

**A.** Modular structure of complex I. Schematic representation of the complex I structure, including the ferredoxin bridge composed, from left to right, of the B14 (blue), SDAP (dark red), C1-FDX (pink), and  $\gamma$  carbonic anhydrase domain (yellow) subunits. The modules of the matrix arm (N and Q modules) and the membrane arm ( $P_p$  and  $P_d$  modules) are shown in blue. IMS: intermembrane space. **B.** Schematic representation of *NDUFAF3-3xFLAG* gene (Cre12.g496800) synthetic gene. Dark blue boxes represent endogenous 5' and 3' UTRs, while light blue boxes depict exons. The 3xFLAG tag insertion site is indicated by the black arrow. The orange line represents the *RBCS2i1* intron engineered in the construct, and the dotted grey line depicts the endogenous promoter (200 bp) (see **Fig. S1** for details). **C.** NADH/NBT staining of native protein complexes from membrane extracts of eight *NDUFAF3-3xFLAG*-expressing transformants (A-H), the recipient mutant (*ndufaf3*), and the wild-type (Control, Ct) strain (600  $\mu$ g of proteins per lane) separated by BN-PAGE. CI: complex I, LC: loading control. **D.** Immunoblots of membrane fractions (20  $\mu$ g of proteins per lane) incubated with antibodies against the FLAG tag of *NDUFAF3-3xFLAG* and NAD7. A Coomassie blue-stained gel loaded with the same extracts is included as a loading control. **E.** Immunoblots of membrane fractions (Memb: 20  $\mu$ g of proteins per lane) and soluble fractions (Sol: 20  $\mu$ g of proteins per lane) incubated with FLAG or TYKY antibodies. A Coomassie blue staining of a gel loaded with the same extracts is included as a loading control. **F.** Immunofluorescence staining of fixed cells of the H transformant visualized by confocal microscopy: upper left: brightfield image; upper right: MitoTracker Orange staining; lower right: Alexa Fluor 488 conjugated FLAG-tag antibodies; lower left: merged image. Scale: 2  $\mu$ m. **G.** Growth rates (number of divisions/24h) in mixotrophy and heterotrophy.  $n = 3$ , error bars are standard deviations, ns = non-significant. Statistical analysis was performed using Ordinary one-way ANOVA for growth in mixotrophy and an unpaired t-test for growth in heterotrophy.

### Figure 2. Analysis of complex I assembly by 2D BN/SDS PAGE reveals that *NDUFAF3* co-migrates with two intermediates.

**A.** Membrane extracts from control (Ct) and H strain were resolved in native conditions (BN-PAGE). The left part (Mb) represents the blotted membrane before incubation with antibodies against the FLAG tag of *NDUFAF3* ( $\alpha$ -FLAG), the middle panel represents the immunoblot, and the right panel (CI) represents NADH staining for complex I activity. The asterisks indicate the position of the *NDUFAF3* containing complexes (~700 and 500 kDa). The black lines indicate the separation on the membrane/blot/gel between the loadings of Ct and H transformant, signifying some samples unrelated to the study **B.** Membrane extracts from control (Ct), *ndufaf3* and H strain were resolved in native conditions (BN-PAGE). The left part (Mb) represents the blotted membrane before incubation with antibodies against the TYKY antibodies, the middle panel represents the immunoblot, the right panel (CI) represents NADH staining for complex I activity. **C.** Purified mitochondria samples were resolved in native conditions (left to right) and denaturing conditions (top to bottom). Starting at the top, the panels display an enriched mitochondrial fraction, NADH/NBT staining of the mitochondrial fraction, and immunoblots against the NAD7 and TYKY complex I subunits and the FLAG tag of *NDUFAF3*. V<sub>2</sub>, I+III<sub>2</sub>, I, and III<sub>2</sub>, correspond to respiratory chain complexes V (dimeric state), supercomplex I+ III<sub>2</sub>, complex I, and complex III (dimeric state), respectively (Cardol et al.,

2006; Cardol *et al.*, 2008, Waltz *et al.*, 2025). The grey arrows identify the position of the NDUFAF3 containing complexes (~700 and 500 kDa).

**Figure 3. NDUFAF3 is associated with subunits of the Q and P modules, including plant-specific proteins.**

**A.** Volcano plot illustrating the global enrichment of proteins in the immunoprecipitation of NDUFAF3-3xFLAG-tagged versus the control strain (n=3). The x-axis represents the  $\log_2$  fold change ( $\log_2FC$ ), while the y-axis displays  $-\log_{10}$  of the adjusted  $p$ -value ( $-\log_{10}(adj)$ ), obtained using the IPInquiry4 R software package. Significantly enriched proteins are defined by a  $\log_2FC > 3$  and a  $-\log_{10}(padj) > 1.3$  (corresponding to an adjusted  $p$ -value  $< 0.05$ ), as indicated by the red axes. NDUFAF3 is marked by a red dot; the N module subunits are shown in violet, the Q module subunits in yellow, the  $P_p$  module subunits in blue, the  $P_d$  module subunits in pink, the ferredoxin bridge subunits in green, and the  $\gamma$  carbonic anhydrase subunits in orange. The complete datasets and analyses are available in the supplemental (Table S1). **B.** Structural model of mitochondrial complex I from *Chlamydomonas* (Waltz *et al.*, 2025). IMS: intermembrane space. The subunits with a  $\log_2FC > 3$  are colored according to  $-\log_{10}(padj)$ . The predicted binding region from the Alphafold2 multimer is shown by dashed blue squares. The Alphafold prediction is available in the supplemental (Fig. S5).

**Figure 4. Comparison of NDUFAF3 and TYKY co-immunoprecipitation experiments.**

Balloon plots illustrate the NDUFAF3 and TYKY co-immunoprecipitation results. The area of each balloon represents the enrichment in  $\log_2$  fold change (FC) of the two co-immunoprecipitations relative to the various subunits of complex I, while the color gradient indicates the  $-\log_{10}$  of the adjusted  $p$ -value (adj). The fold change (FC) and adjusted  $p$ -value (adj) cut-offs are indicated. The different modules of complex I are identified according to the color code in Fig. 3A.