Identification of a novel biogenesis factor for mitochondrial Complex I using *Chlamydomonas reinhardtii* as a model system

DISSERTATION

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ABSTRACT

Mitochondria, the “powerhouse” of the cell, produce energy through the mitochondrial respiratory chain, which comprises five major complexes. Complex I is the first and most complicated enzyme required in this process. With more than 40 subunits, one FMN molecule and eight Fe-S clusters, the assembly of mitochondrial Complex I is highly intricate. Complex I deficiency in humans causes severe metabolic defects leading to fatal diseases such as encephalomyopathy and Parkinson’s disease. In 60% of the patients presenting Complex I dysfunction, there is no molecular explanation and it is assumed that defects in yet-to-be discovered assembly factors are responsible for the Complex I deficiency. In this study, we present the identification of a novel factor required for the biogenesis of Complex I in a green alga.

Study of Complex I biogenesis in humans is very limited due to the lethality of patients with Complex I-related diseases and associated ethical concerns. *Chlamydomonas reinhardtii*, a unicellular photosynthetic alga, is an ideal model organism for the study of Complex I assembly and function. *Chlamydomonas* is genetically tractable and its Complex I subunit composition is similar to its human counterpart. Unlike mammalian systems, Complex I deficient mutants in
*Chlamydomonas* are viable due to their photosynthetic ability. Importantly, *Chlamydomonas* is the only model system whose nuclear and mitochondrial genomes can be easily manipulated for the study of mitochondrial Complex I.

The major goal of the study presented in this thesis is to understand the biogenesis of mitochondrial Complex I using *Chlamydomonas reinhardtii* as a model. In Chapter 2, we describe a forward genetic screen, performed in *Chlamydomonas reinhardtii*, to identify novel loci controlling Complex I biogenesis. Six Complex I mutants *amc8-to-amc13* (assembly of mitochondrial complex I) were isolated from this screen.

In Chapter 3, we determine that the *AMC9* locus is defined by the gene encoding a core 24 kDa Complex I subunit, NUO5. Conserved from bacteria to humans, this subunit is essential for the NADH oxidation activity of Complex I. In Chapter 4, we report the identification of a novel factor, AMC11, involved in Complex I biogenesis. We show that AMC11 is targeted to the mitochondria through its N-terminal sequence and that loss of AMC11 affects mitochondrial gene expression, resulting in a Complex I assembly defect. Finally, in Chapter 5, we describe the molecular characterization of additional *amc* mutants that display reduced levels of assembled Complex I. We describe that, although they maintain the structural integrity of Complex I, these mutants are associated with changes in mitochondrial transcript abundance. Overall, our study is the first to identify a novel Complex I biogenesis factor using a forward genetic screen.
Dedication

To my beloved family
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PUBLICATIONS


FIELDS OF STUDY

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# Table of Contents

Abstract........................................................................................................................................ ii

Acknowledgments........................................................................................................................ v

Publications....................................................................................................................................... vii

Fields of Study.................................................................................................................................. viii

LIST OF Tables................................................................................................................................... xv

LIST OF Figures.................................................................................................................................. xvii

CHAPTER 1.......................................................................................................................................... 1

INTRODUCTION................................................................................................................................. 1

1.1. Mitochondrial Electron Transfer Chain. ................................................................. 1

1.2. Mitochondrial Complex I.......................................................................................... 2

1.3. Core subunits .............................................................................................................. 4

1.4. Non-core subunits of Complex I ........................................................................ 10

1.4.1. GRIM-19 (Gene associated with Retinoic IFN induced Mortality-19). 11

1.4.2. Acyl carrier protein (SDAP/ NDUFAB1) ....................................................... 12

1.4.3. γ - Carbonic Anhydrase (CA/CAL) subunits ............................................. 13

1.4.4. GLDH (L-galactano, 1,4-lactone dehydrogenase)..................................... 15

1.4.5. Post-translational modification of Complex I subunits ............................ 16

1.5. The assembly of Complex I .................................................................................... 18
1.6. Assembly Factors for Mitochondrial Complex I .................. 20

1.6.1. C20ORF7 / NDUFAF5 ............................................. 22
1.6.2. C6ORF60 / NDUFAF3 and C6ORF66 / NDUFAF4. .............. 22
1.6.3. C8ORF38 / NDUFAF6 ............................................. 22
1.6.4. CIA30 / NDUFAF1................................................... 23
1.6.5. Ecsit (Evolutionarily conserved signal intermediate in Toll pathways). .................................................. 24
1.6.6. ACAD9 (Acyl CoA dehydrogenase) ................................ 24
1.6.7. TMEM126B ......................................................... 25
1.6.8. C3ORF1 ............................................................... 25
1.6.9. NDUFAF2 (B17.2L) .................................................. 25
1.6.10. FOXRED1 .......................................................... 26
1.6.11. Apoptosis Inducing Factor (AIF)................................. 26
1.6.12. NUBPL / IND1 / INDH ........................................... 27
1.6.13. MidA / NDUFAF7 .................................................. 28

Chapter 2 .............................................................................. 30

NOVEL COMPLEX I MUTANTS REVEALED FROM A FORWARD GENETIC SCREEN CONDUCTED IN Chlamydomonas reinhardtii .................. 30

2.1. Introduction ............................................................... 30

2.1.1. Chlamydomonas reinhardtii as a model system for the study of mitochondrial Complex I ........................................... 31
2.1.2. Complex I mutants with molecular lesions in mitochondrial genes .... 34
2.1.3. Complex I mutants inactivated for Chlamydomonas nuclear genes... 36
2.1.4. Novel loci involved in Complex I biogenesis ......................... 37

2.2. Experimental Procedures .............................................. 39

2.2.1. Strains and culture conditions ........................................ 39
2.2.2. Insertional mutagenesis ............................................. 39
2.2.3. Phenotypic screening to isolate Complex I mutants ............. 40
2.2.4. Ten-fold Dilution Series .............................................. 40
2.2.5. Complex I activity measurement ..................................... 41
3.3.4. The *amc* mutants can be transformed by biolistics .................................. 78
3.3.5. Transformation of *amc9* with *NUO5* cDNA leads to partial complementation.......................................................... 79
3.3.6. Transformation of *amc9* with *NUO5* genomic DNA results in full phenotypic complementation........................................ 81
3.3.7. Complementation of the *amc5* mutant with the *NUOB10* gene........ 86

3.4. Discussion ........................................................................................................ 90

3.4.1. *NUO5* encodes a core 24 kDa subunit of Complex I............................. 91
3.4.2. Assembly of the 24 kDa subunit (NUO5 / NDUFV2) into Complex I .... 92
3.4.3. Post-translational regulation of the 24 kDa subunit............................ 93
3.4.4. *NDUFV2* is a candidate gene in many mitochondrial disorders......... 94
3.4.5. The *AMC5* locus encodes for the NUOB10 (PDSW) subunit. ........ 98
3.4.6. Other nuclear mutants encoding Complex I subunits in *Chlamydomonas* ................................................................. 100

CHAPTER 4 ............................................................................................................. 102

IDENTIFICATION OF A NOVEL FACTOR INVOLVED IN MITOCHONDRIAL COMPLEX I BIOGENESIS ...................................................................... 102

4.1. Introduction ..................................................................................................... 102

4.2. Experimental Procedures ............................................................................. 104

4.2.1. Strains and culture conditions ................................................................. 104
4.2.2. Genetic analysis ....................................................................................... 105
4.2.3. Nucleic acids extraction ......................................................................... 107
4.2.4. Diagnostic PCR and TAIL-PCR analyses ............................................ 107
4.2.5. RT-PCR analyses .................................................................................. 108
4.2.6. Real-time quantitative PCR ................................................................... 109
4.2.7. Plasmid Construction ............................................................................ 110
4.2.8. PCR-based screening of the Chlamydomonas genomic library and biolistics ................................................................. 111
4.2.9. Assessing growth phenotype by ten-fold dilution series and growth curves ................................................................. 112
4.2.10. Biochemical analyses ........................................................................... 113
4.3. Results ...................................................................................................................... 118

4.3.1. The AMC11 locus is defined by a novel gene. .................................................. 118
4.3.2. The AMC11 gene restores Complex I activity and assembly in the amc11 mutant. ................................................................................................................................. 120
4.3.3. The amc1 and the amc11 mutations are allelic .................................................... 127
4.3.4. The N-terminus of AMC11 carries a mitochondrial targeting signal ................... 132
4.3.5. AMC11 contributes to post-transcriptional regulation of a mitochondrial gene. ................................................................................................................................. 138

4.4. Discussion ................................................................................................................. 149

4.4.1. AMC11 controls the assembly of the distal end of Complex I membrane arm .......... 149
4.4.2. AMC11 is required for post-transcriptional regulation of the nd4 transcript ................................................................................................................................. 150
4.4.3 AMC11, an RNA binding protein? ....................................................................... 152
4.4.4. Proposed mode of action for AMC11 .................................................................... 153

Chapter 5 .......................................................................................................................... 156

Characterization of AMC mutants assembling a mature enzyme ......................... 156

5.1. Introduction ............................................................................................................... 156

5.2. Experimental Procedures ....................................................................................... 158

5.2.1. Strains and culture conditions ............................................................................ 158
5.2.2. Genetic analyses .................................................................................................. 158
5.2.3. DNA and RNA extractions and PCR analyses .................................................... 158
5.2.4. Biochemical and growth analyses ....................................................................... 159
5.2.5. PCR-based screening of the Chlamydomonas genomic library and Biolistics ......................................................................................................................... 159

5.3. Results ....................................................................................................................... 163

5.3.1. The amc12 mutation co-segregates with the iHyg3 cassette. ......................... 163
5.3.2. The amc12 (6E9) mutant carries two unlinked amc mutations, each contributing to Complex I deficiency ................................................................. 165
5.3.3. Analysis of amc12/amcx diploids ..................................................................... 169
5.3.4. The \textit{amc12m2} strain exhibits low abundance of mitochondrial transcripts .................................................................................................................. 170

5.3.5. The \textit{amc8} mutant is characterized by reduced mitochondrial transcript levels .................................................................................................................. 175

5.3.6. Characterization of the \textit{amc10} and \textit{amc13} mutants .................................................................................................................. 179

5.4. Discussion .......................................................................................................................................................... 185

5.4.1. Genetic analysis of the \textit{amc} mutants .................................................................................................................. 185

5.4.2. Effect of nuclear \textit{amc} mutations on mitochondrial DNA and transcript levels .................................................................................................................. 186

Chapter 6 .......................................................................................................................................................... 190

SUMMARY AND CONCLUSIONS .................................................................................................................. 190

6.1. The \textit{nuo5} mutant – a promising platform for studying the relevance of Complex I deficiency to human health .................................................................................................................. 192

6.2. Identification of AMC11 as a novel factor for Complex I biogenesis .................................................................................................................................................................................. 195

6.3. Role for AMC11 in mitochondrial gene expression .................................................................................................................. 196

6.4. Mode of action of AMC11 .......................................................................................................................................................... 199

6.5. Other promising AMC loci? .......................................................................................................................................................... 203

BIBLIOGRAPHY .......................................................................................................................................................... 204
<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Nomenclature of 14 core Complex I subunits in different model systems.</td>
<td>9</td>
</tr>
<tr>
<td>1.2</td>
<td>Assembly factor conservation in plants and mammals.</td>
<td>21</td>
</tr>
<tr>
<td>2.1</td>
<td>Construction of $amc/+$ diploids.</td>
<td>44</td>
</tr>
<tr>
<td>2.2</td>
<td>Summary of phenotype and genetic analysis conducted on $amc8$-to-$13$.</td>
<td>54</td>
</tr>
<tr>
<td>3.1</td>
<td>Construction of $amc9/amcx$ diploids.</td>
<td>69</td>
</tr>
<tr>
<td>3.2</td>
<td>Sequences of primers used in Chapter 3.</td>
<td>70</td>
</tr>
<tr>
<td>3.3</td>
<td>NUO5 has a canonical mitochondrial targeting sequence whereas NUOB10 does not.</td>
<td>95</td>
</tr>
<tr>
<td>4.1</td>
<td>Construction of $amc11/amcx$ diploids.</td>
<td>114</td>
</tr>
<tr>
<td>4.2</td>
<td>Sequence of primers</td>
<td>115</td>
</tr>
<tr>
<td>4.3</td>
<td>Sequence of primers specific to mitochondrial genes used in semi-quantitative RT-PCR.</td>
<td>116</td>
</tr>
<tr>
<td>4.4</td>
<td>Primers used for real-time quantitative PCR experiments.</td>
<td>117</td>
</tr>
<tr>
<td>4.5</td>
<td>The $amc11$ mutation is linked to the insertional cassette.</td>
<td>122</td>
</tr>
<tr>
<td>4.6</td>
<td>Target prediction softwares predict a possible mitochondrial localization for AMC11.</td>
<td>136</td>
</tr>
</tbody>
</table>
Table 5.1. Construction of amc8/amcx diploids. .......................................................... 160
Table 5.2. Generation of amc12/amcx and amc12m2/amcx diploids. ............. 161
Table 5.3. Sequence of primers.............................................................................. 162
Table 5.4. Summary of diploid complementation studies. ............................. 184
LIST OF FIGURES

Figure 1.1. Structure of Mitochondrial Complex I................................................. 8

Figure 1.2. Model of Human Complex I assembly.................................................. 19

Figure 2.1. Six novel mutants exhibit Complex I deficiency................................. 46

Figure 2.2. The amc mutants display a Complex I assembly defect....................... 50

Figure 2.3. The amc8-to-13 mutations are recessive............................................ 52

Figure 3.1. Genetic complementation reveals that the amc9 mutation is not allelic to the mutations in amc1-to-12................................................................. 72

Figure 3.2. amc9 is not genetically complemented by amc13............................... 73

Figure 3.3. The amc9 mutation is monogenic and linked to the insertional cassette. ................................................................................................................. 75

Figure 3.4. The NUO5 gene encoding the 24 kDa Complex I subunit is disrupted in the amc9 strain................................................................. 77

Figure 3.5. Expression of the NUO5 cDNA in amc9 leads to partial complementation of Complex I deficiency................................................. 80

Figure 3.6. Transformation of the amc9 mutant by the wild-type NUO5 genomic DNA........................................................................................................ 83

Figure 3.7. The wild-type NUO5 gene restores heterotrophic growth to the amc9 mutant.................................................................................................. 84
Figure 3.8. The *amc9* mutant, complemented by the *NUO5* gene, is restored for Complex I activity and assembly. ................................................................. 85

Figure 3.9. The *amc5* mutant is complemented with the *NUO5* gene. ............... 88

Figure 3.10. The wild-type *NUOB10* gene restores heterotrophic growth to an *amc5* mutant ................................................................. 89

Figure 3.11. Alignment of NUO5 / 24 kDa subunit............................................. 96

Figure 3.12. Localization of the 24 kDa subunit in *Y.lipolytica*.............................. 97

Figure 3.13. Alignment of NUOB10 / PDSW subunit. ........................................ 101

Figure 4.1. The *AMC11* locus is defined by a novel gene Cre16.g688900. ...... 123

Figure 4.2. The *AMC11* gene restores Complex I proficiency to the *amc11* mutant. .................................................................................. 124

Figure 4.3. The *sid* phenotype of the *amc11* mutant is rescued by the wild-type *AMC11* gene. ................................................................. 125

Figure 4.4. Complex I assembly is restored in the *amc11* mutant upon introduction of the *AMC11* gene. ................................................................. 126

Figure 4.5. Analysis of *amc11/amcx* diploids....................................................... 129

Figure 4.6. The *amc1* and *amc11* mutations are allelic. ................................. 130

Figure 4.7. The *AMC11* gene is disrupted in the *amc1* mutant. ....................... 131

Figure 4.8. Transcript levels in the *amc11* mutant are restored by *AMC11* genomic DNA.................................................................................... 135

Figure 4.9. The N-terminus of AMC11 targets the reporter protein UbiG to the yeast mitochondria........................................................................ 137
Figure 4.10. AMC11 is required for the accumulation of the mitochondrial *nd4* transcript. ................................................................. 143

Figure 4.11. Loss of AMC11 results in the decreased accumulation of multiple mitochondrial transcripts. ................................................. 144

Figure 4.12. The *amc11* mutant contains wild-type levels of mitochondrial DNA. ........................................................................ 145

Figure 4.13. The *amc1* mutant displays reduced levels of mitochondrial transcripts similar to the *amc11* mutant. ........................................ 146

Figure 4.14. Introduction of the reporter gene T-urfl3 at the mitochondrial *nd4* locus ........................................................................ 147

Figure 4.15. AMC11 is required for the post-transcriptional regulation of the *nd4* transcript. ............................................................... 148

Figure 4.16. The secondary structure of AMC11 is predicted to contain a majority of helical structures. ............................................. 151

Figure 5.1. The cassette in the *amc12* mutant interrupts a gene encoding a novel protein. ............................................................... 167

Figure 5.2. The *amc12* mutant contains a second *amc* mutation, unlinked to the cassette. ............................................................... 168

Figure 5.3. Analysis of *amc12/amcx* diploids ................................................................................................................................. 172

Figure 5.4. Biochemical analyses of *amc12/amcx* diploids .............................................................................................................. 173

Figure 5.5. The *amc12 m2* nuclear mutation affects the accumulation of all the protein-coding mitochondrial transcripts .................... 174
Figure 5.6. The amc8 mutant displays down-accumulation of all the protein-coding mitochondrial transcripts. ................................................................. 177

Figure 5.7. The AMC8 locus defines the 8th complementation group. .............. 178

Figure 5.8. The amc10 mutant accumulates reduced levels of nd1 and nd6 transcripts................................................................. 181

Figure 5.9. The amc13 nuclear mutation does not result in the down-accumulation of mitochondrial transcripts. ................................................................. 182

Figure 5.10. Relative mitochondrial DNA content is reduced in amc3. ............ 183

Figure 6.1. The role of the AMC proteins in Complex I assembly process in Chlamydomonas reinhardtii................................................................. 191
CHAPTER 1

INTRODUCTION

1.1. Mitochondrial Electron Transfer Chain.

An energy-transducing membrane involves the sequential transfer of electrons from donor to acceptor molecules, by an electron transfer chain (ETC), via the oxidation of high-energy substrates. Conserved from bacterial to eukaryotic cells, this process results in establishing an electrochemical proton gradient, across a biological membrane, that is crucial for energy production (1).

The mitochondria are an important source of ATP for the eukaryotic cell. They are the site of oxidative phosphorylation (OXPHOS), a type of ETC coupled to ATP synthesis, that takes place in the inner mitochondrial membrane (Figure 1.1 A) (2). OXPHOS involves four major membrane-bound multimeric complexes and two mobile molecules, which mediate electron transfer. The four major complexes are: NADH: ubiquinone oxidoreductase (Complex I); succinate: ubiquinone oxidoreductase (Complex II); ubiquinol: cytochrome c oxidoreductase (Complex III) and cytochrome c oxidase (Complex IV) (3). The mobile components are: i) the lipophilic ubiquinone (or Coenzyme Q) that diffuses through the inner
mitochondrial membrane and, ii) the heme containing cytochrome c, present in the intermembrane space (4,5). The transfer of electrons from NADH to O_2, the terminal electron acceptor, is coupled to the translocation of protons, from the matrix to the intermembrane space, via the energy-transducing Complexes I, III and IV. This electrochemical gradient, established across the inner mitochondrial membrane, acts as the driving force for Complex V (F_1F_0 ATP synthase), to catalyze ATP synthesis in the mitochondrial matrix (3,5).

In land plants, algae, fungi and some parasites, additional components contribute to the ETC (2,3). In addition to Complexes I and II, monomeric type-II NADH dehydrogenases (6), are present on either side of the inner mitochondrial membrane that donate electrons to ubiquinone molecules. On the other hand, alternative oxidases, anchored to the inner mitochondrial membrane, can also accept electrons from ubiquinol. These alternative dehydrogenases and oxidases do not contribute to the proton gradient (7-9).

1.2. Mitochondrial Complex I

NADH: ubiquinone oxidoreductase (Complex I, EC 1.6.5.3) is the largest enzyme of the ETC (3). Conserved from bacteria to humans, this enzyme is considered as the main entry point for electrons in the ETC. Complex I oxidizes NADH and reduces ubiquinone as follows, where Q refers to ubiquinone and QH_2 refers to its reduced form, i.e. ubiquinol (2):

\[
\text{NADH} + \text{Q} + 5\text{H}^+_{\text{matrix}} \rightarrow \text{NAD}^+ + \text{QH}_2 + 4\text{H}^+_{\text{intermembrane space}}
\]
Complex I couples its oxidoreductase activity with the ability to pump protons across the inner mitochondrial membrane, thereby contributing to the proton gradient required for ATP synthesis.

Complex I from different organisms have been used for study of the enzyme structure. Three-dimensional structures for Complex I have been reconstructed from electron micrographs of the Yarrowia lipolytica and Bos taurus holoenzymes. In addition, X-ray crystallographic structures of bacterial Complexes I have also been obtained from Escherichia coli and Thermus thermophilus (10-16). These analyses have revealed the L-shaped structure of this multimeric enzyme, which consists of a membrane arm embedded into the inner mitochondrial membrane and a peripheral arm protruding into the mitochondrial matrix (Figure 1.1 B). Proteomic analyses have shown that Arabidopsis and Chlamydomonas Complex I structures consist of 49 and 42 subunits, respectively (17-20). On the other hand, Complex I from fungi such as Neurospora and Yarrowia contain only 39 and 37 subunits, respectively (21,22). Recent studies have shown that mammalian NDUFA4, previously described as a Complex I subunit, was in fact associated with Complex IV in bovine mitochondria. Hence, the count of subunits for mammalian Complex I has been changed from 45 subunits to 44 subunits (17). In silico analysis of eukaryotic Complex I has uncovered that nearly 41 subunits are conserved amongst plants, fungi and mammals (23).

The topology of Complex I has also been determined by biochemical analyses. Complex I has been fractionated with chaotropic agents and detergents,
and the composition of these fractions has been determined by mass spectrometry (2,24). Fractionation of bovine Complex I, using the detergent \( N,N \)-dimethyldodecylamine \( N \)-oxide (LDAO), resulted in two subcomplexes: the membrane arm subcomplex Iβ and the membrane associated subcomplex Iα. The fraction Iα could be further dissociated into the soluble subcomplex Iλ and the membrane-bound subcomplex Iγ (24) (Figure 1.1 B).

1.3. Core subunits

Mitochondrial Complex I, consisting of more than 40 nuclear and mitochondria-encoded subunits, is nearly 1 MDa in size. On the other hand, its 550 kDa simpler counterpart in \( E. coli \) consists of only 14 subunits (2,3). These 14 evolutionarily conserved subunits are the minimal subunits essential for the catalytic activity of Complex I, and hence referred to as “core” subunits (Table 1.1) (2). These core subunits have orthologous counterparts in all eukaryotic Complex I. Out of the 14 subunits, seven form a part of the peripheral arm protruding into the matrix. This soluble arm harbors the NADH-binding site, one non-covalently bound FMN molecule and eight/nine Fe-S (Iron-sulfur) clusters (25). The membrane arm consists of seven hydrophobic subunits that are required for ubiquinone reduction and proton translocation (10,11). The ubiquinone binding center sensitive to rotenone, a Complex I inhibitor, is present in the membrane arm (26). \( T. thermophilus \) Complex I contains an additional organism-specific subunit Nqo15 present in the soluble arm of the enzyme. Possible roles for this additional
subunit in promoting Fe-S cluster regeneration, storage of iron or enzyme stability have been postulated (12).

Overall, the bacterial Complex I can be divided into three functional modules: the NADH-oxidizing N module, the ubiquinone-reducing Q module and the proton-translocating P module (27). The N module consists of the NADH- and FMN-binding sites and the Fe-S clusters involved in the electron relay from NADH to ubiquinone. The resolution of the crystallographic structure of bacterial Complex I and electron paramagnetic resonance (EPR) studies have provided information regarding the identity and the position of the Fe-S clusters within the enzyme (Figure 1.1 C) (12,28). The identity of core subunits binding the eight Fe-S clusters are provided in Table 1.1. Briefly, two electrons are transferred from NADH to FMN, in the form of a hydride ion. Each electron is transferred one by one from NADH to ubiquinone through the following electron relay: NADH→FMN→N3→N1b→N4→N5→N6a→N6b→N2. From the N2 cluster, electrons tunnel to quinone. The Fe-S cluster N1a (harbored by the 24 kDa subunit Nqo2/NuoE/NUO5), is not part of the main redox chain. However, this subunit is conserved in all species, and was postulated to play a role in preventing reactive oxygen species (ROS) production by Complex I. Although NADH can transfer two electrons to FMN, the redox relay can handle only one electron at a time. In this scenario, the reduced flavosemiquinone, is highly reactive and can easily donate electrons to dissolved oxygen and thus produce ROS. The cluster N1a could temporarily accept one electron from the flavosemiquinone molecule, preventing
an electron leak, until the main relay is once again free to accept the second electron (10,12). Recently, the Hirst group has proposed that the redox state of the N1a cluster does not affect the relative amounts of ROS, and that, the main function of this cluster may be in the assembly and stability of the enzyme (29). A ninth Fe-S cluster N7, is present only in bacterial Complex I. EPR studies have confirmed that the N7 cluster has no role in electron relay in Complex I. Instead, it may be required for the stability of the enzyme (30).

The seven hydrophobic subunits, referred to as ND subunits, do not harbor redox groups and comprise the proton-translocating P module and the quinone-reducing Q module. The subunits ND1, PSST, ND7, ND4 and ND5 have been postulated to have roles in quinone binding and reduction (27,31), although the precise quinone binding site is yet to be defined. The membrane subunits ND2, ND4 and ND5 are possibly involved in proton translocation due to their similarity to Na⁺/K⁺ antiporters (10,27). Two models for coupling of electron transfer and proton translocation have been proposed. One model suggested by the Sazanov group involves a conformational change driven by the redox relay system resulting in the simultaneous pumping of four protons. In such a model, three protons are translocated by the ND2, ND4, ND5 subunits (each subunit translocating one proton) and the fourth proton is translocated at the interface of the ND2, ND4L and ND6 subunits (10). The second model proposed by the Brandt group postulates that the catalytic reaction drives two rounds of pumping, each involving two proton
translocation events, one proton pumped by the ND4/ND5 subunits and the second proton pumped by the ND1/ND2/ND3/ND4L/ND6 subunits (32).

In mammals and fungi, these seven hydrophobic subunits are encoded by the mitochondrial genome. Mitochondria from vascular plants, such as Arabidopsis, encode nine Complex I subunits (including ND7 and ND9, in addition to the seven found in mammals) in the mitochondria. Reclinomonas americana, a flagellate protist, is the only known organism that encodes a maximum of eleven Complex I subunits in the mitochondria (33).
The general scheme of the electron transfer chain (ETC) in plant and fungal mitochondria. Complex I is the first enzyme (shown in blue) and binds one FMN molecule (green hexagon) and eight Fe-S clusters (yellow squares). Complexes II, III, IV and ATP synthase are the other major complexes involved in the ETC and are depicted with their relevant substrates. Alternative type II NADH dehydrogenases (ND), present on both sides of the inner mitochondrial membrane, and alternative oxidase (AOX) are found in plant, fungal and some parasite mitochondria, but absent in mammalian mitochondria. (Source: Remacle et al, 2008) (2).

Human Complex I topology depicts its L-shaped structure. The subunits belonging to a specific subcomplex depicted as Iα, Iβ, Iλ and Iγ are indicated (Source: Vogel et al, 2007) (34).

A schematic representation of the electron relay from NADH to quinone (Q) through FMN and eight Fe-S clusters. The distances between the co-factors were estimated from the crystal structure of *T. thermophilus* (12) (Source: Friedrich et al, 2012) (31).
Table 1.1. Nomenclature of 14 core Complex I subunits in different model systems.

<table>
<thead>
<tr>
<th>E. coli</th>
<th>T. thermophilus</th>
<th>C. reinhardtii</th>
<th>A. thaliana</th>
<th>H. sapiens</th>
<th>B. taurus</th>
<th>Cofactor</th>
</tr>
</thead>
<tbody>
<tr>
<td>NuoF</td>
<td>Nqo1</td>
<td>NUO6</td>
<td>NDUFV1</td>
<td>51 kDa</td>
<td>FMN, N3</td>
<td></td>
</tr>
<tr>
<td>NuoE</td>
<td>Nqo2</td>
<td>NUO5</td>
<td>NDUFV2</td>
<td>24 kDa</td>
<td>N1a</td>
<td></td>
</tr>
<tr>
<td>NuoG</td>
<td>Nqo3</td>
<td>NUS1</td>
<td>NDUS1</td>
<td>75 kDa</td>
<td>N1b, N4, N5, N7*</td>
<td></td>
</tr>
<tr>
<td>NuoI</td>
<td>Nqo9</td>
<td>NUO8</td>
<td>TYKY</td>
<td>NDUS8</td>
<td>TYKY</td>
<td></td>
</tr>
<tr>
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<td>Nqo6</td>
<td>NUO10</td>
<td>PSST</td>
<td>NDUS7</td>
<td>PSST</td>
<td></td>
</tr>
<tr>
<td>NuoA</td>
<td>Nqo7</td>
<td>ND3/NUO3</td>
<td>ND3\textsuperscript{m}</td>
<td>ND3\textsuperscript{m}</td>
<td>Nd3\textsuperscript{m}</td>
<td></td>
</tr>
<tr>
<td>NuoC*</td>
<td>Nqo5</td>
<td>NUO9/ND9</td>
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<td>NDUS3</td>
<td>30 kDa</td>
<td></td>
</tr>
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<td>Nqo4</td>
<td>NUO7/ND7</td>
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<td>NDUS2</td>
<td>49 kDa</td>
<td></td>
</tr>
<tr>
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<td>ND1\textsuperscript{m}</td>
<td>ND1\textsuperscript{m}</td>
<td>ND1\textsuperscript{m}</td>
<td></td>
</tr>
<tr>
<td>NuoJ</td>
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<td>ND6\textsuperscript{m}</td>
<td>ND6\textsuperscript{m}</td>
<td>ND6\textsuperscript{m}</td>
<td></td>
</tr>
<tr>
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<td>Nqo11</td>
<td>NUO11/ND4L</td>
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<td>ND4L\textsuperscript{m}</td>
<td>ND4L\textsuperscript{m}</td>
<td></td>
</tr>
<tr>
<td>NuoL</td>
<td>Nqo12</td>
<td>ND5\textsuperscript{m}</td>
<td>ND5\textsuperscript{m}</td>
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<td>ND5\textsuperscript{m}</td>
<td></td>
</tr>
<tr>
<td>NuoM</td>
<td>Nqo13</td>
<td>ND4\textsuperscript{m}</td>
<td>ND4\textsuperscript{m}</td>
<td>ND4\textsuperscript{m}</td>
<td>ND4\textsuperscript{m}</td>
<td></td>
</tr>
<tr>
<td>NuoN</td>
<td>Nqo14</td>
<td>ND2\textsuperscript{m}</td>
<td>ND2\textsuperscript{m}</td>
<td>ND2\textsuperscript{m}</td>
<td>ND2\textsuperscript{m}</td>
<td></td>
</tr>
</tbody>
</table>

The nomenclature of the core subunits is provided for six organisms E. coli (10), T. thermophilus (10), C. reinhardtii (3), A. thaliana (2), H. sapiens (3), B. taurus (3). The FMN molecule and the Fe-S clusters bound by each core subunit is also provided (31).

The subunits are referred to as Nuo (NADH:ubiquinone oxidoreductase) in E. coli and C. reinhardtii; Nqo (NADH:quinone oxidoreductase) in T. thermophilus; NDUF (NADH dehydrogenase ubiquinone flavoprotein) in H. sapiens; based on mass in kDa or a stretch of four conserved residues from the N-terminus in B. taurus and A. thaliana.

*: NuoC and NuoD are fused as one single subunit in E. coli (2) and the N7 Fe-S cluster is found only in a few bacteria such as E. coli and T. thermophilus (31).

\textsuperscript{m} indicates mitochondrially encoded subunits, that generally have an ‘ND’ nomenclature.
1.4. Non-core subunits of Complex I

In addition to the core subunits, mitochondrial Complex I consists of numerous non-core subunits that are all nuclear-encoded. Also referred to as supernumerary or accessory subunits, their precise role in Complex I is not understood because they are not a direct part of the main redox chain or proton-translocating channels (2). While several mutations in the core subunits have been well documented to be the cause of Complex I deficiency in humans, mutations in non-core subunits are also known to cause mitochondrial disorders such as Leigh syndrome, MELAS (Mitochondrial Encephalomyopathy, lactic acidosis and stroke-like episodes) and Lethal infantile mitochondrial disease (35), thereby emphasizing the importance of non-core subunits for Complex I function. To date, roles in regulating Complex I activity and assembly or protection against ROS activity have been postulated as possible function for the non-core subunits (24,36). For instance, loss of the accessory subunits NDUFA3, NDUFA5 and NDUFA12 in human cell lines causes the accumulation of a 460 kDa intermediate. This phenotype is diagnostic of a defect in the assembly of the membrane arm of Complex I (37), underscoring the requirement of non-core subunits in Complex I assembly. In yet another example, non-core subunits have been implied in mediating transition between active/deactive state of Complex I (38,39). This transition has been observed in vertebrate species, where the ‘idle’ or ‘deactive’ enzyme is the dominant form, which becomes ‘active’ in the presence of substrates, through conformational changes. Recently, it has been observed that
Complex I is present in the ‘deactive’ form under hypoxic conditions, such as ischemia (38). It has been postulated that the 39 kDa subunit, located proximal to the core PSST and ND3 subunits, has a possible function in mediating conformational changes in Complex I, to switch to this ‘deactive’ state (13).

Intriguingly, a few non-core subunits have also been implied in mediating regulatory roles in metabolic pathways, in addition to their function in mitochondrial Complex I. The following section describes such non-core subunits.

1.4.1. GRIM-19 (Gene associated with Retinoic IFN induced Mortality-19)

The GRIM-19 (NDUFA13) subunit is localized to the membrane arm of Complex I in the subcomplex Iγ (Figure 1.1 B) (40). GRIM-19 knock-out in mice results in embryonic lethality (41), due to loss of Complex I function. Mutation in GRIM-19 has been observed in patients with early onset hypotonia, dyskinesia and sensory deficiencies (42). These patients exhibit severe Complex I deficiency wherein stalled assembly intermediates are not even detected (42), thereby highlighting the importance of GRIM-19 in the early stages of Complex I assembly. The C-terminus (134-144 amino acids) of GRIM-19 has been shown to be required for its assembly into Complex I (43). On the other hand, deletion of GRIM-19 at 70-80 amino acids or 90-100 amino acids does not affect its assembly into Complex I. However, the mitochondrial membrane potential is disrupted in these GRIM-19 deletion mutants, although they are still capable of Complex I assembly (43). In comparison, C-terminal deletion of other Complex I subunits, such as NDUFA9 and NDUFS3, allows their assembly into Complex I without affecting the
mitochondrial membrane potential (43). Therefore, it has been suggested that GRIM-19 has special roles in controlling mitochondrial membrane potential. However, the molecular mechanism of this control remains unknown.

In mammalian cells, GRIM-19 has dual localization in the nucleus and the mitochondria as part of Complex I. In the nucleus, it regulates interferon-beta and retinoic acid-induced cell death by binding to and inhibiting the STAT3 transcriptional factor. STAT3 (Signal Transducer and activator 3) is involved in activating the transcription of genes encoding anti-apoptotic factors and genes involved in cell proliferation (44,45). A conditional skin-specific knock-out of GRIM-19 in mice causes tumorigenesis, mediated through STAT3 action (46). Furthermore, it has been shown that GRIM-19 interacts with invading viral proteins and non-coding transcripts. These interactions are proposed to sequester GRIM-19 and prevent induction of apoptosis, a normal cellular response to viral infection, through inhibition of STAT3 activity (2). Although GRIM-19 homologs have been found in vascular plants, fungi and green alga (18,20,22,23), the possibility of dual function in these organisms remains unexplored.

1.4.2. Acyl carrier protein (SDAP/NDUFAB1)

Acyl carrier proteins (ACPs) are normally involved in the synthesis of type-II fatty acids and lipoic acid (47). In N. crassa, an ACP-like Complex I subunit has been demonstrated to possess de novo fatty acid synthetic ability (48). It has been postulated that it might function in either producing and/or delivering myristic acid, a fatty acid associated with the core hydrophobic subunit ND5. Only Neurospora
ND5 is known to interact with a fatty acid molecule, and hence this proposed function is species-specific (40). Two subunits, resembling acyl carrier proteins, have been found in both bovine and Yarrowia Complex I, one localized to the proximal membrane arm and the second present in the distal membrane arm (13,49). Loss of ACP results in defects in Complex I assembly and mitochondrial membrane lipid content in these organisms.

An ortholog of the ACP subunit has been identified in the green alga Chlamydomonas, but the subunit has not been detected in Complex I (18,23). Similarly, although two soluble forms of mitochondrial ACP, are localized to the mitochondrial matrix of Arabidopsis (50), they were not detected in Arabidopsis Complex I via proteomic analysis (20). Hence, a role for acyl carrier proteins in plant Complex I is yet unconfirmed.

1.4.3. γ - Carbonic Anhydrase (CA/CAL) subunits

Electron micrographs of Complex I from potato, Arabidopsis, Zea mays and the alga Polytomella, have shown the presence of a spherical soluble domain present in the membrane arm, protruding into the matrix, which is absent in mammalian and fungal Complex I (51-53). Although, Arabidopsis consists of five CA subunits (CA1, CA2, CA3 have canonical carbonic anhydrase domains, whereas CAL1 and CAL2 have non-conserved residues in these domains) (18,51,54), the spherical domain is proposed to consist of only two CA subunits and one CAL subunit (carbonic anhydrase-like subunit) (52,55).
*Chlamydomonas*, putative orthologs have been identified as *bona fide* Complex I subunits (CAG1, CAG2 and CAG3) (56).

γ - Carbonic anhydrases belong to a family of Zn-containing enzymes that interconvert CO₂ and HCO₃⁻ (2). However, recombinant CALs from Complex I fail to exhibit carbonic anhydrase activity by themselves (57). Interestingly, *Arabidopsis* CAL2 knock-out lines display 80% reduction in mature Complex I (51), highlighting the role of these subunits in Complex I assembly.

Recently, the CA subunits were postulated to play a role in plant development and photomorphogenesis, although the connection to Complex I function remains unclear (58,59). For instance, overexpression of CA2 results in male sterility in *Arabidopsis* (60), whereas CAL2 knock-outs do not display any defect in seed germination (52). On the other hand, RNAi knock-down of the genes encoding these subunits affects the light dependent regulation of seedling growth and flowering.

Interestingly, double mutants of CA2/CAL1 and CA2/CAL2 exhibit delay in seedling growth, which is rescued upon growth in high CO₂ (58). Although the Complex I deficiency observed in the single and double mutants is equivalent, the double mutants exhibited a 40% reduction in carbon assimilation rates with respect to single mutants (58). Reduced carbon assimilation and restoration of growth in the presence of high CO₂, is indicative of a role for the CA subunits in mediating photorespiration (58). Photorespiration refers to the phenomenon by which RuBisCo binds O₂ instead of CO₂, causing reduced carbon assimilation and
resulting in the production of a toxic product. This product is broken down to release CO₂, which is recycled for carbon assimilation. As CA2 homotrimers can bind inorganic carbon (61), the CA module was postulated to function in recycling CO₂, generated from respiration and photorespiration, by an unknown mechanism.

Although CA subunits were first characterized as specific to the plant-lineage, they have been recently found in a non-photosynthetic organisms such as *Acanthamoeba castellannii* (a free living, soil and freshwater amoeba) and *Polytomella* (a non-photosynthetic unicellular alga) (51,62,63). These results suggest that the role of the CA subunits in Complex I extends beyond the plant kingdom.

1.4.4. GLDH (*L*-galactono, 1,4-lactone dehydrogenase)

GLDH is an enzyme that catalyzes the final step in ascorbic acid biosynthesis (64). A small fraction of *Arabidopsis* mature Complex I forms a higher molecular weight complex by association with GLDH. In *Arabidopsis*, loss of GLDH results in lethality of seedlings, a phenotype that can be rescued by ascorbate supplementation (65). On the other hand, in *N. benthamiana*, homozygous *gldh* mutants are not viable even in the presence of ascorbate and GLDH is not associated to the mature complex. Instead, GLDH is found to be associated with a 800 kDa subcomplex in an *nd4* null mutant (66). It has been hypothesized that in *N. benthamiana*, GLDH is bound to the membrane arm subcomplex in order to mediate the assembly of the distal membrane arm and is later replaced by the core ND5 subunit in the final stages of Complex I assembly (66). On the contrary,
Arabidopsis GLDH has been confirmed to associate with the mature Complex I, and also with the 470 kDa and 420 kDa assembly intermediates. Within the 420 kDa intermediate, GLDH is associated with the core membrane arm subunit ND2 and carbonic anhydrase subunits CA2, CA3 and CAL2. The 470 kDa intermediate includes association with the GRIM-19 non-core subunit. In-gel activity assays have revealed that GLDH exhibits ascorbate activity in the 420 kDa and 470 kDa subcomplexes and the mature holoenzyme (67). However, the role of ascorbate synthesis in Complex I function remains undeciphered.

1.4.5. Post-translational modification of Complex I subunits

The occurrence of several post-translational modifications in Complex I subunits is now documented. However, in most cases, the contribution of such modifications to Complex I assembly/activity is unclear. Among the post-translational modifications, phosphorylation has been reported in several instances. The phosphorylation of serine/threonine residues in the following non-core subunits, mediated via cAMP dependent protein kinase, have been determined – NDUFS4, NDUFA10, ESSS, MWFE, B14.5a, B14.5b, B16.6 (2,68). Recently, the mitochondrial c-src kinase has been implied in phosphorylation of a tyrosine residue present in the core subunit NDUFV2 and the non-core subunit NDUFB10 (69,70). Loss of phosphorylation of NDUFV2 and NDUFB10 causes reduction in the levels of the mature Complex I and NADH dehydrogenase activity (69,70). Hence, it has been proposed that phosphorylation of these subunits is essential for assembly into Complex I and/or mediating its enzymatic activity.
Mutations in PINK1 (PTEN-induced putative kinase 1), a serine/threonine kinase, is an established cause of Parkinson disease (PD), and all patients with mutations in PINK1 exhibit Complex I deficiency (71). Homozygous pink1 mutants in Drosophila also display Complex I defects (72). Interestingly, expression of Saccharomyces cerevisiae type II NADH dehydrogenase (Ndi1p) alleviates many of the functional defects caused by the pink1 mutation (73). These results suggest that the pink1-associated phenotypes are caused by loss of Complex I function. Therefore, bypassing the Complex I deficiency, by the ectopic expression of an alternative NADH dehydrogenase, can improve the efficiency of the ETC (73).

Analysis of the phosphoproteome of the pink1 mutants revealed the loss of phosphorylation at Ser250 of the non-core NDUFA10 subunit. Introducing a phosphomimetic NDUFA10 into pink1 null mutants in mice and Drosophila, alleviated the pink1-associated phenotypes, including Complex I deficiency and ATP synthesis (72). Therefore, phosphorylation of the NDUFA10 subunit is required for ubiquinone reductase activity of Complex I (72), and these findings emphasize the role of PINK1 in Complex I function.

In addition to phosphorylation, two core subunits 49 kDa and PSST, present near the quinone binding site, have also been found to be methylated and hydroxylated, respectively (74). Although the identity of the corresponding methylase and hydroxylase are not known, these modifications are proposed to play crucial roles in mediating the redox potential of the N2 cluster coordinated by PSST (74).
1.5. The assembly of Complex I

Mitochondrial disorders due to OXPHOS dysfunction occur in 1:5000 newborns. Amongst these, isolated Complex I dysfunction accounts for 25%-35% of the disorders (75). Mutations in mitochondrial- and nuclear- encoded subunits are a prevalent cause of Complex I deficiencies in humans, manifesting in a variety of disorders such as neurodegenerative diseases, myopathies and Parkinson’s disease (35). Surprisingly, 60% of the Complex I deficiencies cannot be explained by molecular lesions in structural subunits of Complex I. In these cases, it was postulated that defects in yet-to-be discovered assembly factors prevent the formation of a functional holoenzyme (2,76).

The dual genetic origin of mitochondrial Complex I and its large size makes the assembly process very complicated. Briefly, the process of Complex I assembly involves the addition of hydrophobic modules and hydrophilic modules in a precise step-wise manner as illustrated in Figure 1.2 (34,77).

Models for Complex I assembly have been gathered from the genetic and biochemical studies in different organisms, such as vascular plants (Arabidopsis, Nicotiana), algae (Chlamydomonas), fungi (Neurospora, Yarrowia), nematodes (C. elegans), mammals (mice, Chinese hamster cell lines, human patient cell lines) and bacteria (E. coli, T. thermophilus) (3,16,21,60,66,68,78-86). This section shall describe the different assembly factors isolated for mammalian Complex I. However, this section is not intended for elaborating on the different models of Complex I assembly or the associated mitochondrial diseases (refer to reviews
A simplified scheme of human Complex I assembly is shown. Subunits (ND1-to -6, NDUFS1-to -8 ; NDUFA1,2,6,9,10,12,13 ; NDUFV1,2,3 and NDUFB6 are indicated in black font and assembly factors (C20ORF7, NDUFAF1 to 4, IND1, AIF, Ecsit) in colored font. The different subcomplex intermediates (1-to-5 and a-to-d) are indicated. Hydrophilic subunits come together to form assembly intermediate 3. Hydrophobic subunits form assembly intermediate ‘a’, with the help of C20ORF7 factor. Subcomplexes 3 and ‘a’ are assembled together with the help of NDUFAF3 and NDUFAF4 to form the membrane bound assembly intermediate 4, which is ~400 kDa in size. Membrane bound intermediate b assembles with the help of Ecsit and NDUFAF1 factors. Intermediate 4 combines with the independently formed membrane-bound intermediate b to form the subcomplex 5. Subcomplex 6 (830 kDa) is formed after a subcomplex c, containing the core subunits ND4 and ND5, is added to intermediate 5. Soluble subunits in subcomplex d are then added to subcomplex 6, with the function of NDUFAF2, to form the final holoenzyme. The factors ACAD9, TMEM126B and C3ORF1 and NDUFAF7 are not represented in this figure. (Source: McKenzie and Ryan, 2010) (77).
1.6. Assembly Factors for Mitochondrial Complex I

Traditionally, assembly factors have been defined as proteins required for the post-translational assembly of various Complex I subunits. While they may be associated with assembly intermediates, assembly factors do not form a part of the final holoenzyme. In 1998, the first factors CIA30 and CIA84, associated with a Complex I assembly intermediate, were discovered in Neurospora (89). A patient with a mutation in a candidate assembly factor (B17.2L) was first reported in 2005, thereby commencing the study of assembly factors in Complex I-deficient patients (90). In recent years, additional factors have been discovered using different approaches such as biochemical identification in assembly intermediates, candidate gene approach via subtractive phylogenetic analysis and, mapping of mutations via linkage analysis in families of patients with Complex I defects (75, 90-92).

In this section, fourteen mammalian Complex I assembly factors shall be described, focusing on their specific function in Complex I assembly (Table 1.2). Although the identity of these factors and their role in Complex I assembly has been experimentally validated, their exact biochemical activity is yet to be deciphered. While some of the assembly factors are conserved among Complex I containing eukaryotes, others appear to be lineage specific. Most of the known factors function in Complex I assembly after mitochondrial localization and are localized to the matrix or embedded in the inner membrane. C8ORF38 forms a special case because its essential function appears to be dependent upon
cytosolic localization despite the fact that the protein is also found in the mitochondria. The other exception is AIF, which is the only assembly factor in the mitochondrial intermembrane space. Figure 1.2 provides a pictorial representation of different assembly intermediates involved in Complex I assembly, for easy reference.

<table>
<thead>
<tr>
<th>Alternative names</th>
<th>Mammalian</th>
<th>Higher plants</th>
<th>C. reinhardtii</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>C20orf7/NDUFAF5</td>
<td>NDUFAF5</td>
<td>At1g22800</td>
<td>XP_001693605</td>
<td>Early stages of ND1 assembly</td>
</tr>
<tr>
<td>NDUFAF3/C6orf60</td>
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<td>At3g60150</td>
<td>XP_001702394</td>
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</tr>
<tr>
<td>NDUFAF4/C6orf66</td>
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<td>-</td>
<td>XP_001701912</td>
<td>Early and intermediate stages of assembly</td>
</tr>
<tr>
<td>C3orf1</td>
<td>C3orf1</td>
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<td></td>
<td></td>
</tr>
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<td>At1g17350</td>
<td>XP_001701850, NuoAF1</td>
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<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
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<td>ACAD9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>TMEM126B</td>
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<td></td>
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<td>-</td>
<td>-</td>
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</tr>
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<td>At4g19540 (INDL)</td>
<td>XP_001702721</td>
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</tr>
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<tr>
<td>AIF</td>
<td>AIF</td>
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</tr>
</tbody>
</table>

Table 1.2. Assembly factor conservation in plants and mammals.

The mammalian Complex I assembly factors discussed in this Chapter are listed here along with their homologs from vascular plants (Arabidopsis) and green alga (Chlamydomonas) as reported in Salinas et al, 2014 (3).
1.6.1. **C20ORF7 / NDUFAF5**

C20ORF7 is involved in the very early stage of Complex I assembly (93). Upon loss of this factor, the core ND1 subunit is no longer detected. This results in a drastic reduction of the membrane-bound ~400 kDa intermediate required for the completion of the early stages of assembly (94). Therefore, C20ORF7 is essential for the formation of the first assembly ~400 kDa intermediate. This factor contains an S-adenosylmethionine (SAM) dependent methyltransferase fold (93). It has been postulated that C20ORF7 could be required for the methylation of the non-core subunit NDUFB3, although this is yet to be experimentally proven (89,95).

1.6.2. **C6ORF60 / NDUFAF3 and C6ORF66 / NDUFAF4.**

These two assembly factors are also associated with the ~400 kDa assembly intermediate, containing the NDUFS2, NDUFS3, NDUFS7, NDUFS8 and NDUFS9 subunits (96,97). NDUFAF3 and NDUFAF4 are localized to the inner mitochondrial membrane and interact closely with each other. A possible role in anchoring the ~400 kDa intermediate to the membrane has been proposed (96,97).

1.6.3. **C8ORF38 / NDUFAF6**

C8ORF38 also functions in the early stages of Complex I assembly. It performs dual roles as a Complex I chaperone in both the cytoplasm and the mitochondria. In the mitochondria, C8ORF38 is required for the stability of the ND1 subunit and its subsequent incorporation into the ~400 kDa intermediate, but not
for ND1 translation (98,99). The *Drosophila* C8ORF38 ortholog was shown to interact with the cytosolic Hsp90 to help chaperone the Complex I subunits NDUFS3 and ND42 from the cytoplasm to the mitochondria (100). In addition, C8ORF38 was shown to protect the ND42 subunit against proteolytic degradation (100). In *Drosophila*, expression of a cytosolic form of C8ORF38 can rescue a c8orf38-null mutant, an indication that, unlike other complex I assembly factors, the essential function of C8ORF38 in Complex I biogenesis is in the cytoplasm.

1.6.4. **CIA30 / NDUFAF1**

CIA30 and CIA84 were first discovered in *N. crassa* as factors associated with an intermediate subcomplex (89). Together, they are required for the successful assembly of the membrane arm of Complex I in *Neurospora* (80). While CIA84 seems to be a fungal-specific assembly factor, CIA30 is conserved among all Complex I-containing organisms. In human fibroblasts, knock-down of *NDUFAF1*, encoding the ortholog of CIA30, results in the loss of the mature complex (86,101). Instead, a ~460 kDa subcomplex accumulates, further underscoring the role of NDUFAF1 in the assembly of the membrane arm. NDUFAF1 is known to be associated with newly synthesized ND1, ND2 and ND3 subunits, and hence, a stabilizing role has been proposed (102). In addition, NDUFAF1 also associates with other assembly factors Ecsit and ACAD9 described below (101). NDUFAF1 remains associated to the assembly intermediates from the ~460 kDa subcomplex to the penultimate ~830 kDa subcomplex, but dissociates upon formation of the final holoenzyme (27,34,35).
1.6.5. **Ecsit (Evolutionarily conserved signal intermediate in Toll pathways)**

Ecsit is a mammalian-specific mitochondrial protein. It interacts with the NDUFAF1 factor (Section 1.4.4) and is associated with the ~460 kDa, ~600 kDa and ~830 kDa assembly intermediates (101). Since knockdown of Ecsit results in reduced levels of NDUFAF1, it has been proposed that they act as co-chaperones (94).

1.6.6. **ACAD9 (Acyl CoA dehydrogenase)**

ACAD9 displays similarity to enzymes involved in mitochondrial β-oxidation of fatty acids (103). Loss of ACAD9 results in severe Complex I deficiency. ACAD9 co-purifies with NDUFAF1 and Ecsit and knock-down of ACAD9 results in decreased levels of the associated NDUFAF1 and Ecsit proteins. Hence, together with NDUFAF1 and Ecsit, ACAD9 associates with the ~460 kDa assembly intermediate (104,105).

Initial studies in patients showed that loss of ACAD9 does not affect fatty acid oxidation and the primary defect was only in Complex I assembly (104). Recently, fatty acid oxidation by ACAD9 has been demonstrated *in vivo* (106). Patients carrying mutations that affect both Complex I assembly and ACAD9’s enzymatic activity display drastic neurological symptoms (103). The severity of the symptoms has been attributed to the possibility that ACAD9 is the only enzyme in the brain tissue, capable of generating long-chain fatty acids. Hence, its role in fatty acid oxidation in certain tissues is more crucial than others, which lack
alternative enzymes that may contribute to fatty acid oxidation (103). Currently, a
dual role for ACAD9 in Complex I assembly and fatty acid oxidation has been
proposed.

1.6.7. **TMEM126B**

TMEM126B, a hydrophobic protein localized in the inner mitochondrial
membrane, associates with the ~460 kDa assembly intermediate, along with
NDUFAF1, ACAD9 and Ecsit (107). It was initially identified in association with
C3ORF1 assembly factor described below, by biochemical profiling of the rat
mitochondrial complexes.

1.6.8. **C3ORF1**

C3ORF1 initially associates with the early ~400 kDa assembly intermediate,
containing the ND1 subunit. It co-purifies with the NDUFAF3 and the NDUFAF4
assembly factors that are also found with this assembly intermediate (107,108). In
addition, C3ORF1 is associated with the ~460 kDa subcomplex and with subunits
from the N (NADH oxidizing) and Q (ubiquinone reducing) modules (109). This
factor is localized to the inner mitochondrial membrane, and together with
TMEM126B, is proposed to function as membrane anchors for the membrane-bound subcomplexes (109).

1.6.9. **NDUFAF2 (B17.2L)**

A mammalian factor, NDUFAF2 is involved in the late stages of assembly,
where it is associated with the ~830 kDa subcomplex (90). Loss of NDUFAF2
results in accumulation of the ~400 kDa and ~460 kDa assembly intermediates (2).

### 1.6.10. **FOXRED1**

FOXRED1, belonging to the FAD-dependent oxidoreductase family, is localized to the inner mitochondrial membrane facing the mitochondrial matrix (110,111). Loss of FOXRED1 results in the formation of an initial unstable ~830 kDa intermediate. However, failure to assemble a mature complex results in the accumulation of a ~460 kDa subcomplex. FOXRED1 co-immunoprecipitates with various Complex I subunits, further corroborating its function in Complex I assembly (111). Although FOXRED1 has a putative oxidoreductase activity and contains an FAD-binding domain, its function in Complex I assembly is yet to be elaborated (110).

### 1.6.11. **Apoptosis Inducing Factor (AIF)**

AIF, a FAD dependent NADH oxidase (112), is localized to the mitochondrial intermembrane space and exhibits dual roles in Complex I assembly and apoptosis. Upon receiving apoptotic signals, AIF migrates to the nucleus from the mitochondria and causes chromatin condensation and DNA degradation in a caspase independent manner (35). AIF depletion results in reduced accumulation of Complex I subunits, decreased Complex I activity and mitochondrial respiration (113). Although the requirement of AIF for Complex I function is confirmed, its mode of action in Complex I assembly remains a mystery (114).
1.6.12.  **NUBPL / IND1 / INDH**

Human Ind1 protein is a member of P-loop NTPases involved in Fe-S cluster binding and was first identified in *Y.lipolytica* (115). Loss of IND1 in *Yarrowia* results in the accumulation of a ~460 kDa intermediate, lacking the peripheral arm containing Fe-S binding subunits (115). Interestingly, IND1 is capable of transferring Fe-S cluster to an acceptor protein *in vitro*, although this function needs to be confirmed *in vivo* (116). Further, it has been shown that C-terminal end mutations, where the Fe-S cluster is purported to bind, results in drastic reduction of mature Complex I (116,117). Based on these results, one hypothesis for IND1 function is a role in Fe-S cluster delivery to one or several core subunits of the soluble arm.

Studies of IND1 in *Arabidopsis* led to the proposal for an additional function for this assembly factor, where loss of IND1 results in the accumulation of a 650 kDa intermediate. It was observed that the lack of IND1 in *Arabidopsis* affects mitochondrial translation (118). Loss of mitochondrially-encoded Complex I subunits would also result in an assembly defect, thereby explaining the accumulation of a ~650 kDa assembly intermediate. In bacteria, overexpression of human IND1 results in the increased expression of a reporter gene encoding β-lactamase (119). This has been attributed to enhanced translation of the β-lactamase encoding transcript. The proposed hypothesis for this phenotype is that IND1 binds to the 3′ UTR of TEM-1 mRNA, preventing its endonucleolytic cleavage and thereby improving its translation (119).
Taken together, these studies postulate an alternative role for IND1 in RNA binding and translation. Examples of factors functioning in translation and assembly of respiratory complexes have been documented for Complex III and IV (120). Although the two proposed functions of IND1 are not mutually exclusive, further studies need to be conducted to establish the exact role of IND1 in Complex I assembly.

1.6.13. *MidA/NDUFAF7*

Originally characterized in *Dictyostelium*, NDUFAF7 is a protein localized to the mitochondrial matrix (121). NDUFAF7 is recruited in the early stages of Complex I assembly, where it stabilizes the ~400 kDa intermediate. Yeast two hybrid experiments revealed that NDUFAF7 interacts with NDUFS2, a subunit of the soluble arm of Complex I, containing a di-methylated arginine residue (121). NDUFAF7 harbors a methyltransferase domain, implying a possible function in methylating NDUFS2. Indeed, transient depletion of NDUFAF7 results in loss of methylation in the NDUFS2 subunit (122). Therefore, it has been concluded that NDUFAF7 is an assembly factor that mediates its function through dimethylation of the Arg-85 residue of NDUFS2 (122). So far, this is the only assembly factor whose precise mode of action in Complex I assembly has been established.
In summary, the identity of factors involved in Complex I assembly and their mechanism of action are only now emerging. So far, factors involved in the biogenesis of Complex I have been identified through mutations in patients, biochemical analyses of assembly intermediates and the candidate gene approach. An overarching goal of this thesis is to expand our knowledge of Complex I assembly by using a forward genetic approach for a rapid identification of hitherto unknown biogenesis factors. It has been observed that loss of Complex I subunits also blocks Complex I assembly (27,35). Synthesis and mitochondrial import of these subunits are also critical for successful assembly of Complex I. However, for the purposes of this thesis, we define assembly/biogenesis factors as proteins required for the synthesis, stability and post-translational assembly of Complex I subunits that do not form a part of the final holoenzyme.
CHAPTER 2

NOVEL COMPLEX I MUTANTS REVEALED FROM A FORWARD GENETIC SCREEN CONDUCTED IN CHLAMYDOMONAS REINHARDTII

2.1. Introduction

Complex I is an L-shaped enzyme, with its membrane arm attached to the inner mitochondrial membrane and its hydrophilic arm protruding into the matrix (11,12,123). This multimeric enzyme is of dual genetic origin with only a few subunits synthesized in the mitochondria while the majority of its constituents are imported from the cytosol (2). Complex I acts as a NADH: ubiquinone oxidoreductase, pumping protons from the matrix to the intermembrane space, thereby creating a proton gradient necessary for ATP synthesis (20). Oxidative phosphorylation disorders are a major cause of mitochondrial diseases in humans, out of which approximately 44% are characterized by isolated and combined Complex I deficiencies (124). Indeed, if molecular lesions in the genes encoding for the structural subunits of Complex I are responsible for 40% of Complex I-linked diseases, 60% of Complex I defects observed in patients have no molecular
explanation (27,124). Studies in various experimental systems indicate that the membrane and soluble arms assemble as independent subcomplexes containing the structural components of Complex I (2,27). It is now apparent that additional factors, referred to as assembly/biogenesis factors, which are not part of the holoenzyme, are also required for the assembly of Complex I into its active form. However, the identity and function of such assembly factors in the manufacture of Complex I is only beginning to emerge.

2.1.1. Chlamydomonas reinhardtii as a model system for the study of mitochondrial Complex I

Many model systems have been used for the study of Complex I including fungi, vascular plants and animals (2,20,21,84,125). However, to our knowledge, these organisms have not been used to perform a genetic screen to isolate novel Complex I mutants. Recently, Chlamydomonas reinhardtii, a unicellular photosynthetic alga, has been successfully used as a model system for the study of mitochondrial Complex I. Chlamydomonas Complex I, made up of 42 subunits, has at least 34 subunits conserved with the counterparts forming the mammalian enzyme (18,23). Hence, it is reasonable to expect that the Complex I assembly process will also be largely conserved. Chlamydomonas also provides several advantages as an experimental model system (126). A fully annotated genome (version 5.5), with gene models generated from RNA-seq experiments, has been established (127,128). Although Chlamydomonas is considered a “plant-like” organism because of its ability to perform photosynthesis, it also retains many of
the animal-like characteristics from the last plant-animal common ancestor (128). For instance, the mitochondrial genome of *Chlamydomonas* is similar in size and gene structure to animal mitochondrial genomes (1).

Another advantage is that the haploid vegetative phase of *Chlamydomonas* has a rapid generation time of 8-12 h (128). In addition, it is a model system with established genetic and molecular biology techniques (126,129). Interestingly, this is the only system where nuclear, chloroplast and mitochondrial genomes have all been successfully manipulated (2,130). Forward and reverse genetic screens, through insertional mutagenesis of the nuclear genome, have been successfully conducted (131-133), and methods for epigenetic targeted gene silencing have also been established (134,135). Although nuclear transformation in *Chlamydomonas* mostly takes place by random, non-homologous recombination (136), methods for specifically targeting nuclear genes are now being explored. Zinc-finger nuclease (ZFN) was recently demonstrated as a strategy for targeted gene disruption (137) and application of CRISPR/Cas9 technology has also been attempted through transient expression of Cas9 and single guide RNA genes (138). However, stable transformants were not recovered due to the presumed toxicity of constitutively expressed Cas9 in *Chlamydomonas* (138). Thus, targeted nuclear gene mutagenesis is a promising endeavor that is in progress. On the other hand, targeted manipulation of organellar genes is already well established because transformation of chloroplast and mitochondrial genomes occurs by homologous recombination (139,140).
Importantly, the photosynthetic and mitochondrial membrane systems are separate in *Chlamydomonas*. Hence, it is an ideal system to study mutants of mitochondrial biogenesis as they are still viable under phototrophic conditions (2), unlike mammalian mitochondrial mutants. In particular, Complex I mutants have a specific phenotype under respiratory conditions compared to Complex III and Complex IV mutants. Complex III and Complex IV mutants are arrested for growth in the dark, even in the presence of a reduced carbon source such as acetate, and are referred to as *dark* die (dk) mutants (141). On the other hand, Complex I mutants have a slow growth in the dark (sid) phenotype, thus allowing for specific identification of Complex I mutants amongst other respiratory mutants. This unique phenotype is attributed to the presence of alternative type-II NADH dehydrogenases (6). These FAD-containing monomeric enzymes are rotenone insensitive and are present on the surface of the inner mitochondrial membrane, exposed to either the matrix or the intermembrane space (142). They are able to partially compensate for lack of Complex I in the electron transfer chain, by oxidizing NAD(P)H and reducing ubiquinone. However, they are not proton-pumping enzymes and hence cannot fully compensate for Complex I function (143). Finally, Complex I mutants in *Chlamydomonas* also retain their ability for sexual reproduction, enabling extensive genetic studies (2,131). The following Complex I mutants have been generated in *Chlamydomonas*. 
2.1.2. Complex I mutants with molecular lesions in mitochondrial genes

The mitochondrial genome of *Chlamydomonas* encodes seven genes involved in the electron transfer chain: *nd1, nd2, nd4, nd5, nd6*, encode core Complex I subunits located in the membrane arm of Complex I and, *cob* and *cox1*, encode Complex III and Complex IV subunits respectively (144) (see Chapter 4, Figure 4.10). To understand the role of these mitochondrial subunits in Complex I assembly, mitochondrial mutants were isolated by the Remacle group. Chemical mutagenesis with acriflavine, a DNA intercalating dye, was conducted and mutants defective for respiratory growth were isolated and named as *dark uniparental minus (dum)* mutants (143,145). This screen revealed Complex I mutants with molecular lesions in *nd1 (dum20, dum25), nd5 (dum5, dum23)* and *nd6 (dum17)*, which display severe reduction in NADH: dehydrogenase activity (56,143,145,146). Although these mutants display wild-type levels of Complex II, III and IV activities, they all exhibit an increased Complex II+III activity, which is hypothesized to be a compensatory effect for the loss of Complex I. In addition, Complex I and Complex III mutants were also isolated that contain deletion of multiple mitochondrial genes such as *dum24 (deletion of cob and the 3′-end of the nd4 gene)* and *dum22 (deletion of cob, nd4 and the 3′-end of the nd5 genes)* (143,145). Loss of ND1 or ND6 results in total absence of Complex I assembly. Interestingly, a frameshift mutation in *nd1* causes loss of Complex I assembly, whereas, deletion of only two amino acids in *nd1* coding sequence still enables the formation of a mature complex at lower levels compared to wild-type (145,146).
On the other hand, loss of ND4 (in dum22, dum24) or ND5 (in dum22, dum23) is characterized by the accumulation of a 700 kDa subcomplex instead of a fully assembled 950 kDa complex (56,145). The characterization of the above mitochondrial mutants enabled us to determine the order of assembly of different Complex I subunits. It is now clear that among the membrane arm subunits, ND1 and ND6 need to be assembled first to form the 700 kDa subcomplex, to which, ND4 and ND5 are later added (3).

In recent years, targeted mitochondrial manipulation, by homologous recombination, has been successfully conducted in Chlamydomonas by biolistic transformation (139). To date, stable mitochondrial transformation is possible only in two organisms: Saccharomyces cerevisiae and Chlamydomonas reinhardtii. However, Saccharomyces lacks mitochondrial Complex I and hence, Chlamydomonas remains the sole model system for mitochondrial manipulation of Complex I subunits. First, a deletion in the nd4 coding sequence was generated as a proof of concept, with the resulting mutant displaying Complex I deficiency and accumulating a 700 kDa subcomplex, consistent with previous observations in other nd4 deletion mutants such as dum24 (130). This tool has been further expanded to test a “provisional” nd4 point mutation discovered in a human patient with chronic progressive external ophthalmoplegia (147). This human mutation was categorized as “provisional” because its true contribution to Complex I deficiency was difficult to assess due to the heteroplasmic state of the mutation in the patient (only 39.6% mutant mtDNA was found in muscle) (147). Hence, this
provisional mutation was introduced into \textit{Chlamydomonas} mitochondria by biolistics and \textit{nd4} homoplasmic mutants containing the L157P change were obtained (148). Although the L157P mitochondrial transformants were able to assemble a mature Complex I, as opposed to \textit{nd4-null} mutants which accumulate a 700 kDa subcomplex (145), their NADH:ubiquinone oxidoreductase activity was severely reduced. Thus, it was confirmed that the L157P change in ND4 causes Complex I deficiency, although there was no apparent defect in Complex I assembly. This example demonstrates that Complex I studies in \textit{Chlamydomonas} can provide insights into the molecular bases of CI-linked human diseases.

2.1.3. \textit{Complex I mutants inactivated for Chlamydomonas nuclear genes}

\textit{Chlamydomonas} mitochondrial genome encodes for only five Complex I subunits, whereas, mammalian and angiosperm mitochondria encode for seven and nine subunits respectively (149,150). It has been hypothesized that the endosymbiotic event led to the transfer of four genes, encoding for the membrane arm subunits ND3, ND4L, ND7 and ND9, from the mitochondria to the nucleus (149,150). The genes encoding these Complex I subunits have acquired traits for appropriate expression in the nuclear genome, and the corresponding gene products are characterized by lower hydrophobicity and mitochondrial targeting sequences for successful mitochondrial localization (149,150). Reverse genetic studies were conducted through RNA interference (RNAi) to determine the role of these subunits in Complex I activity and assembly. ND3, ND4, ND7 and ND9 knock-down mutants are characterized by severe reduction in NADH: duroquinone
oxidoreductase activity and no mature or 700 kDa subcomplexes for Complex I were detected (149,150). This suggests that ND3, ND4L, ND7 and ND9 need to be assembled prior to ND4 and ND5, similar to the order of assembly in mammalian and plant Complex I (20,151).

2.1.4. Novel loci involved in Complex I biogenesis

Our laboratory was interested in identifying novel factors required for Complex I biogenesis, which are not retained in the final holoenzyme. In humans, only fourteen biogenesis factors have been identified so far (Chapter 1), out of which eight are currently known to be conserved in *Chlamydomonas* (152). As more than 40 subunits need to be assembled together to yield a functional enzyme, it is reasonable to hypothesize that yet-to-be discovered factors are also involved in Complex I biogenesis. To identify novel biogenesis factors, a forward genetic approach was undertaken (131) via insertional mutagenesis using antibiotic resistance cassettes. Six nuclear mutants named as assembly of mitochondrial complex I (*amc1, amc3*-to-7) were isolated in this screen. Another nuclear Complex I mutant isolated by an independent screen via acriflavine mutagenesis (146) was added to this collection and named *amc2* (131). These Complex I mutants, isolated by screening for the characteristic *sid* phenotype, displayed severe Complex I deficiency except for *amc3* which exhibited only partial deficiency. The mutants can be categorized into three types based on biochemical analyses: a) accumulation of a stable 700 kDa subcomplex, as observed in *amc5, amc6* and *amc7*, b) accumulation of a labile 700 kDa subcomplex in *amc1* and
amc2, and c) accumulation of reduced levels of a 950 kDa mature complex in amc3 and amc4, although the mature complex in amc4 is not always detectable. All seven mutants have no defect in Complex II, III or IV activity and display the characteristic increase in Complex II+III activity already reported for the Complex I mutants in mitochondrial nd genes (56,145,146). Genetic analysis of amc1-to-7 revealed that the AMC locus in each mutant was monogenic. However, only the amc5 locus was tightly linked to the insertional cassette. Further genetic analysis revealed that amc5 and amc7 mutations were allelic and corresponded to a molecular lesion in NUOB10, which encodes a bona-fide membrane arm Complex I subunit (131).

Positional cloning via linkage analysis of the amc2 mutant lead to the mapping of the amc2 mutation within a 350 kb region in Chromosome 10 (153). This region does not contain any of the known Complex I subunits or assembly factors and hence, AMC2 is likely to be a novel locus involved in Complex I biogenesis. Further efforts to identify the mutant gene in amc2 are underway. The molecular identity of AMC1, AMC2, AMC3, AMC4 and AMC6 loci are yet to be determined and could either encode Complex I subunits or biogenesis factors.

This chapter deals with the characterization of six additional Complex I mutants isolated by continuation of the forward genetic screen as described in (131).
2.2. Experimental Procedures

2.2.1. Strains and culture conditions

Chlamydomonas strains were grown in Tris-Acetate-Phosphate (TAP), with 20 mM Tris-base and 17 mM acetic acid, or TAP supplemented with arginine (400 µg/mL) (TARG) liquid or solid medium at 25°C, in continuous light at 50 µE.m\(^{-2}\).s\(^{-1}\) (129). Wild-type strain 4C\(^{-}\) (mt arg7-8) was used for transformation (Dr. Rochaix, University of Geneva, Switzerland). Strains amc1 (4C10), amc2 (169dn26), amc3 (17G7), amc4 (A4G7), amc5 (87D3), amc6 (96A5), amc7 (111A4) (131,146) and their derivatives were used in this thesis.

2.2.2. Insertional mutagenesis

Transformation of 4C\(^{-}\) (mt arg7-8) strain was conducted after autolysine treatment by electroporation as in (154). The strain was grown in liquid TARG medium for 2-3 days until it reached exponential phase (3 – 6 x 10\(^6\) cells/mL). For each transformation, 2.5 x 10\(^7\) cells were electroporated at 1.3 kV and 10 µF with 100 ng of hygromycin B resistance cassette (iHyg3) and 20 µg of herring sperm DNA. The iHyg cassette consists of the aph7” gene with an RBCS2 intron, under the control of the β2 tubulin-encoding gene (TUB2) promoter and the RBCS2 terminator (Figure 1). This cassette was amplified from pHyg3 plasmid (155) using the primers: APH7-F (5’- TCATATCAAGCCTCTTTTCG-3’) and APH7-R (5’- AAGCTTCCATGGGATGACG-3’). The transformants were selected on TARG solid medium supplemented with hygromycin B (HyB, 25 µg/mL).
2.2.3. Phenotypic screening to isolate Complex I mutants

A phenotypic screen to identify candidate mutants with Complex I deficiency was conducted by scoring for slow growth in the dark (sid) phenotype as described in (131). Hygromycin B resistant (HyB\textsuperscript{R}) colonies, which appear after 10 days of incubation, were transferred into 400 µl of TARG + HyB liquid media in 96-well plates, each well containing a single colony. The 96-well plate was incubated for five days at 25°C in continuous light at 50 µE.m\textsuperscript{-2}.s\textsuperscript{-1}. The liquid cultures were then replica-plated onto two solid TARG media, incubated in the light and dark for five days. Transformants with a sid phenotype were selected and sub-cloned three times to obtain a single colony and used for further analysis. The following transformants were confirmed as Complex I mutants through subsequent phenotypic analyses: amc8 (1H5), amc9 (41D9), amc10 (42A10), amc11 (10G11), amc12 (6E9) and amc13 (4C3).

2.2.4. Ten-fold Dilution Series

One loop of cells grown on solid TARG plates (for 3-5 days) were resuspended in 500 µl of liquid TARG medium. The cell density was measured spectrophotometrically at A\textsubscript{750} and normalized to an OD\textsubscript{750} = 2 by dilution. This normalized suspension was used as the starting material [1] for making five serial ten-fold serial dilutions [10\textsuperscript{-1}, 10\textsuperscript{-2}, 10\textsuperscript{-3}, 10\textsuperscript{-4} and, 10\textsuperscript{-5}]. A volume of 8 µl for each dilution was plated on solid TARG plates. For scoring the sid phenotype, two plates were prepared simultaneously and incubated at 25°C, one in continuous light and
another in the dark, for at least 7 days. The light-incubated plate served as a control for confirming normalized cell density amongst multiple strains.

2.2.5. Complex I activity measurement

Complex I activity measurement was conducted as described previously in (145,146,156) by using NADH and duroquinone as substrates. Cells grown for 2-3 days on solid medium were harvested and resuspended in Tris-HCl extraction buffer (10 mM Tris-HCl pH 6.8-7.0, 5.2 g Mannitol, 100 mg BSA, 0.5 mM PMSF, 5 mM EDTA). Cells were lysed by sonication and a crude membrane extract was obtained by differential centrifugation. Complex I activity was determined as the rate of NADH oxidation, which was measured spectrophotometrically at A$_{340}$. NADH (100 µM) and duroquinone (100 µM) were used as substrates. Specific activity was calculated using molar extinction coefficient for NADH at $\varepsilon_{340\text{nm}} = 6.22$ mM$^{-1}$ cm$^{-1}$ in the absence and presence of 15 µM rotenone, a Complex I-specific inhibitor rotenone.

2.2.6. Blue Native PAGE (BN-PAGE) and in-gel activity assays

Partially purified membranes were extracted as described for Complex I activity measurement, except that MOPS-KOH extraction buffer was used (10 mM MOPS-KOH pH 7.4, 5.2 g Mannitol, 100 mg BSA, and 0.5 mM PMSF). Complexes were separated by BN-PAGE using 4-12% (w/v) acrylamide gradient gels as described in (157). Membranes were partially solubilized as follows. Membrane proteins (500 µg) were pelleted at 18,000 $g$ for 20 min at 4°C. The membranes were then resuspended in 180 µl of 2% (w/v) sodium n-dodecyl-β-D maltoside
(DDM) and 20 µl of 10% (w/v) sodium taurodeoxycholate hydrate (TDC). Both DDM and TDC were dissolved in ACA buffer (750 mM aminocaproic acid, 0.5 mM EDTA, 50 mM Bis Tris, pH 7). Partially solubilized membrane protein (200 µg) were loaded per lane. In-gel NADH oxidase (Complex I) activity was visualized as purple bands after incubating the gels in 100 mM MOPS buffer, pH 8, containing 1 mg/ml $\rho$-nitro blue tetrazolium chloride (NBT) and 0.2 mM NADH. Following in-gel Complex I staining, in-gel ATPase (Complex V) activity was detected by incubating the gels in the dark for 2-3 h, in 50 mM HEPES KOH pH 8 buffer containing 30 mM CaCl$_2$ and 5 mg/ml ATP, until a white precipitate was visible. This precipitate revealed the ATPase activity of Complex V.

2.2.7. Immunoblotting analysis

Partially purified membrane extracts were prepared as described for BN-PAGE and protein complexes were separated as described above. For SDS-PAGE, 10 µg of proteins was separated by 12.5% acrylamide gel and immunoblotting was performed according to established protocols (158). The separated proteins were electro-blotted onto PVDF membranes and custom-made primary antibodies specific for *Chlamydomonas* Complex I subunits (from Genescript, as described in (131)), were used. Membranes separated in BN gels were probed with 1:3000 $\alpha$-51 kDa. SDS-PAGE immunoblots were probed with 1:3000 $\alpha$-51 kDa, 1:3000 $\alpha$-49 kDa, 1:2000 $\alpha$-TYKY and 1:12,000 $\alpha$-cyt f.
2.2.8. Genetic analysis of the amc mutants

Genetic crosses were conducted according to (129). Gametogenesis was induced by resuspending vegetative cells in TAP liquid medium lacking nitrogen (TAP-N), at 25°C in low light, with shaking for 5 h. The gametes were mixed in equal proportions and incubated in high light at 25°C overnight. In some cases, 10 mM dibutyryl-cAMP and/or 1 mM IBMX were added to the mixture to stimulate mating. To isolate meiotic zygotes, the mixture was plated on TAP-N solid medium (containing 3% (w/v) select agar) and incubated in high light at 25°C for 5 days. The meiotic progeny was obtained through bulk germination or tetrad dissection of zygotes on TARG solid medium, in high light at 25°C. For obtaining meiotic progeny, the wild-type strain CC125 (137C+) or 1' (mt+) strains were crossed with the original amc mutants. Meiotic amc progeny derived from these crosses have also been used for further analyses.

For constructing amc/+ diploids, the amc mutants (mt, amc, aph7", arg7-8) were crossed with CC125 (Table 2.1) and the mating mixture was directly plated on selective medium (TAP + HyB). Individual diploids were subcloned to a single colony and their mating type was determined by diagnostic PCR (159), to confirm diploidy.
The mating type minus (mt\(^-\)) strains amc\(^8\)-to-13 are arginine auxotrophs (arg\(-\)) and hygromycin B resistant (HyB\(^R\)) and crossed with mating type plus (mt\(^+\)) Complex I proficient strain CC125, which is an arginine prototroph (arg\(+\)) and hygromycin B sensitive (HyB\(^S\)). The amc\(^+/\) diploids were selected for arginine prototrophy and hygromycin B resistance.

<table>
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<th>Diploid</th>
<th>mt(^-) Strain</th>
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<th>Strain</th>
<th>Phenotypes</th>
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<td>amc(^13+/)</td>
<td>arg(+), HyB(^R)</td>
</tr>
</tbody>
</table>

**Table 2.1. Construction of amc\(^+/\) diploids.**

The mating type minus (mt\(^-\)) strains amc\(^8\)-to-13 are were arginine auxotrophs (arg\(-\)) and hygromycin B resistant (HyB\(^R\)) and crossed with mating type plus (mt\(^+\)) Complex I proficient strain CC125, which is an arginine prototroph (arg\(+\)) and hygromycin B sensitive (HyB\(^S\)). The amc\(^+/\) diploids were selected for arginine prototrophy and hygromycin B resistance.
2.3. Results

2.3.1. Forward genetic screen leads to the isolation of novel Complex I mutants

Previously, a forward genetic screen was conducted in our laboratory to isolate Complex I mutants using *Chlamydomonas reinhardtii* as a model system. This screen was not saturated as it yielded only six nuclear Complex I mutants that were characterized (131). To reveal additional AMC loci encoding novel Complex I biogenesis factors, this forward genetic screen was continued. Insertional mutagenesis was conducted on wild-type strain (4C: *arg7*-8) by electroporation with the iHyg3 cassette (1.743 kb) encoding the *aph7*” gene that confers hygromycin B (HyB) resistance. The resulting transformants were screened by replica-plating for a slow growth in the dark (*sid*) phenotype, a characteristic phenotype of Complex I mutants in *Chlamydomonas* (56,131,145,146). Approximately 4200 independent transformants were screened, out of which 30 transformants with a potential *sid* phenotype were selected. Subsequent phenotypic analysis by ten-fold serial dilutions revealed that only 10 transformants displayed a true *sid* phenotype (data not shown and Figure 2.1 A). To confirm that the *sid* phenotype was caused by a Complex I deficiency, activity measurements were conducted by determining rotenone-sensitive NADH: dehydrogenase activity in crude membrane extracts. Complex I activity measurements confirmed that six out of ten transformants were true Complex I mutants (data not shown and Figure 2.1 B).
Figure 2.1. Six novel mutants exhibit Complex I deficiency.

A. The growth phenotype of the wild-type (WT, 4C-) and amc8-to-13 (original mutants as described in Section 2.2.3) was analyzed by ten-fold dilution series. The dilutions were plated on media containing acetate as a carbon source and incubated in the light or in the dark for seven days. B. Complex I (rotenone-sensitive NADH: dehydrogenase) activity was determined from partially purified membranes. The activities are represented as the average of five biological replicates with the error bars indicating standard deviation of the mean. The amc mutants display significantly reduced Complex I activities compared to WT as determined by two-tailed unequal variances t-test. * indicates p<0.05, ** indicates p<0.01 and *** indicates p<0.001.
These newly isolated Complex I mutants were added to the existing library of assembly of mitochondrial complex I (amc) mutants (131) and were named amc8-to-amc13. While the amc8, amc9 and amc11 strains display highly reduced Complex I activity, the amc10, amc12 and amc13 display partial Complex I deficiency (Figure 2.1 B). Consistent with that phenotype, the amc9 and amc11 mutants exhibit slower growth in the dark compared to the amc8, amc10, amc12 and amc13 strain, thereby reflecting their relative levels of Complex I activity. Thus, six Complex I mutants have been successfully isolated from a forward genetic screen by insertional mutagenesis.

2.3.2. The Complex I mutants display defects in Complex I assembly

Mitochondrial Complex I is one of the largest enzymes of the respiratory chain. In Chlamydomonas, 42 subunits need to be assembled in a precise step-wise process to generate a mature 950 kDa complex (23). Previous characterization of nuclear and mitochondrial Complex I mutants in Chlamydomonas have revealed different levels of Complex I assembly in these mutants (56,131,145,146,149,160). Hence, the level of Complex I assembly in the newly isolated amc8-to-13 mutants was determined by separating protein complexes from crude membrane extracts by BN-PAGE. Mature Complex I and partially assembled subcomplexes were visualized by in-gel NBT staining that reveals NADH oxidase activity as a purple band. Note that Complex I mutants that have an assembled soluble arm are capable of NADH oxidation, even if the ubiquinone reductase activity is impaired. In the wild-type (WT) strain, a purple
band at 950 kDa indicates a fully assembled Complex I (Figure 2.2 A). Purple bands seen at higher molecular weights could be due to differential solubilization of membranes or association of Complex I with other respiratory complexes such as Complex I+III2 (56). In the amc8-to-13 strains, three distinct levels of assembly were detected: i) no assembled and active complex in the amc9 mutant, ii) accumulation of a labile 700 kDa subcomplex in the amc11 strain and, iii) fully assembled complex in the amc8, amc10, amc12 and amc13 strains. Assembled Complex I detected by immunoblotting analysis following BN-PAGE, with polyclonal antibody against the soluble arm 51 kDa subunit, correlated with the in-gel activity assay (Figure 2.2 B). No assembled complex was observed for amc9, whereas fully assembled complexes accumulating to a lesser degree than WT were observed for amc8, amc10, amc12 and amc13. The 700 kDa subcomplex observed in amc11 is similar to the subcomplex accumulating in the nuob10 nuclear mutant (131) and the mitochondrial nd4 and nd5 mutants (145), which do not accumulate membrane subunits of the distal arm of Complex I. It must be noted that the subcomplex detected in the amc11 mutant is highly labile and its detection varied among independent extractions.

It has been previously observed that some Complex I mutants accumulate reduced levels of Complex I subunits (96,131). Hence, the steady-state accumulation of selected Complex I subunits was tested by immunoblotting analysis of proteins from crude membrane extracts separated by SDS-PAGE (Figure 2.2 C). Three subunits from the soluble arm of Complex I, 49 kDa, 51 kDa
and TYKY (23), were chosen for analysis. Although the amc8, amc12, amc10 and amc13 strains assemble reduced levels of mature Complex I, only amc8 and amc12 accumulate reduced levels of 49 kDa, 51 kDa and TYKY subunits whereas amc10 and amc13 accumulate these subunits to wild-type levels. Although no assembled complex was observed for amc9, it still accumulates wild-type levels of 49 kDa and TYKY subunits, but has decreased levels of the 51 kDa subunit. Finally, the amc11 mutant accumulates reduced levels of all three subunits.

2.3.3 The amc mutations are recessive

In order to identify if the amc mutations causing the Complex I deficient phenotype are recessive or dominant with respect to the wild-type allele, heterozygous vegetative diploids (amc+/+) were constructed by crossing amc mutants with wild-type strain according to Table 2.1. Diploids were verified for the presence of genetic markers from both mating types by diagnostic PCR (data not shown). Two independent diploids from each cross were tested for growth in the dark by ten-fold dilution series (Figure 2.3). It was observed that heterozygous diploids of amc8-to-13 were restored for growth in the dark, thereby indicating that all the amc mutations are recessive.
A. (Top panel) Blue-Native PAGE was conducted on 200 µg of partially purified membrane fraction. In-gel Complex I activity was detected by NBT staining. The purple bands indicate in-gel staining of NADH oxidase activity in mature (950 kDa) and partially assembled subcomplexes of Complex I. In some cases, multiple purple bands larger than 950 kDa are detected. The identity of these bands is unclear, although they could be due to the solubilization of the membrane or could be Complex I in association with other complexes (56). The green bands correlate to the photosynthetic complexes copurified in the membrane fractions. (Bottom panel) In-gel ATPase staining to detect Complex V was conducted to confirm equal protein loading. The reduced ATPase staining in WT compared to the amc8-to-13 mutants is not always observed. B. Immunoblotting was conducted, using α-51 kDa on complexes separated by BN-PAGE, to detect assembled soluble arm of Complex I. Two hundred µg of protein was loaded per lane. This image is a composite of two gels: with the WT lane from one gel and the lanes corresponding to the amc mutants from another. C. SDS-PAGE immunoblotting was conducted on 10 µg of partially purified membranes using polyclonal antibodies to detect soluble arm Complex I subunits: α- 49 kDa, α- 51 kDa, α- TYKY. α- cyt f was used to confirm equal loading. In A, B and C, the WT strain is 4C- and the amc8-to-13 strains are the original mutants as described in Section 2.2.3, except the amc10 (12C) strain, which is a meiotic spore derived from the original amc10 strain.

Figure 2.2. The amc mutants display a Complex I assembly defect.
Although the Complex I mutants were generated by insertional mutagenesis, it has been previously observed for *amc1*-to-7 that the insertional cassette is not always linked to Complex I deficiency (131). Hence, analysis of the meiotic progeny of *amcxWT* crosses by bulk germination, was conducted to test whether the insertional cassette segregates with the Complex I deficient phenotype (Table 2.2). All the recombinant HyBuR spores obtained from genetic crosses of *amc9*, *amc11* and *amc12* displayed a *sid* phenotype, indicating that the Complex I deficiency in these mutants is tightly linked to the insertional cassette. On the other hand, for *amc8*, *amc10* and *amc13*, only a fraction of the HyBuR recombinant spores displayed the *sid* phenotype indicating that the *AMC* locus was segregating away from the iHyg3 cassette. Furthermore, bulk germination and tetrad analysis of the meiotic progeny indicated that the mutations in the *amc8*, *amc9*, *amc10*, *amc11*, *amc13* strains are monogenic (data not shown), whereas the mutation in the *amc12* mutant is not monogenic (see Chapter 5).
The amc mutants were crossed with WT strain to obtain heterozygous amc/+ diploids as described in Table 2.1. Two independent diploids obtained from each cross were tested for complementation of growth in the dark by ten-fold dilution series. The dilution series was plated on acetate containing medium and incubated in the light or dark for 10 days. The growth of the following diploids are depicted panels a) to f) wherein WT (4C-) and the respective amc mutants, used for constructing the diploids, were used as controls: a) amc8/+, b) amc9/+, c) amc10/+: the amc10 strain shown is amc10 (12C), d) amc11/+, e) amc12/+, f) amc13/: the amc13 strain shown is amc13 (16).

Figure 2.3. The amc8-to-13 mutations are recessive.
2.4. Discussion

Mitochondrial Complex I, the first and largest enzyme of the mitochondrial electron transfer chain, is a proton-pumping NADH: ubiquinone oxidoreductase (152). The identity of the factors involved in the biogenesis of Complex I are only beginning to emerge and could account for the 60% of Complex I deficiencies in humans lacking a molecular explanation (27). In an effort to identify novel Complex I biogenesis factors in a quick and easy manner, a forward genetic approach in *Chlamydomonas* was undertaken. Unlike mammalian cells, *Chlamydomonas* mutants completely lacking Complex I are still viable by their ability to photosynthesize. Additionally, these mutants display a slow growth in the dark (*sid*) phenotype that easily distinguishes them from other respiratory mutants (2,131,145).

This screen describes the isolation of six Complex I mutants *amc8* - to -13 (Figure 2.1), in addition to previously characterized *amc1* - to -7 (131). Similar to *amc1* - to -7, the *amc8* - to -13 mutations are recessive (Figure 2.3) and monogenic (except *amc12*). While the mutations in the *amc8*, *amc10* and *amc13* strains are not linked to the insertional cassette, the mutations in the *amc9*, *amc11* and *amc12* mutants appear to be linked (Table 2.2). The *amc12* strain seems to harbor two mutations, each contributing to Complex I deficiency, out of which one is tightly linked to the cassette. The genetic analysis of the *amc12* mutant will be described in detail in Chapter 5. Thus, out of 13 *amc* mutations isolated, only four are linked to the insertional cassette.
Table 2.2. Summary of phenotype and genetic analysis conducted on amc8-to-13.

Complex I-specific activity for the amc mutants was determined by measuring NADH-d duroquinone oxido-reductase activity (Figure 2.1 B) and is represented as percentage of WT activity (WT set to 100%). The detection of fully assembled complex and the 700 kDa subcomplex is determined from Blue-Native PAGE in-gel activity and immunoblottings. ++++, +++, ++, +, - indicate relative levels of detected complex. Genetic analysis was conducted by obtaining meiotic zygotes and analyzing the 2:2 segregation of Complex I phenotype in at least 8 independent tetrads. In cases where tetrad analysis was not successful, bulk germination of zygotes was conducted and the resulting spores were scored for Complex I deficient phenotype. Key: arg+: arginine prototrophy, HyB<sup>R</sup>: hygromycin B resistant.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Other names</th>
<th>Cassette</th>
<th>CI activity</th>
<th>Fully assembled complex</th>
<th>700 kDa Subcomplex</th>
<th>Genetic analysis</th>
<th>Recombinant meiotic progeny (arg+ / HyB&lt;sup&gt;R&lt;/sup&gt;)</th>
<th>CI deficiency linked to cassette</th>
<th>Monogenic</th>
</tr>
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<tr>
<td>WT</td>
<td></td>
<td></td>
<td>100%</td>
<td>+++</td>
<td>-</td>
<td>Bulk</td>
<td>230 / 50</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>amc8</td>
<td>1H5</td>
<td>iHyg3</td>
<td>21%</td>
<td>++</td>
<td>-</td>
<td>Bulk, Tetrad</td>
<td>50 / 50</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>amc9</td>
<td>41D9</td>
<td>iHyg3</td>
<td>14%</td>
<td>-</td>
<td>-</td>
<td>Bulk, Tetrad</td>
<td>100 / 48</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>amc10</td>
<td>42A10</td>
<td>iHyg3</td>
<td>57%</td>
<td>+++</td>
<td>-</td>
<td>Bulk, Tetrad</td>
<td>100 / 100</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>amc11</td>
<td>10G11</td>
<td>iHyg3</td>
<td>21%</td>
<td>+</td>
<td>++</td>
<td>Bulk</td>
<td>85 / 85</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>amc12</td>
<td>6E9</td>
<td>iHyg3</td>
<td>40%</td>
<td>+</td>
<td>-</td>
<td>Bulk, Tetrad</td>
<td>112 / 51</td>
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<td>Yes</td>
</tr>
<tr>
<td>amc13</td>
<td>4C3</td>
<td>iHyg3</td>
<td>59%</td>
<td>+++</td>
<td>-</td>
<td>Bulk, Tetrad</td>
<td>85 / 85</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Although, the insertional mutagenesis approach has been popularly used in *Chlamydomonas* (126), the occurrence of unlinked mutations is not uncommon and has been previously reported. For instance, in a study by the Niyogi group (161), nine out of 17 photosynthetic mutants were not linked to the insertional cassette. However, only 2 out of 15 motility mutants, analyzed by the Lefebvre group, were not linked to their insertional cassette (162). The mutations that are not linked to the insertional cassette were proposed to be due to the integration of extracellular genomic DNA from lysed cells uptaken during electroporation (163), insertion of cleaved and non-functional pieces of the cassette, or insertion of the carrier DNA used as part of the electroporation protocol (154). So far, the expected frequency of linked mutations generated by insertional mutagenesis is not clear. Recently, a high-throughput insertional mutagenesis with subsequent screening and genotyping has been created by the Jonikas group (163). Phenotypic screening and detailed genetic analysis of such a large library will be more informative in accurately determining the expected frequency of linked vs unlinked mutations, in mutants created by insertional mutagenesis in *Chlamydomonas*.

Only 0.02% of 54,000 transformants screened from the forward genetic screen were Complex I mutants. The screen is still clearly not saturated, and the screening of a larger insertional mutant library, similar to the Jonikas group (163), will yield additional novel AMC loci. Also, the development of a screen to positively select for Complex I mutants or enrich for this class of mutants might increase the recovery of amc mutations. Although such a screen is not readily available for
Complex I mutants, a similar concept has been successfully applied for others. Enrichment for photosynthetic mutants has been achieved through the treatment of the mutagenized population with metronidazole (161,164). Metronidazole accepts electrons from reduced ferredoxin resulting in superoxide formation that negatively affects the viability of photosynthetically proficient strains, while allowing photosynthetically impaired mutants to survive. Such an enrichment has been shown to yield 50% of photosynthetically impaired mutants (164). Another example is TTC (2,3,5-triphenyltetrazolium chloride), which is converted to red formazan via the activity of the respiratory enzymes. This can be used as a colorimetric test to confirm functional Complex III and IV activity in *Chlamydomonas* in the dark via an overlay method (130).

The Complex I mutants isolated from the current screen display different levels of Complex I activity and assembly as determined by NADH: duroquinone oxidoreductase activities (Figure 2.1 B) and analysis via BN-PAGE and immunoblotting (Figure 2.2 A and B). Steady-state levels of the Complex I subunits were also assessed by SDS-PAGE immunoblotting analysis (Figure 2.2 C). The hydrophilic subunits analyzed by immunoblotting are the FMN containing 51 kDa subunit in the NADH oxidizing module, and the 49 kDa and TYKY subunits localized in the Q module close to the ubiquinone reduction center (165).

The *amc9* mutant is severely reduced in Complex I activity, has no detectable mature Complex I and also displays reduced levels of the 51 kDa soluble subunit (Figure 2.2). Hence, it is possible that the *amc9* mutant is blocked
for Complex I assembly at an early stage. On the other hand, the amc11 strain accumulates a labile 700 kDa subcomplex while displaying drastic reduction in Complex I activity. This phenotype is characteristic of mutants impaired for assembly of the distal membrane arm as observed in the nuclear mutants amc1, amc2, amc5-to-7 (131) and the mitochondrial nd4 and nd5 mutants (56). Specifically, loss of membrane arm subunits such as NUOB10 (in the amc5 mutant), ND4 (in dum24) and ND5 (in dum23) results in the accumulation of the 700 kDa subcomplex. The amc11 mutant also exhibits reduced accumulation of the 51 kDa, 49 kDa and TYKY subunits. Such pleiotropic reduction in subunits of the soluble arm has been previously observed in Complex I mutants unable to assemble the membrane arm (131). It is possible that failure to assemble the holoenzyme results in either reduced synthesis or increased degradation of soluble subunits.

The amc8, amc10, amc12 and amc13 strains assemble reduced levels of mature complexes, similar to previously characterized amc3 and amc4 mutants (131). In these mutants, the following explanations are possible: a) the reduced accumulation of Complex I subunits results in reduced levels of mature complex in amc4, amc8 and amc12, b) subunits accumulate to wild-type levels (as in amc10 and amc13), but their assembly is impeded by the absence of an unknown biogenesis factor, c) other untested subunits act as limiting factors for the assembly of Complex I, or d) a mutation in a subunit results in reduced activity, but does not block assembly of mature complex. Two categories of mutants
accumulating a mature complex have been isolated from these two screens: i) amc4, amc8 and amc12, which display lower accumulation of 49 kDa, 51 kDa and TYKY subunits and, ii) amc3, amc10 and amc13 which are characterized with partial Complex I deficiency and accumulate wild-type levels of these subunits.

In conclusion, the forward genetic screen has successfully yielded 13 Complex I mutants. While the amc9 strain seems to be blocked for early stages of Complex I assembly, the amc11 mutant is blocked in the later stages of assembly where the final hydrophobic subunits are incorporated to complete the holoenzyme. On the contrary, amc8, amc10, amc12 and amc13 are still able to assemble a mature complex albeit to reduced levels, compared to wild-type. Further characterization of the amc9, amc11 and amc12 mutants will be described in Chapters 3, 4 and 5 respectively.
CHAPTER 3

CHLAMYDOMONAS MUTANTS HAVE MOLECULAR LESIONS IN NUCLEAR GENES ENCODING COMPLEX I SUBUNITS.

3.1. Introduction

A forward genetic screen was conducted to isolate nuclear Complex I mutants. The first screen, described in Barbieri et al, 2011 (131), led to the isolation of seven Complex I mutants, referred to as assembly of mitochondrial complex I (amc1-to-7). Among the seven mutants, genetic analysis revealed that only the amc5 mutation was linked to the insertional cassette and that amc5/7 were allelic. Molecular analysis of amc5 conducted by TAIL-PCR, mapped the insertional cassette to intron 3 of the NUOB10 gene encoding the PDSW subunit of Complex I. The insertion was accompanied by a deletion of the NUOB10 genomic sequence, downstream of the site of insertion. NUOB10 is a non-core subunit of the membrane arm. Loss of NUOB10 in amc5 and amc7 is characterized by the accumulation of a 700 kDa subcomplex, a trait of mutants impaired for membrane arm assembly (2,56). However, the identity of the AMC5 locus could not be
confirmed by complementation with the wild-type NUOB10 gene, because the initial attempts at transforming the amc5 strain were unsuccessful (131).

In the second screen (described in Chapter 2), six novel Complex I mutants amc8-to-13 were isolated. This chapter deals with the molecular characterization of the amc9 mutant, which displays severely reduced rotenone-sensitive NADH: duroquinone oxidoreductase activity (Figure 2.1). Analysis via BN-PAGE and subsequent in-gel Complex I staining revealed no active Complex I, and immunoblotting experiments failed to detect assembled complexes or subcomplexes in this mutant (Figure 2.2). This loss of Complex I assembly could be due to mutations in structural subunits of Complex I or biogenesis factors. For example, in humans, mutations in the assembly factor C20ORF7 results in failure to accumulate the initial ~400 kDa assembly intermediate (35) and hence, no subcomplexes are detected. Therefore, we were interested in identifying the gene defining the AMC9 locus and delineating the cause of the Complex I assembly defect. This chapter describes the genetic analysis of the amc9 mutant and the molecular identification of the AMC9 locus. In addition, we report the successful nuclear transformation of Complex I mutants.
3.2. Experimental Procedures

3.2.1 Strains and Culture Conditions

*Chlamydomonas* strains were grown in liquid or solid medium at 25°C, in continuous light at 50 µE.m\(^{-2}\).s\(^{-1}\) (1). Four types of media were used: Tris-Acetate-Phosphate (TAP), TAP supplemented with (400 µg/mL) arginine (TARG), TARG supplemented with 25 µg/ml hygromycin B (TARG+HyB), or TARG supplemented with 25 µg/ml paromomycin (TARG + Pm). Strains described in Chapter 2 were used here. In addition, the wild-type strain 141 (\(mt^+\), \(arg9\)-2) was also utilized in genetic crosses (166).

3.2.2 Genetic Analysis

Genetic crosses were conducted as described in Section 2.2.8. For easy selection of *amc9/amcx* diploids, *amc9* spores with different selectable markers were generated by the following cross. The original *amc9* strain 41D9 (\(mt\), *aph7*", \(arg7\)-8) was crossed with the Complex I proficient strain 141 (\(mt^+\), \(arg9\)-2) to yield meiotic zygotes. These zygotes were germinated on TAP + HyB solid medium in high light at 25°C, to select for recombinant meiotic progeny that is arginine prototrophic and hygromycin B resistant. One such spore *amc9* (3) (\(mt^+\), *nuo5::aph7"*) was back crossed with 141 (\(mt^+\), \(arg9\)-2) to obtain meiotic zygotes that were subsequently used for tetrad dissection and bulk germination. For tetrad dissection, the meiotic zygotes were transferred to TARG solid media and the germinated tetrads were dissected within 16-24 h of transfer. For bulk germination, the zygotes were transferred to TARG + HyB solid media to allow for selection of
spores that carry the iHyg3 cassette. The relevant phenotypes of spores obtained from tetrad dissection and bulk germination were deduced by replica-plating as described in Section 2.2.3. The following haploid spores (nuo5::aph7\(^{7}\), arg9-2) were then selected for use in constructing amc9 diploids: amc9 (8B\(^{+}\)), amc9 (3A\(^{+}\)) and amc9 (23\(^{+}\)).

amc9/amcx diploids were constructed as described in Table 3.1. Individual diploids were subcloned to a single colony and their mating type was determined by diagnostic PCR, to confirm diploidy (159).

3.2.3 Nucleic acid extraction

Genomic DNA was extracted from *Chlamydomonas* by phenol-chloroform method (158). One or two loops of cells, grown for two to three days in continuous light at 50 µE.m\(^{-2}\).s\(^{-1}\) on TARG solid medium, was harvested and resuspended in buffer (10 mM Tris HCl, pH 8, 10 mM EDTA, 10 mM NaCl, 15% (w/v) glycerol). Cells were lysed by sonication for 5 sec at 9 watts output. Proteins were degraded by treatment with 100 µg proteinase K and the extract was incubated at 55 °C for one hour. RNA was degraded with 50 µg RNase A. Nucleic acids were extracted twice by phenol/ chloroform and DNA was precipitated by adding 2.2 volumes of ethanol and 0.1 volume of 3 M Na acetate pH 5.5.

For semi-quantitative RT-PCR of the NUO5 transcript, RNA extraction was conducted by TRIzol (Invitrogen) according to the manufacturer’s protocol with a few modifications. Cells were grown for two or three days (on solid TARG medium for strain 4C\(^{-}\) and TARG + HyB for the strains amc9 and amc9 (NUO5) strains) in
continuous light 50 µE.m².s⁻¹ at 25°C. Cells (100-200 mg) were harvested and resuspended in 500 µl of sterile water, vortexed and pelleted at 16,873 g for 5 min. The pellet was resuspended in 750 µl of TRIzol, and incubated for 5 min at 22 ºC, following which 0.2 ml of chloroform was added, mixed vigorously by shaking and incubated for 2-3 min at 22 ºC. Phase separation was achieved by centrifuging the samples (18,001 g, 15 min) at 4°C. The supernatant was subjected to a second chloroform extraction (equal volume) to remove traces of phenol. RNA was precipitated with 0.75 ml of isopropanol and pelleted at 4°C (18,001 g, 10 min). The pellet was washed with 70% ethanol and resuspended in RNase-free water.

For real-time quantitative PCR of the NUOB10 cDNA, RNA was prepared using the Isolate II Plant RNA Kit (Bioline). Cells (100 mg) grown for 2-3 days on TARG solid medium, with 25 µg/ml paromomycin for amc5 and amc5 (NUOB10), was harvested. Cells were lysed by vortexing with 9/10th volume of glass beads for 5 min at 22 ºC. RNA extraction was completed according to manufacturer’s protocols.

3.2.4. Diagnostic PCR and TAIL-PCR

For diagnostic PCR analysis, GoTaq Polymerase (Promega) was used, with addition of 2.5% (v/v) DMSO and 1 µM each of forward and reverse primers for standard reactions. Note that a denaturation temperature of 98ºC (instead of 95ºC) was used for Chlamydomonas genomic DNA / cDNA templates. For sequencing analyses, PCR products were gel-purified using the NucleoSpin Gel Extraction Kit (Clontech) as per the manufacturer’s instructions. The purified PCR product was
then cloned into pGEM-T Easy Vector Systems (Promega) and then sequenced with T7 and SP6 primers flanking the cloning site. The sequence of the primers used for diagnostic PCR is provided in Table 3.2.

TAIL-PCR (Thermal Asymmetric Inter-Laced PCR) was used to identify the sequence flanking the iHyg3 cassette in the amc9 mutant as in (167). The partially degenerate primer AD1 (5′-NTCASTWTSGW TT-3′) was used for TAIL-PCR (167,168). The following iHyg3-specific primers, APH7R3 (5′-AGAATTCCTGTCGTTCCGCAG-3′), APH7R4 (5′-TAGGAATCATCCGAATCAATACG-3′) and APH7R5 (5′-CGGTCGAGAAGTAACAGG-3′) were used for the primary, secondary and tertiary PCR reactions respectively. Similar reactions were conducted using wild-type genomic DNA and purified iHyg3 cassette to identify non-specific amplification of DNA.

3.2.5. Semi-quantitative and real-time quantitative PCR.

For semi-quantitative RT-PCR of the NUO5 transcript, 10 µg of TRIzol extracted RNA was treated with RQ1 RNase-free DNase I (Promega). Reverse transcription was achieved with 800 units of M-MLV Reverse transcriptase (Life Technologies) using 500 ng of Random Hexamers (Promega), following the manufacturer's protocol. Semi-quantitative PCR was conducted using GoTaq Polymerase (Promega) as described in Section 3.2.4. Two-fold dilutions of the cDNA template (cDNA amount equivalent to 400 ng, 200 ng, 100 ng and 50 ng of input RNA) were subjected to PCR amplification for 35 cycles. A region of the
NUO5 cDNA was amplified across the site of insertion using the primer pair NUO5E2L2 / NUO5E3R and another region downstream of the insertion site was amplified using the primers NUO5E4L / NUO5E5R (Table 3.2). CBLP, used as the reference, was amplified using the primer pair CBLP-F (5′-ATGTGCTGTCCGTGGCTTTC-3′) and CBLP-R (5′-CAGACCTTGACCATCTTTGTCCTC-3′) (169).

For real-time quantitative PCR, 2 µg of RNA was treated with RQ1 RNase free DNase I (Promega). Reverse transcription was achieved with 400 units of M-MLV Reverse transcriptase (Life Technologies) using 250 ng of Random Hexamers (Promega), following the manufacturer’s protocol. cDNA equivalent to 50 ng of total input RNA, was used as template for real-time quantitative PCR (qPCR) using SensiMix (Bioline) on a Mastercycler ep realplex thermocycler (Eppendorf). The primers NUOB10E4L / NUOB10E4R were used to amplify the NUOB10 mRNA and the transcript abundance of CBLP, a house keeping gene, was determined using the primers CBLP-F / CBLP-R described in Section 3.3.12. Transcript abundance of E3 ubiquitin ligase UBI (Cre03.g159200) was determined by using primers Ubiupper (5′-GTACAGCGCGCGGTACAGGCAC-3′) and Ubilower (5′-AGCGTCAGCGCGCGGTACAGGCAC-3′) (170). Primer efficiencies for primers binding to NUOB10, CBLP, UBI, determined by calibration curves, were 100%, 108% and 93% respectively. Three independent biological replicates were performed, each analyzed using three technical replicates. qPCR reactions were denatured at 98°C and annealed at 60°C. Relative fold change in
amc5 and amc5 (NUOB10) mutants was calculated by normalizing to the average of the isogenic WT (3A⁺) strain (set to 1.0).

3.2.6. Plasmid Construction

The pSL18 plasmid (171), containing the aph8” gene conferring paromomycin resistance under the control of RBCS2/HSP70A promoter and the RBCS2 terminator, was used as the vector. The cloned NUO5 cDNA (Accession AV639079) was obtained from the Kasuza EST database (172). The pSL18 vector was digested with restriction enzymes Ndel and Notl leading to the excision of the PSAD terminator (609 bp). The 5.519 kb fragment of the digested vector was purified for cloning. The NUO5 ORF and 3’ UTR were amplified from the NUO5 cloned cDNA, using primers IPNUO5 (5’-CTGCTACTCAACAACAAGCCCATATGCTGTCTAGG-3’) and IPNUO5R (5’-GCTCCACCGCGGTGGCGGCCGTCGTGTGGCCTCCATTC-3’), with the high fidelity Pfu Turbo DNA Polymerase (Agilent). These primers provide 20 bp ends overlapping with the digested vector. Cloning of the amplified PCR product was achieved using the In-Fusion HD Cloning Kit (Clontech) according to the manufacturer’s protocol.

3.2.7. PCR-based screening of Chlamydomonas genomic library

An ARG7-based indexed cosmid library of Chlamydomonas genomic DNA, created by Dr. Jean David Rochaix’s laboratory (University of Geneva, Switzerland) (173), was screened for the presence of cosmids carrying the NUO5 and NUOB10 genes by diagnostic PCR. The NUO5 containing cosmid (referred to
as 9A2) was identified using the primer pairs NUO5 E1L / NUO5 E2R and NUO5 E2L / NUO5 E3R (Table 3.2). The NUOB10 containing cosmid (referred to as cosmid 7D10), was identified using the primer pairs NUOB10E1L / NUOB10E4R (Table 3.2). The region of Chlamydomonas genomic DNA inserted into these cosmids was sequenced to confirm the presence of the gene of interest.

3.2.8. Biolistic Transformation

The recipient arginine auxotrophic strains amc9 (41D9) (mt, nuo5::aph7”, arg7-8) or amc5 (87D3) (mt*, nuob10::aph8, arg7-8) were subjected to biolistic transformation using a homemade particle delivery device. The ARG7 gene in the cosmids 9A2 and 7D10 was used as a selection marker. The strains were grown in liquid TARG medium for 2-3 days until they reached exponential phase (3 – 6 x 10^6 cells/mL). The cells were plated on selective TAP medium at 10^8 cells/plate. For each bombardment, DNA was coated on sterile 0.6-0.9 μm tungsten particles (STREM Chemicals, Newburyport, MA, USA, # 93-7437) by using 2 μg of cosmid, 0.1 M Spermidine and 2.5 M CaCl₂. The bombardment was conducted at a helium pressure of 250 psi and vacuum of 27.5 inches Hg. The plate was positioned 10.5 cm away from the nozzle containing the coated particles. The bombarded plates were first incubated at low light overnight for recovery and then transferred to continuous high light (50 μE.m².s⁻¹).

For transforming the amc9 (41D9) (mt, arg7-8) strain with the plasmid containing the NUO5 cDNA, the same biolistic protocol was used, except the transformants were selected on TARG + paromomycin (20 μg/mL).
3.2.9. Growth curves

Liquid cultures were inoculated with a starting cell density of $10^5$ cells / ml in 50 mL TARG cultures. For each strain, three biological replicates were inoculated in continuous light at 50 µE.m$^{-2}$.s$^{-1}$ and in the dark. Cell density was evaluated every 8 h by measuring optical density at A$_{750}$, over a period of 10 days. We experimentally confirmed that the optical density at 750 nm accurately reflects cell number (data not shown). Growth rate $\mu$ was calculated as $3.3^{\ast}[\log_{10}N - \log_{10}N_0] / (t_N - t_0)$, where N is the final cell density at time $t_N$ and $N_0$ is the initial cell count at time $t_0$. The generation time was calculated as $1/ \mu$.

3.2.10. Biochemical analyses and Ten-Fold Dilution series

Ten-fold dilution series was conducted as described in Section 2.2.4. Complex I activity measurement was done as detailed in Section 2.2.5. Blue-Native PAGE and *in-gel* activity assays were conducted as reported in Section 2.2.6 and SDS-PAGE and immunoblotting analysis was completed as elaborated in Section 2.2.7.
Table 3.1. Construction of amc9/amcx diploids.

The mating type minus (mt⁻) strains were crossed with mating type plus (mt⁺) strains to obtain diploids with specific phenotypes for selection. amc9 diploids were generated by using either the original amc9 (41D9) strain or meiotic progeny of the original strain, such as amc9 (8B⁺), amc9 (3A⁺) or amc9 (23⁻).

Key: arg+: arginine prototrophs; arg-: arginine auxotrophs; HyB R: hygromycin B resistant; HyB S: hygromycin B sensitive; Pm R: paromomycin resistant. All arginine auxotrophic strains have the arg7-8 mutation, except for amc9 (8B⁺), amc9 (3A⁺) and amc9 (23⁻) strains that harbor the arg9-2 mutation.

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<th>Strain</th>
<th>Phenotype</th>
<th>mt⁺</th>
<th>Strain</th>
<th>Phenotype</th>
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<td>amc9 (23⁻ arg9-2)</td>
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Table 3.2. Sequences of primers used in Chapter 3.

The sequences of primers specific for target genes is provided.
3.3. Results

3.3.1. *The AMC9 locus defines a 7th complementation group*

To determine if the *amc9* mutation was allelic to the mutations in the *amc1*-to-13 mutants, *amc9/amcx* diploids were obtained as described in Table 3.1. Two independent diploids from each cross were tested for growth in the dark by ten-fold dilution series. Since both behaved the same, only one representative diploid is shown for majority of the crosses (Figure 3.1). All *amc9/amcx* diploids were restored for growth in the dark, thereby indicating that *AMC9* defines a locus distinct from *AMC1-12*. However, the *amc9/amc13* diploids were not complemented (Figure 3.1, panel k). To confirm this phenotype, two additional *amc9/amc13* diploids, obtained from an independent cross (Table 3.1), were tested and all displayed slow growth in the dark similar to *amc13*. Analysis by BN-PAGE and *in-gel* Complex I activity assay (Figure 3.2 A) revealed that the *amc9/amc13* diploid assembles a mature complex, similar to *amc13*, but still exhibits Complex I deficiency as determined by NADH: duroquinone oxidoreductase activity (Figure 3.2 B). On the contrary, *amc1/amc9* and *amc2/amc9* diploids are clearly restored for growth in the dark (Figure 3.1: panels a, b) and Complex I assembly (Figure 3.2 A). Further genetic analyses need to be conducted to determine the cause of non-complementation between *amc9* and *amc13*. 
Figure 3.1. Genetic complementation reveals that the amc9 mutation is not allelic to the mutations in amc1-to-12.

The amc9/amcx diploids were generated as described in Table 3.1. One diploid obtained from each cross was tested for complementation of growth in the dark by ten-fold serial dilution. The dilution series were plated on acetate containing medium and incubated in the light or dark for 16 days. Only two dilutions are shown here. The growth of the following diploids are depicted in panels a) to k). WT (4C-) and the respective amc mutants, used for constructing the diploids were used as controls. In panels c, d and h, the amc9 strain shown is amc9 (41D9): a) amc1xamc9, b) amc2xamc9, c) amc3xamc9, d) amc4xamc9, e) amc6xamc9, f) amc7xamc9, g) amc8xamc9, h) amc10xamc9, i) amc11xamc9, j) amc12xamc9, k) amc13xamc9.
Figure 3.2. *amc9* is not genetically complemented by *amc13*.

A. Blue-Native PAGE was conducted on 200 µg of partially purified membrane fraction and *in-gel* Complex I activity was detected by NBT staining. B. Complex I (rotenone-sensitive NADH: dehydrogenase) activity was determined with partially purified membranes. The activities are represented as the average of three biological replicates with the error bars indicating standard deviation of the mean. Two independent *amc9/amc13* diploids from a single cross (see Figure 3.1 and Table 3.1) were tested here and they both display significantly reduced CI activities compared to WT, as determined by two-tailed unequal variances t-test. ** indicates p<0.01 and *** indicates p<0.001.

In both A and B, the WT strain shown is 4C and the *amc* mutants shown are the parental strains used for constructing the diploids as described in Table 3.1.
3.3.2. *The amc9 mutation is monogenic and linked to the insertional cassette*

In Chapter 2, we reported that the *amc9/+* diploids were restored for growth in the dark, thereby confirming that the *amc9* mutation is recessive with respect to the wild-type allele. In order to test if the *amc9* mutation is monogenic and linked to the insertional cassette, the transmission of the *sid* phenotype was monitored in the meiotic progeny of an *amc9* × *WT* cross via tetrad analysis. In each of the seven tetrads, a 2:2 segregation of the iHyg3 cassette (*aph7” gene*) and *sid* phenotype (*amc9* mutation) was observed, confirming Mendelian inheritance of each trait. The 2:2 segregation of the *sid* phenotype of two representative tetrads, tested by ten-fold dilution series, is shown in Figure 3.3 A. This 2:2 segregation of the *sid* phenotype confirms that the *amc9* mutation is monogenic. In all the tetrads, we observed that the *sid* phenotype always segregated with the HyB resistance phenotype (data not shown), an indication that the *amc9* mutation is linked to the insertional iHyg3 cassette harboring the *aph7” gene*. To further substantiate this result, recombinant HyB<sup>R</sup> spores obtained from bulk germination of meiotic zygotes, issued from the same genetic cross, were tested for growth in the dark. Out of 50 HyB<sup>R</sup> spores that were tested, all of them displayed a *sid* phenotype (Figure 3.3 B), confirming that the *amc9* mutation is tightly linked to the insertional cassette.
Figure 3.3. The *amc9* mutation is monogenic and linked to the insertional cassette.

Meiotic zygotes were obtained by crossing the Complex I proficient strain 141 (*mt*\(^+\) arg9-2) with the *amc9* (3\(^-\)) strain (*mt*, *amc9*). The meiotic zygotes, obtained from the same genetic cross, were used for both tetrad dissection (A) and bulk germination (B).

**A.** The growth phenotype of two out of seven tetrads dissected from the above cross are shown here by ten-fold dilution series. The dilutions were plated on acetate containing medium and incubated in the light and in the dark for 15 days. The WT strain and the *amc9* strain shown here are the original parental strains. **B.** Fifty recombinant hygromycin B resistant spores, were analyzed from bulk germination. The spores were replica-plated on TARG solid medium in the light or dark and the growth phenotype was scored after 15 days of incubation. The ratio of arginine prototrophic: auxotrophic spores was 20:30. The WT and *amc9* strains shown here are 4C:\(^-\) and *amc9* (41D9), respectively.
3.3.3. *The AMC9 locus is defined by the NUO5 gene*

The tight linkage between the insertional cassette and the *sid* phenotype in the *amc9* mutant suggests that the latter could be caused by integration of the cassette in a gene controlling Complex I (Figure 3.3). Therefore, to identify the disrupted gene in the *amc9* mutant, we sought to recover the sequence flanking the insertional cassette via TAIL-PCR (167) as described in Section 3.2.4. The insertional cassette was mapped to exon 1 of the *NUO5* gene, which encodes a Complex I subunit (Figure 3.4 A). The location of the insertion was confirmed by amplification of the genomic region spanning the insertion site using primer pairs NUO5E2L / NUO5E3R. A band of expected size (715 bp) was amplified in the WT and not in the *amc9* mutant (Figure 3.4 B).

The insertion of the full iHyg3 cassette was confirmed by amplifying across the *NUO5/iHyg3* junctions in *amc9*. Primer pairs amplifying from the 5′-end of *aph7"* gene with the *TUB2* promoter (NUO5E2L / APH7-R5) and 3′-end of *aph7"* gene with the *RBCS2* terminator (APH7-F8 / NUO5E3R) yielded products of expected size, 524 bp and 955 bp respectively, in *amc9* but not in WT (Table 3.2, Figure 3.4 B). Diagnostic PCRs conducted upstream and downstream of the insertion site amplified wild-type PCR products (Figure 3.4 B and data not shown). These results confirm the insertion of a full-sized iHyg3 cassette into exon2 of the *NUO5* gene with no other major rearrangements in the *amc9* mutant. Thus, *amc9* has a molecular lesion in the *NUO5* gene that encodes the 24 kDa subunit of Complex I.
Figure 3.4. The *NUO5* gene encoding the 24 kDa Complex I subunit is disrupted in the *amc9* strain.

A. The position of the insertional cassette in the *NUO5* gene of the *amc9* mutant is indicated. The gray rectangles indicate the 5’ and 3’ UTRs of the gene. The blue bars indicate regions of the exons corresponding to the coding sequence and the thin lines represent introns. The yellow arrow indicates the extent of the genomic sequence retrieved by TAIL-PCR, flanking the cassette insertion site. The cassette is in the sense orientation with respect to the *NUO5* gene. Brown arrows indicate the iHyg3 specific primers that were used for TAIL-PCR (APH7R3, APH7R4, APH7R5) and for diagnostic PCR (APH7F8). Black arrows indicate the *NUO5*-specific primers. B. Diagnostic PCR confirms the location of the iHyg3 cassette in the *amc9* mutant. DNA amplification across the insertional site (NUO5 E2L/NUO5 E3R) occurs only in the WT (4C-) and not in the *amc9* (41D9) strain. On the other hand, amplification of the genomic sequence flanking the cassette occurs only in the *amc9* mutant using primer pairs (NUO5 E2L/APH7R5) and (APH7F8/NUO5 E3R).
3.3.4. The amc mutants can be transformed by biolistics

Electroporation and glass bead transformation methods have been successfully used for complementation of *Chlamydomonas* photosynthetic and flagellar mutants (162,174,175). However, initial attempts to transform *amc1*, *amc2* and *amc5* mutants with wild-type genes were unsuccessful (153). Although, mitochondrial Complex III mutants could be transformed by electroporation, mitochondrial and nuclear Complex I mutants were particularly resistant to transformation by both electroporation and glass beads for unknown reasons (131,153). For instance, the transformation of the wild-type strain by electroporation to generate the insertional mutants for this forward genetic screen yielded an average of 120 colonies per 100 ng of DNA. On the other hand, ten independent transformations of *amc9* with 2 µg of DNA, by electroporation, resulted in no transformants in 9 cases, and only 3 transformants in one case. These results further confirm that Complex I mutants are recalcitrant to traditional methods of nuclear transformation.

Hence, transformation by biolistic gun was attempted as an alternative. Although biolistic transformation is regularly used for chloroplast and mitochondrial transformation in *Chlamydomonas* (139,140), this method has also been previously used for nuclear transformation (161,162,176,177). Biolistic transformation of *amc* mutants yielded as many as 44 transformants per µg of transforming DNA. The following parameters were standardized for high efficiency transformation: amount of transforming DNA (2 µg), distance between the DNA-
containing nozzle and target cells (10.5 cm), and selection for complemented transformants in high light, low light and the dark. Although the selection in the dark yielded 63% of complemented transformants compared to 14-20% in high light, the selection time in the dark was nearly one month compared to 10 days in the light. Hence, for rapid screening, selection of transformants in high light was pursued.

3.3.5. Transformation of amc9 with NUO5 cDNA leads to partial complementation

Functional complementation of Chlamydomonas photosynthetic mutants has been previously accomplished in our laboratory by transforming with the cDNA of the gene of interest (174,178). Hence, a similar approach was undertaken to complement the amc9 mutant. The sequence of the NUO5 ORF was cloned under the control of the PSAD promoter, in the pSL18 vector containing aph8” gene as a selection marker. The plasmid was introduced into amc9 (41D9) (mt-, arg7-8) strain by biolistic gun and transformants were selected on TARG + paromomycin solid medium.

Out of 259 transformants that were screened for restoration of growth phenotype by replica-plating, only one transformant showed faster growth in the dark compared to the amc9 mutant (data not shown). One possibility is that, the transformants integrated the selection marker but not the NUO5 cDNA. However, diagnostic PCR of the transformants revealed the presence of the transforming NUO5 cDNA in majority of the transformants. Thus, although the transforming cDNA was successfully inserted, the efficiency of complementation was very poor.
Figure 3.5. Expression of the NUO5 cDNA in amc9 leads to partial complementation of Complex I deficiency.

Complementation of the amc9 mutant with NUO5 cDNA was attempted by biolistics. Only one single transformant from more than 200 colonies, referred to as amc9 (NUO5), showed faster growth in the dark compared to amc9. The WT and amc9 strains shown here are 4C and amc9 (41D9). A. The growth phenotype of the transformed amc9 (NUO5) strain was tested by ten-fold dilution series. The dilution series was plated on acetate containing medium and incubated in the light or dark for 21 days. B. Complex I (rotenone-sensitive NADH: dehydrogenase) activity was determined using partially purified membranes. The activities are represented as the average of three biological replicates with the error bars indicating standard deviation of the mean. The amc9 (NUO5) strain is partially complemented for Complex I activity, although still significantly decreased compared to WT as determined by two-tailed unequal variances t-test. *** indicates a p value = 0.000372 and ** indicates a p=0.004583. C. Blue-Native PAGE was conducted on 200 µg of partially purified membranes. In-gel Complex I activity was detected by NBT staining. In-gel ATPase activity was conducted as a control for protein loading.
Further phenotypic characterization of the single *amc9* transformant, displaying faster growth in the dark in the initial tests by replica plating, revealed that this transformant is only partially restored for growth in the light (Figure 3.5 A), partially complemented for Complex I activity (Figure 3.5 B) and displays low levels of assembled Complex I as detected by BN-PAGE *in-gel* activity (Figure 3.5 C). These results are a qualitative confirmation that the *NUO5* cDNA, although integrated into the *amc9* nuclear genome, is not being efficiently expressed.

3.3.6. *Transformation of amc9 with NUO5 genomic DNA results in full phenotypic complementation.*

Transformation of the *NUO5* cDNA did not result in full restoration of Complex I proficiency, due to inefficient expression. Hence, transformation of the *amc9* strain with wild-type genomic DNA containing the *NUO5* gene was attempted. The *amc9* (41D9) (*mt*, *arg7*-8) strain was transformed with an *ARG7*-containing cosmid carrying the *NUO5* gene and transformants were selected based on the restoration of arginine prototrophy. Out of 461 transformants that were screened by replica-plating, 80 transformants were restored for growth in the dark. Amongst the 80 transformants, 16 were tested for growth in the dark by ten-fold dilution series (data not shown) and all displayed restoration of growth phenotype. Four transformants (out of 16) were tested for NADH: duroquinone oxidoreductase activity (data not shown) and all of them showed complete restoration of Complex I activity. One of these four transformants was chosen for further analyses and shall be referred to as *amc9 (NUO5)*.
Diagnostic PCR of the *amc9 (NUO5)* transformant revealed the presence of the wild-type *NUO5* gene, in addition to the endogenous mutant *NUO5* gene (Figure 3.6 A). Semi-quantitative RT-PCR (Figure 3.6 B) showed that the *amc9* mutant lacks transcript spanning the insertion site (amplified by primers NUO5 E2L2 / NUO5 E3R) resulting in loss of wild-type *NUO5* transcript. These transcript levels are restored upon complementation. Interestingly, the *amc9* mutant is apparently producing a chimeric *NUO5* transcript downstream of the insertion site. It is possible that the chimeric transcript originates from the 3'-end of the cassette or downstream of the cassette. Generation of chimeric transcripts from the 3'-end of the insertional cassette has been previously reported in *Arabidopsis* T-DNA lines (179). However, this chimeric transcript does not produce a functional 24 kDa subunit as evidenced by loss of Complex I activity and assembly in the *amc9* strain (Chapter 2).

The restoration of growth phenotype in the *amc9 (NUO5)* strain was demonstrated by assessing growth in liquid and solid medium (Figure 3.7). The *amc9* mutant clearly displayed a slower growth rate in the dark, compared to wild-type, whereas, the *amc9 (NUO5)* transformant exhibited a growth rate similar to that of wild-type (Figure 3.7 A, right panel). The generation time in the dark for wild-type and *amc9* is 17.5 h and 52 h, respectively. Upon complementation, the *amc9 (NUO5)* strain displays a wild-type like growth in the dark as evidenced by the generation time of 16.8 h (Figure 3.7 B) and ten-fold dilution series (Figure 3.7 C).
The amc9 strain was transformed with a cosmid containing the NUO5 gene by biolistics. The molecular analysis of one transformant [amc9 (NUO5)] is represented here. The WT and amc9 strains shown here are 4C + and amc9 (41D9) respectively. A. A diagram of the NUO5 gene with the approximate position of the insertional cassette in the amc9 mutant is depicted here. The brown arrows indicate primers APH7R5 and APH7F8 that bind to the aph7" gene in iHyg3 cassette. The black arrows represent primers that are specific for the NUO5 gene: NUO5E1L, NUO5E2R, NUO5E2L, NUO5E3R, NUO5E4L and NUO5E5R. B. Diagnostic PCR of NUO5 gene in amc9 indicates that amc9 is interrupted in exon 2 of NUO5 and that there are no major genomic rearrangements before or after the site of insertion. The amc9 (NUO5) strain contains a wild-type copy of NUO5 gene as expected. C. Semi-quantitative RT-PCR analysis shows restoration of NUO5 transcript levels in the complemented strain. PCR was conducted with primers that amplify across the insertion site (NUO5E2L2 / NUO5E3R). The faint lower molecular weight band, observed in the amc9 strain using the primer pair (NUO5E2L2 / NUO5E3R), are primer-dimers formed in the absence of amplification. PCR was conducted to amplify transcript downstream the insertion site, using the primer pair (NUO5E4L / NUO5E5R). PCR amplification was performed on two fold dilutions of the total cDNA (Gray triangles represent dilutions of cDNA). CBLP, a housekeeping gene, was used as a reference gene. All primer sequences are detailed in Table 3.2.
Figure 3.7. The wild-type NUO5 gene restores heterotrophic growth to the amc9 mutant.

A. The growth of WT, amc9 and amc9 (NUO5) was recorded by measuring optical density at 750 nm, in the light or in the dark, over a period of 10 days in light or in the dark. The average of three biological replicates is reported here, with error bars indicating standard deviation of the mean. B. The average generation time for each strain calculated from growth curves in A is indicated here. The error bars represent standard deviation of the mean. C. Restoration of the growth phenotype in amc9 (NUO5) was confirmed by ten-fold dilution series plated on acetate containing medium and incubated in the light for seven days and in the dark for 16 days. In sections A, B and C, the WT and the amc9 strain used were 4C- and amc9 (41D9) respectively.
Figure 3.8. The *amc9* mutant, complemented by the *NUO5* gene, is restored for Complex I activity and assembly.

**A.** Complex I (rotenone-sensitive NADH: duroquinone oxidoreductase) activity was determined with partially purified membranes. The activities are represented as an average of three biological replicates with the error bars indicating standard deviation of the mean. The *amc9* mutant displays a significant reduction in Complex I activity with respect to WT, as determined by two-tailed unequal variances *t*-test with a *p* value = 0.03. The *amc9* (*NUO5*) strain is rescued for Complex I activity. While there is no significant difference between activities measured for the WT and *amc9* (*NUO5*) strains, there is a significant difference between *amc9* and *amc9* (*NUO5*) with a *p* = 0.019. **B.** Blue-Native PAGE was conducted on 200 µg of partially purified membrane fraction. *In-gel* Complex I activity was detected by NBT staining. **C.** SDS-PAGE Immunoblotting was conducted on 10 µg of partially purified membranes using polyclonal antibodies to detect soluble arm CI subunits: α- 49 kDa, α- 51 kDa, α- TYKY. α- cyt f was used to confirm equal loading.
Further biochemical analyses of \textit{amc9 (NUO5)} revealed wild-type levels of NADH: duroquinone oxidoreductase activity (Figure 3.8 A), Complex I assembly (Figure 3.8 B) and steady-state levels of the Complex I subunits (Figure 3.8 C). From these results, we concluded that the \textit{AMC9} locus corresponds to the \textit{NUO5} gene encoding the 24 kDa Complex I subunit.

3.3.7. \textbf{Complementation of the amc5 mutant with the NUOB10 gene.}

We undertook the same approach for transforming the \textit{amc5} strain, which was previously unsuccessful (131). The \textit{amc5 (87D3) (mt\textsuperscript{+}, arg7\textsuperscript{-})} strain was transformed with an ARG7-based cosmid containing the \textit{NUOB10} gene (see Section 3.2.7), and arginine prototrophic transformants were selected. Out of 318 transformants, 104 transformants were restored for growth in the dark. Five independent transformants showed complete restoration of NADH: duroquinone oxidoreductase activity (data not shown). One of these five transformants was chosen for further analyses and shall be referred to as \textit{amc5 (NUOB10)}.

Diagnostic PCR of the \textit{amc5 (NUOB10)} transformant revealed the presence of the wild-type \textit{NUOB10} gene, in addition to the interrupted \textit{NUOB10} locus in the \textit{amc5} strain (Figure 3.9 A and B). Real-time qPCR (Figure 3.6 C and D) confirmed the loss of the wild-type \textit{NUOB10} mRNA in the \textit{amc5} mutant, which is restored in the \textit{amc5 (NUOB10)} strain. Interestingly, the \textit{amc5 (NUOB10)} strain is expressing five-fold increase in the \textit{NUOB10} transcript with respect to wild-type. This increased expression of \textit{NUOB10} mRNA could be either due to multiple integrated copies of the cosmid or a positional effect of the integrated copy, resulting in higher
expression. The amc5 (*NUOB10*) strain displays wild-type levels of Complex I activity (Figure 3.9 E) and also displays wild-type growth in the dark as evidenced by growth curves (Figure 3.10 A and B) and ten-fold dilution series (Figure 3.10 C). Interestingly, both amc9 and amc5 mutants require a slightly longer generation time even in the light (16 h for amc9 vs 12 h for WT) (Figure 3.7 and 3.10). This observation is in accordance with previous reports where some Complex I mutants showed a slight reduction in mixotrophic growth (light + acetate containing medium) (2). The reason for this is not known as Complex I mutants in *Chlamydomonas* do not exhibit reduced photosynthetic capacity (180). In conclusion, the AMC5 locus has been confirmed to correspond to the *NUOB10* gene encoding the PDSW Complex I subunit.
Figure 3.9. The amc5 mutant is complemented with the NUO5 gene.

The amc5 strain was transformed with a NUOB10 containing cosmid by biolistics. The phenotypic rescue in one transformant amc5 (NUOB10) is represented here. The WT and amc5 strains shown here are 3A* and amc5 (87D3), respectively. A. A diagram of the NUOB10 gene with the position of the insertional cassette in the amc5 mutant is depicted here. The gray and red rectangles represent UTRs and the coding sequence of NUOB10, respectively. The introns are denoted by thin lines. The X mark indicates deletion of the NUOB10 genomic region downstream of the insertion site. The black arrows indicate the primer binding sites for the NUOB10 specific primers. B. Diagnostic PCR of the NUO5 gene in the amc5 strain exemplifies the molecular lesion as previously described in (131). The amc5 (NUOB10) strain contains an intact copy of NUOB10 gene as expected. C. Semi-quantitative RT-PCR analysis shows restoration of NUOB10 transcript levels in the complemented strain. PCR was conducted with primers amplifying exon 4 (NUOB10E4L / NUOB10 E4R), and exon 2, upstream of the insertion site (NUOB10E12L / NUOB10E2R). CBLP transcript levels were used as a reference. D. Real-time quantitative PCR was used to assess the quantity of NUOB10 mRNA relative to two reference genes CBLP and UBI. The average was obtained from three biological replicates, each including three technical replicates. The error bars represent standard deviation of the mean. The results are represented as fold change relative to WT (WT set to 1.0). E. Complex I (rotenone-sensitive NADH: dehydrogenase) activity was determined with partially purified membranes. The activities are represented as the average of three biological replicates with the error bars indicating standard deviation of the mean. The activities for the WT and amc5 strains are significantly different according to the two-tailed unequal variances t-test with a p = 0.000116. The amc5 and amc5 (NUOB10) strain have significantly different activities with a p = 0.000199. All primer sequences described in A, B, C and D are detailed in Table 2.3.
Figure 3.10. The wild-type NUOB10 gene restores heterotrophic growth to an amc5 mutant

A. The growth of the WT, amc5 and amc5 (NUOB10) strains was recorded by measuring optical density at 750 nm, in the light or in the dark, over a period of 10 days. The average of three biological replicates is reported here, with error bars indicating standard deviation of the mean. B. The average generation time for each strain calculated from growth curves in A is indicated here. The error bars represent standard deviation of the mean. C. Restoration of the growth phenotype in amc5 (NUOB10) was confirmed by ten-fold dilution series plated on acetate containing medium and incubated in the light for seven days and in the dark for 16 days. In sections A, B and C, the WT and the amc5 strain used were 3A+ and amc5 (87D3) respectively.
3.4. Discussion

The forward genetic screen conducted in *Chlamydomonas* resulted in the isolation of 13 Complex I mutants *amc*1- to-13. The *amc*5 and *amc*7 mutations were discovered to be allelic with molecular lesions in the *NUOB10* gene encoding the PDSW subunit of Complex I (131). This chapter describes the molecular identification of the *AMC*9 locus. The monogenic and recessive *amc*9 mutation was linked to the insertional cassette (Figure 3.3). Through TAIL-PCR analysis, the cassette was found to interrupt the *NUO5* gene encoding the 24 kDa subunit of Complex I (Figure 3.4). Thus, three out of 13 *AMC* loci encode for Complex I subunits and provide an ideal proof of concept that the forward genetic screen yields *bona fide* Complex I mutants.

Test of allelism with *amc*9/*amc*x diploids revealed that the *amc*1-to-12 mutations are not allelic to *amc*9 (Figure 3.1). Thus, it is unlikely that the *amc*1-to-12 strains carry mutations in the *NUO5* gene. It must be noted that *amc*5/*amc*9 diploids could not be generated. However, *amc*7/*amc*9 diploids exhibited wild-type growth in the dark and since the *amc*5 and *amc*7 mutations are allelic, we inferred that *amc*5 and *amc*9 are not allelic. In addition, molecular analysis has revealed that the *amc*5 and *amc*9 strain carry mutations in two different genes encoding Complex I subunits, further confirming that they are not allelic. On the other hand, *amc*9/*amc*13 diploids, obtained from two independent crosses, could not be complemented for the *sid* phenotype (Figure 3.1). Further testing of Complex I activities on diploids confirmed decreased NADH: dehydrogenase activity (Figure
The non-complementation could imply a case of allelism. However, the molecular identity of the AMC13 locus remains unknown because the mutation is unlinked to the insertional cassette (Table 2.2). To test the possibility of allelism, the sid phenotype of the amc9xamc13 meiotic progeny needs to be examined. A similar analysis has been previously conducted for amc2 x amc4 mutants to conclude that they belong to different complementation groups (153).

Due to unknown reasons, the Complex I mutants were recalcitrant to transformation by electroporation and glass beads (153). This chapter reports the first successful transformation of Complex I mutants in Chlamydomonas by biolistics. The amc5 and amc9 mutants were transformed with a cosmid containing the corresponding wild-type gene, resulting in complementation of the growth phenotype, Complex I activity and assembly (Figures 3.6, 3.7, 3.8, 3.9 and 3.10). This phenotypic rescue with the wild-type gene confirmed that the Complex I deficiency in the amc5 and amc9 mutants was indeed caused by the loss of the subunits NUOB10 and NUO5 respectively.

3.4.1. NUO5 encodes a core 24 kDa subunit of Complex I

The amc9 mutant is characterized by loss of the 24 kDa Complex I subunit, a core subunit, which is essential for NADH:ubiquinone oxidoreductase activity (Figure 3.11). This subunit harbors a 2Fe-2S cluster, co-ordinated by four conserved cysteines (highlighted in green in Figure 3.11). This Fe-S cluster is purported to play a crucial role in NADH oxidation and/or structural stability of the enzyme (12). In the T.thermophilus ortholog Nqo2, an additional pair of cysteines
form a disulfide bond to increase the stability of the C-terminal fold (12) (highlighted in magenta, Figure 3.11). However, these stabilizing cysteines do not appear to be conserved in other orthologs of \textit{NUO5}.

Electron micrography and three-dimensional reconstruction of Complex I holoenzyme and subcomplex in \textit{Y. lipolytica} has shown that the 24 kDa subunit, is in close interaction with the 51 kDa subunit, in the N module (equivalent to the subcomplex Iλ) of the soluble arm protruding into the matrix ((165), Figure 1.1 B and Figure 3.12). This 51 kDa subunit harbors a FMN and NADH binding domain in addition to a 4Fe-4S cluster essential for NADH oxidation. Interestingly, while the \textit{amc9} mutant accumulates specifically exhibits lower levels of the 51 kDa subunit, which is restored upon complementation with the \textit{NUO5} gene (Figure 3.8 C). This could imply that steady-state levels of the 51 kDa subunit in \textit{Chlamydomonas} is dependent upon its association with the 24 kDa subunit in the holoenzyme, a finding consistent with the physical interaction evidenced from the structural data.

\textbf{3.4.2. Assembly of the 24 kDa subunit (NUO5 / NDUFV2) into Complex I}

Different model systems have been used for the study of mitochondrial Complex I (2,35,125) and assembly of the 24 kDa subunit deduced from these studies is described below. In \textit{E.coli}, the incorporation of the 24 kDa subunit is dependent on the assembly of the Q (ubiquinone-reducing) module (181), after which the N (NADH oxidizing) and Q modules are assembled with hydrophobic modules to form a membrane bound subcomplex (125). In \textit{Chlamydomonas}, the
NUO5 subunit has been purified as part of the 700 kDa subcomplex (56), implying early assembly of NUO5. In *N. crassa*, the 24 kDa subunit is assembled as part of the peripheral arm subcomplex, independent of the membrane arm subcomplex (125, 182). In *Arabidopsis*, the 24 kDa subunit is part of the 370 kDa subcomplex that consists of the N module and the Q module (20). This intermediate is in turn assembled with hydrophobic subunits to form a loosely bound membrane subcomplex (125), similar to the 700 kDa subcomplex in *Chlamydomonas*. Thus, in most model systems, including *Chlamydomonas*, this soluble subunit is assembled as part of the peripheral arm, in the early stages of Complex I assembly. On the contrary, the human 24 kDa ortholog (NDUFV2), along with few other subunits of the N module, is added to a membrane bound 830 kDa subcomplex at only the final stage of Complex I assembly (35, 183). The reason for the different order of assembly in human Complex I is not clearly understood.

3.4.3 Post-translational regulation of the 24 kDa subunit

Post-translational modification of NDUFV2 through phosphorylation of the conserved Tyr$^{193}$ (highlighted in blue in Figure 3.11) by mitochondrial c-Src kinase has been demonstrated in human cell lines (69). Loss of NDUFV2 phosphorylation at this site resulted in a 42% decrease in Complex I activity and reduced accumulation of the holoenzyme. It is hypothesized that phosphorylation of NDUFV2 is necessary for electron transfer and NADH oxidation (69). Other targets of mitochondrial c-Src kinase include the NDUFB10 subunit (NUOB10 ortholog) of Complex I (70). Detailed analysis of the mitochondrial phosphoproteome of
Chlamydomonas may provide new insights into post-translational modifications required for Complex I function.

3.4.4. **NDUFV2 is a candidate gene in many mitochondrial disorders.**

NDUFV2 has been identified as a critical gene in many disorders. For instance, a deletion of the N-terminus of NDUFV2 has been found in patients with Leigh’s syndrome, hypertrophic cardiomyopathy and encephalomyopathy (184,185). NDUFV2 is also a candidate gene in other diseases where Complex I deficiency is suspected to be an underlying cause. For example, patients with Alzheimer’s disease (186), Parkinson’s disease (187,188), bipolar disorders and schizophrenia have polymorphisms in the NDUFV2 gene (189-191). Most of these mutations have been deemed “provisional” because their contribution to the respective disease is not clearly understood at the molecular level. For instance, a conversion of the amino acid residue Lysine (K) to Arginine (R) at position 209 (K209R) of human NDUFV2, has been observed in the familial and sporadic patients of PD (187). This lysine residue is highly conserved in most eukaryotic species, including Chlamydomonas (highlighted in yellow in Fig 3.11). Hence, it would be interesting to delineate the contribution of this mutation to CI activity. Interestingly, although this lysine residue is conserved in animals, mosses and alga, land plants such as Arabidopsis and Vitis already harbor this K-to-R change (Figure 3.11). Since, we now have a null mutant for the 24 kDa subunit in Chlamydomonas, it is an ideal system for assessing the effect of this K-to-R
mutation on Complex I activity and assembly. Experiments are on-going in this endeavor.

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Table 3.3. NUO5 has a canonical mitochondrial targeting sequence whereas NUOB10 does not.

Online target prediction softwares such as TargetP (192), MitoProt II (193), Predotar (194) and PredAlgo (195) were used to predict the localization of NUO5 and NUOB10. Although both proteins are known subunits of Complex I and have been found to be part of mitochondrial proteome, only NUO5 has a recognizable mitochondrial targeting sequence. For the 282 amino acid long NUO5 in *Chlamydomonas*, TargetP predicts a cleavage site at 40 amino acid, Mitoprot II predicts a cleavage site at 13 amino acid and PredAlgo at 33 amino acid. No cleavage sites are predicted for NUOB10.
Figure 3.11. Alignment of NUO5 / 24 kDa subunit.

NUO5 / 24 kDa subunit proteins were aligned using Clustal Omega (BLOSUM 62 scoring matrix) and Bioedit. The conserved cysteines that co-ordinate the 2Fe-2S cluster in NUO5 are highlighted in green. The two cysteines that provide increased stability by a disulfide link in T. thermophilus ortholog are highlighted in magenta. The conserved lysine residue that is mutated to arginine in a Parkinson’s patient is highlighted in yellow. Accession numbers are as follows: T. thermophilus (AAA97942), E. coli (EDV69206), Y. lipolytica (XP_502254), N. crassa (XP_961535), D. melanogaster (NP_573228), D. rerio (NP_957041), H. sapiens (NP_066552), B. taurus (NP_776990), M. musculus (NP_082664), C. reinhardtii (XP_001698508), D. melanogaster (NP_082664), P. patens (XP_001759238), A. thaliana (NP_066552), V. vinifera (XP_002281655).
Figure 3.12. Localization of the 24 kDa subunit in *Y.lipolytica*.

This image is reproduced from the work of Clason *et al.*, 2007 (165). Surface representation of the three-dimensional structure reconstructed from transmission electron microscopy in **a**) the subcomplex and **b**) the holoenzyme and, **c**) overlay of surface of subcomplex (in gray) and surface of bacterial 24 kDa (in orange) and 51 kDa (in red) from the X-ray structure from Sazonov and Hinchliffe, 2006 (12).
3.4.5. The AMC5 locus encodes for the NUOB10 (PDSW) subunit.

NUOB10/NDUFB10 is a poorly characterized non-core subunit conserved in eukaryotes. It is also referred to as the PDSW subunit, named after the N-terminal amino acids conserved in mammals (Figure 3.13). This subunit has been identified in purified Complex I in *Chlamydomonas* (18), *Arabidopsis* (20) and Bovine (24). It is highly conserved among eukaryotes (Figure 3.13). However, no mutations for PDSW subunit have been reported in patients so far. Although it is localized to the distal end of the membrane arm subcomplex Iβ and is required for the assembly of the membrane arm, NUOB10 does not contain any transmembrane helices (24). Biochemical analyses have shown that NUOB10, although a hydrophilic subunit, is associated with the inner mitochondrial membrane probably facing the matrix side (196). It is possible that it is localized to the membrane by interactions with neighboring membrane subunits. In addition, unlike NUO5, NUOB10 does not have a canonical mitochondrial targeting sequence (Table 3.3 and (24)) that is cleaved upon import. Instead, it must be localized to the mitochondria by virtue of unknown internal targeting sequence(s) present in the protein. Such internal targeting sequences for mitochondrial localization have been observed in BCS1, a factor required for Complex III maturation (197).

The NUOB10 subunits harbor a C(X)$_{11}$C motif (yellow highlight in Figure 3.13). The role of these conserved cysteines is not clearly understood. A possible role in Fe-S cluster binding or metal transport to mitochondria has been proposed.
(18), although it is not known to contain an Fe-S cluster. It is also possible that these cysteines form a disulfide link that increases the stability of the protein, similar to the conserved cysteines in the C-terminal end of Nqo2 complex I subunit in *T.thermophilus* (12). The availability of a NUOB10 null mutant *amc5* in *Chlamydomonas* provides an ideal opportunity to test the role of these conserved residues in Complex I activity and assembly. FLAG-tagged *NUOB10* genomic constructs carrying cysteine to serine substitutions, when introduced into the *amc5* mutant, will provide a platform for assessing the contribution of these residues in Complex I assembly/activity.

Phosphorylation of the human NDUFB10 subunit by mitochondrial c-Src kinase has been found to be essential for Complex I enzymatic activity, but not assembly (70). The inhibition of c-Src kinase in cancer cells causes reduced Complex I activity, resulting in decreased mitochondrial respiration and proliferation. Furthermore, analysis of the mitochondrial phosphoproteome supports the presence of phosphorylated residues Tyr$^{55}$ and Tyr$^{142}$ in NDUFB10 (198). Thus, phosphorylation of Complex I subunits is now emerging as an important role in regulating the activity of the respiratory chain.
3.4.6. **Other nuclear mutants encoding Complex I subunits in Chlamydomonas.**

Interestingly, three additional *Chlamydomonas* CI mutants, carrying molecular lesions in genes encoding Complex I subunits, have been isolated from a similar forward genetic screen conducted by the Remacle group (150,180): i) one mutant carries a deletion in the promoter region the *ND9* gene, ii) the second mutant has a molecular lesion in the coding sequence of a gene encoding the 39 kDa non-core NUOA9 subunit, and iii) the third mutant has a mutation in the 3’ UTR of gene encoding the *Chlamydomonas*-specific NUOP4 subunit.

Thus, we now have strains with mutations in five endogenous Complex I subunits. While the mutations in *NUO5*, *NUOB10* and *NUOA9* are loss-of-function mutations leading to an assembly defect (131,150,180). The disruption in the sequence elements of the *ND9* and *NUOP4* genes appears to affect the level of expression of the corresponding gene products, causing a decrease in the detected levels of mature Complex I (150,180). This data further corroborates the Complex I assembly defects observed due to mutations in Complex I subunits found in bacteria, land plants, fungi and mammals (21,31,35,196,199-201). Therefore, successful synthesis of Complex I subunits are the first requirement for completion of Complex I biogenesis.
Figure 3.13. Alignment of NUOB10 / PDSW subunit.

NUOB10 / PDSW subunit proteins were aligned using Clustal Omega (BLOSUM 62 scoring matrix) and Bioedit. The conserved PDSW sequence at the N-terminus is highlighted in green. The two cysteines are highlighted in yellow. Accession numbers are as follows: D. melanogaster (NP_651972), G. gallus (XP_414844), H. sapiens (O96000), B. taurus (O02373), M. musculus (Q9DCS9), X. tropicalis (NP_001017250), D. rerio (Q6PC16), C. reinhardtii (XP_001694041), P. patens (XP_001770976), A. thaliana (NP_566608), V. vinifera (XP_002276810), N. crassa (CAA48768), Y. lipolytica (XP_002142991).
CHAPTER 4

IDENTIFICATION OF A NOVEL FACTOR INVOLVED IN MITOCHONDRIAL COMPLEX I BIOGENESIS

4.1. Introduction

Mitochondrial Complex I, the first and largest enzyme of the electron transfer chain, is of dual genetic origin (2). The study of mutants, in various model systems, has revealed a precise modular assembly for Complex I (125). However, little is known about biogenesis factors that are required for the synthesis of the nuclear- and mitochondrially-encoded subunits. In this study, we define biogenesis factors as factors that are required for the formation of the subunits and/or their subsequent incorporation to form the mature enzyme. A few factors, involved in a post-translation step of Complex I assembly, have been identified (86,92,102,104,105,107,108,111,115,121,202) (see Chapter 1). However, apart from the Fe-S cluster binding IND1 (116) and the methyltransferase NDUFAF7 (122), the precise function of the factors in Complex I assembly remains unknown.

To identify novel factors involved in Complex I biogenesis, we performed a forward genetic screen to isolate Complex I mutants (described in Chapter 2). We
utilized *Chlamydomonas reinhardtii*, a unicellular biflagellate green alga, as a model system to study mitochondrial Complex I (2). *Chlamydomonas* Complex I mutants display a unique slow growth in the dark (*sid*) phenotype (2,145,146). We used this phenotype as the basis for a forward genetic screen and isolated Complex I mutants, *amc1*-to-13, that display different levels of Complex I assembly defects (Chapter 2 and (131)). Using molecular genetics approach, we discovered that the *AMC9* and the *AMC5/7* loci are defined by the *NUO5* and *NUOB10* genes, respectively, which encode for Complex I subunits (Chapter 3 and (131)). The isolation of mutants with molecular lesions in the structural subunits of Complex I, established a proof of concept and prompted us to pursue this approach to reveal novel loci controlling Complex I biogenesis. We focused on the molecular characterization of the remaining *amc* mutants as their loci might correspond to genes encoding factors required for Complex I biogenesis.

The mutants *amc11* (described in Chapter 2) and *amc1* (131) display a *sid* phenotype and severe reduction in rotenone-sensitive NADH: duroquinone oxidoreductase activity. In both mutants, analysis by Blue-Native PAGE has evidenced the accumulation of a 700 kDa subcomplex, diagnostic of a block in the assembly of the membrane arm of Complex I. However, this subcomplex is labile and not always detected in independent extractions. In addition, the steady-state levels of the 51 kDa, 75 kDa and TYKY subunits, which are localized in the soluble arm of Complex I, is decreased. This is a trait previously noted in Complex I mutants unable to assemble the membrane arm (131).
This chapter describes the identity of the *AMC1* and the *AMC11* loci. Study of the *amc1* meiotic products revealed that the mutation is not linked to the insertional cassette (131), thereby making the molecular identification of the *AMC1* locus a challenging prospect. It is possible that the *amc1* and *amc11* mutants carry a mutation in a hydrophobic subunit, which is part of the membrane arm, thereby causing the assembly defect. However, sequencing analyses confirmed that the *AMC1* locus does not correspond to any of the 14 nuclear genes that encode the hydrophobic subunits of the membrane arm or the CIA30 assembly factor whose loss leads to a similar assembly defect (131). In this chapter, we examine the *AMC11* locus. We report that the *amc1* and *amc11* mutations map to a novel gene encoding a previously unrecognized protein with a mitochondrial localization. Our results suggest that the AMC11 protein regulates a post-transcriptional step of mitochondrial gene expression.

4.2. Experimental Procedures

4.2.1. Strains and culture conditions

*Chlamydomonas* strains were cultured as described in Section 3.2.1. Experiments described in this chapter employ the original *amc11* (10G11) strain (*mt*, *amc11::aph7*, *arg7-8*). In addition, the following haploid spores, derived from 10G11, *amc11* (65) (*mt*, *amc11::aph7*) and *amc11* (70) (*mt*, *amc11::aph7*), were used in genetic crosses. In addition, a Complex I proficient strain 9A (*mt*, *arg9-2*), isolated from genetic crosses described in Section 3.2.2, was utilized in genetic analyses.
S. cerevisiae strains CWO4 (MATα ade2-1 his3-11,15 leu2-3,11 trp1-1 ura3-1) (203) and JM45 Δcoq3 (MATα, his4-580, leu2-3, leu2-112, trp1-289, ura3-52, coq3::LEU2) (204) were grown at 28°C in liquid or solid medium, containing glucose as the fermentable substrate or ethanol as the respiratory substrate (205). Strains were transformed by the one-step transformation method (206,207). The CWO4 strain was transformed with pEMBLYe30/2urf13-TW plasmid carrying the T-urf13 ORF and LEU2 as a selectable marker (208). The transformant, referred to as Urf13-C2, was used as positive control for immuno-detection of T-Ur13 protein.

Chemo-competent E. coli DH5α strains were used for molecular cloning. E. coli was grown at 37°C in Luria-Bertani (LB) broth and LB agar according to (209).

4.2.2. Genetic analysis

Genetic crosses were conducted as described in Sections 2.2.8 and 3.2.2. To determine the segregation of the sid phenotype in the amc11 mutant, two independent crosses were conducted (Table 4.5). Cross I involved the mating of the wild-type strain CC125 (mt+) with amc11 (10G11) (mt, amc11::aph7”, arg7-8). Bulk germination was conducted as described in Section 3.2.2. Arginine prototrophic (arg+) haploid spores were selected for HyB resistance (HyB^R) by plating the germinated spores on TAP+HyB solid medium. For Cross II, wild-type strain 1’ (mt+) was mated with amc11 (10G11) (mt, amc11::aph7”, arg7-8). After bulk germination of the meiotic zygotes, haploid spores were plated on TARG
medium. The relevant phenotypes of the spores were deduced from replica-plating as described in Section 2.2.3.

The amc11 haploid spores carrying the arg9-2 mutation were generated as follows. The amc11(70) strain (mt+, amc11::aph7") was mated with the Complex I proficient strain 9A (mt, arg9-2). The following haploid spores obtained from the cross, carrying the arg9-2 and the amc11 mutations, were chosen for construction of diploids: amc11(90) (mt+, amc11::aph7", arg9-2) and amc11(5) (mt, amc11::aph7", arg9-2). These spores were used to generate the amc11/amcx diploids as described in Table 4.1. Individual diploids were sub-cloned to a single colony on solid medium and their mating type was determined by diagnostic PCR to confirm diploidy (210).

To test whether the amc1 and amc11 mutations are allelic, amc1(2) (mt+, amc1) was crossed with amc11(65)(mt, amc11::aph7"). The meiotic zygotes were bulk-germinated and 98 haploid spores were tested for their relevant phenotypes by replica-plating.

The amc11(nd4::T-urf13, amc11::aph7") strains were generated by mating amc11(70) (mt+, amc11::aph7") with nd4::T-urf13 (mt). Here, all the meiotic progeny will inherit mitochondrial DNA (nd4::T-urf13) from the mating type minus strain (211). The HyB R haploid spores carrying the amc11 nuclear mutation were selected and sequenced to confirm the presence of the mitochondrial nd4::T-urf13 mutation.
4.2.3. Nucleic acids extraction

Genomic DNA extraction was performed as described in Section 3.2.3. RNA was prepared using the Plant RNeasy Kit (Qiagen) according to the manufacturer’s protocols, with the following modifications. Cells were grown for 2-3 days on either TARG solid medium or antibiotic containing medium. Cells (100 mg) were harvested and lysed by vortexing, with 9/10th volume of glass beads, for 5 min at 22 °C.

4.2.4. Diagnostic PCR and TAIL-PCR analyses

Diagnostic PCRs and TAIL-PCRs were performed as described in Section 3.2.4. The sequences of primers used in diagnostic PCRs are provided in Table 4.2. PCR products were separated by agarose gel electrophoresis, stained by ethidium bromide and imaged using the Fotodyne - FOTO/analyst(R) imaging system.

TAIL-PCR was used to identify the sequence flanking the insertional cassette in the amc11 mutant as in (167). The following partially degenerate primers were used for TAIL-PCR: AD1 (5’-NTCASTWTSGWGTT-3’), AD2 (5’-NGTCGASWGANAWGAA-3’) and RMD228 (5’-WGNTCWGNCANGCG-3’) (167,168). The iHyg3-specific primers, APH7F3, APH7F5 and APH7F8, were used to amplify the genomic DNA flanking the cassette at its 3’-end. The sequence flanking the 5’-end of the cassette was obtained by conventional PCR, using the AMC11-specific primer au5.g6830 exon2F3 and the iHyg3-specific primer APH7R5.
TAIL-PCR was also used to identify the sequence disrupting the **AMC11** gene in the *amc1* mutant. The partially degenerate primer RMD228 was used to retrieve the sequence of the inserted DNA, in combination with the following **AMC11**-specific primers (for primary, secondary and tertiary reactions, respectively): A) Cre16.g688900 E2F32, Cre16.g688900 E2F33 and au5.g6830 exon2F2, and B) Cre16.g688900 E2F33, au5.g6830 exon2F2 and 10G11 exon2F (1). The complete sequence was retrieved by amplification with the primer pair amc1insertF1/amc11del-R. The inserted DNA, disrupting the **AMC11** gene, was confirmed by sequencing two independent *amc1* strains: the original *amc1* (4C10) and its haploid spore *amc1*(27).

**4.2.5. RT-PCR analyses**

To determine the **AMC11** gene model, 2-5 µg of WT (4C-) RNA was treated with RQ1 RNase free DNase I (Promega) and reverse-transcribed with 400 units of M-MLV reverse transcriptase (Life Technologies) with either OligodT primers or random hexamers as per manufacturer’s protocol. The **AMC11** cDNA was amplified using GoTaq Polymerase (Promega), Pfu Turbo DNA Polymerase (Agilent), Platinum Pfx DNA Polymerase (Life Technologies), Phusion High-Fidelity DNA Polymerase (NEB) or Velocity DNA Polymerase (Bioline). The following primers were successful in amplifying the **AMC11** cDNA: Cre16.g688900 5’UTR-F1/E2R10 (amplicon was cloned into Promega pGEM-T easy Vector System and used in Section 4.2.7 to determine the sequence corresponding to the **AMC11** N-terminus); 10G11 exon2F (1) / amc11-del1R; Cre16.g688900
exon2F4/exon3R2; Cre16.g688900 exon3F / exon4R; Cre16.g688900 exon4F2/exon4 3R; Cre16.g688900 exon5-F3/exon-8R; au5.g6830 exon 6F2/Cre16.g688900 3'UTR-1R; Cre16.g688900 exon8-1F/3'UTR-2R. Finally, the amplicons were sequenced.

For semi-quantitative RT-PCR analyses, 2.5 µg of RNA was reverse-transcribed as described above. Semi-quantitative PCR was conducted as described in Section 3.2.4. To determine the transcript abundance of AMC11, two-fold dilutions of the cDNA template (equivalent to 200ng, 100ng, 50ng, 25ng, 12.5 ng and 6.25ng of input RNA) were subjected to PCR amplification for 35 cycles. For determining mitochondrial transcript abundance, two-fold dilutions of the cDNA template (equivalent to 50ng, 25ng, 12.5ng, and 6.25 ng of input RNA) were subjected to PCR amplification for 30 cycles. CCDA transcript abundance was used as a control. Sequences of primers specific for nuclear genes and mitochondrial genes are provided in Tables 4.2 and 4.3, respectively.

4.2.6. Real-time quantitative PCR.

For real-time quantitative PCR (qPCR) of the mitochondrial transcripts, 2 µg of RNA was treated with RQ1 RNase free DNase I (Promega). Reverse transcription was achieved with 400 units of M-MLV Reverse transcriptase (Life Technologies) using 250 ng of Random Hexamers (Promega), according to the manufacturer’s protocol. Amount of cDNA equivalent to 50 ng of total input RNA was used as template for qPCR using SensiMix (Bioline) on a Mastercycler ep realplex thermocycler (Eppendorf). qPCR reactions were denatured at 98ºC and
annealed at 60°C. Relative transcript levels were determined by normalizing the levels of target transcripts to the geometric mean of two reference transcripts, *CBLP* and *UBI*. For determining the relative mitochondrial DNA (mtDNA) content, 8 ng of total genomic DNA was used as template for qPCR. The mitochondrial *nd4* gene was used as the target gene and the nuclear *TUA2* gene was used as the reference. In all cases, normalization of target transcript or gene levels to the reference was performed by the Pfaffl method (212). Relative fold change was calculated by normalizing to the average of the isogenic WT strain (set to 1.0). All qPCR analyses were conducted on two or three independent biological replicates, each including three technical replicates.

Primers used for qPCR analysis are reported in Table 4.4. Primer efficiency was determined by generating calibration curves for each pair of primers using two-fold dilutions of the template (WT (4C-) cDNA or genomic DNA). The $r^2$ value and amplification efficiency was calculated from these curves and is reported in Table 4.4.

4.2.7. Plasmid Construction

The AMC11 N-terminal mitochondrial targeting sequence was heterologously expressed as a translation fusion with the UbiG protein in yeast. For this purpose, plasmids pAHG, pQMG, and pAH3 were kindly provided by Dr. Catherine Clarke (University of California, Los Angeles) (213). The sequence encoding the AMC11 N-terminus (1-59 amino acids) was amplified, using the primers PHA-F (5'-
CACACACGAATTGATATCAAGCGGTAAGCTTTATGCACCGGACCTGCTGTGC-3') and PHA-R (5'-AAATACCCGCAGTTTTTGCCATTACAAGCTTTAACCCCCCTCGGTTGCGAGC-3'), from a plasmid containing a cloned fragment of the AMC11 cDNA (from Section 4.2.5). The PCR product was cloned into the HindIII site of the plasmid pAHG (213), carrying the leaderless ubiG gene under the control of the constitutive CYC1 promoter, by In-Fusion Cloning (In-Fusion HD cloning kit, Clontech). The resulting construct, containing 1-59 amino acids of AMC11 as leader sequence, is referred to as pAHG59. The pAHG, pQMG, pAH3, and pAHG59 plasmids, each containing the URA3 selectable marker, were introduced into Δcoq3 by one-step transformation (206).

The T-urf13 ORF was codon optimized for expression in Chlamydomonas mitochondria (Genescript) (144) and provided as a cloned fragment in plasmid Mitourf13m2. The T-urf13 ORF was amplified with Pfu DNA Polymerase from the Mitourf13m2 plasmid using primers IVU-F and IVU-R (Table 4.3). Ndel-digested pND4-LP plasmid (148) was used as the vector and the desired clone was obtained by In-Fusion HD system (Clontech). The cloned plasmid (referred to as pVU2) was sequenced to confirm the in-frame substitution of the nd4 ORF with the T-urf13 ORF.

4.2.8. PCR-based screening of the Chlamydomonas genomic library and biolistics

The ARG7-based indexed cosmid library (described in Section 3.2.7) was screened for the presence of cosmids carrying the AMC11 gene, by diagnostic
PCR. The *AMC11*-containing cosmid (referred to as 1H10) was identified using the primer pairs au5.g6830 exon2F3/10G11 AD1-F(2) and Cre16.g688901-exon24F/-3'UTR-1R. In order to generate a construct containing only the *AMC11* full-length gene, the cosmid 1H10 was digested with BamH1 restriction enzyme and recircularized by T4 DNA ligase. The region of *Chlamydomonas* genomic DNA, present in the original cosmid 1H10 (referred to as cosmid A) and the BamHI digested and religated cosmid (referred to as cosmid B) were sequenced and are represented in Figure 4.2 A.

Cosmids A and B were used for transforming the original *amc11* (10G11) (*mt*, *amc11::aph7*", *arg7*-) strain by biolistics as described in Section 3.2.8. The transformants containing the full and truncated cosmid 1H10 shall be referred to as *amc11* (AMC11)-A and *amc11* (AMC11)-B, respectively.

Mitochondrial transformation was also conducted as described in Section 3.2.8, by transforming the *dum11* strain (214) with 1 µg of pVU2 plasmid DNA (130).

4.2.9. Assessing growth phenotype by ten-fold dilution series and growth curves

Growth curves were performed and generation times calculated as described in Section 3.2.9. Ten-fold dilution series on *Chlamydomonas* and yeast strains was conducted as described in Section 2.2.4, except the cell density for yeast strains was measured spectrophotometrically at $A_{600}$ and normalized to an $OD_{600} = 1$ by dilution.
4.2.10. Biochemical analyses

Respiratory activities were measured as described in Section 2.2.5. Complex II+III activity was calculated using molar extinction coefficient for cytochrome $\text{c}$ at $\varepsilon_{550\text{nm}} = 19.6\text{mM}^{-1}\text{cm}^{-1}$, in the absence and presence of Complex III specific inhibitor myxothiazol (7.5 µM) and Complex IV inhibitor KCN (1mM) (146). Blue-Native PAGE, in-gel Complex I activity staining, SDS-PAGE and immunoblotting were conducted as described in Sections 2.2.6 and 2.2.7. Immuno-detection of T-Urf13 reporter protein was performed using 1:500 $\alpha$-T-urf13 antibody (215), which was kindly provided by Dr. Christine Chase (University of Florida, Gainesville). Total cellular protein was extracted from Chlamydomonas using the freeze/thaw method (216), from two-three day old light-grown plates. The yeast strain URF13-C2, expressing T-Urf13, was used as a positive control for immuno-detection with $\alpha$-T-urf13. Crude protein extract was prepared with 0.2M NaOH, from the URF13-C2 strain, as described in (217).
Table 4.1. Construction of *amc11/amcx* diploids.

The mating type minus (*mt*) strains were crossed with mating type plus (*mt*+) strains. From each cross, diploids were specifically selected for their unique phenotypes. The *amc11/amcx* diploids were generated by using either the original *amc11 (10G11)* strain or haploid spores derived from the original strain, such as *amc11 (90)* and *amc11 (5)*. The crosses highlighted in gray were unsuccessful in yielding diploids.

Key: arg+ : arginine prototrophic strain; arg- : arginine auxotrophic strain; HyB^R^ : hygromycin B resistance; HyB^S^ : hygromycin B sensitivity; Pm^R^ : paromomycin resistance. All arginine auxotrophic strains carry the arg7-8 mutation, except for *amc11 (90)* and *amc11 (5)*, which harbor the arg9-2 mutation.
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<th>Primer Name</th>
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<td>10G11 exon2F (1)</td>
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<td>10G11 AD1-F (2)</td>
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**Table 4.2. Sequence of primers**

List of primers used for TAIL-PCRs, diagnostic PCRs, and RT-PCRs used for experiments described in Chapter 4.
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Table 4.3. Sequence of primers specific to mitochondrial genes used in semi-quantitative RT-PCR.
Table 4.4. Primers used for real-time quantitative PCR experiments.

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<td>Chlamy nd4 fw</td>
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<td>nd4</td>
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The amplification efficiency for gene-specific primers, for the mitochondrial targets \(\text{cox1}, \text{nd4}, \text{nd5}, \text{cox1}, \text{nd2}, \text{nd6}, \text{nd1}, \text{rtl}\) (218), and nuclear targets \(\text{CBLP}\) (169), \(\text{TUA2}\) (219), \(\text{UBI}\) (220), was identified by generating calibration curves. The $r^2$ value and PCR efficiency, calculated from each calibration curve, are reported here. These PCR efficiencies were used in calculating the relative expression ratio by the Pfaffl Method (212). The Phytozome IDs of nuclear genes: \(\text{TUA2}\) - Cre04.g216850, \(\text{UBI}\) - Cre03.g159200, \(\text{CBLP}\) - Cre13.g599400.
4.3. Results

4.3.1. The AMC11 locus is defined by a novel gene.

In Chapter 2, we reported the isolation of the \textit{amc11} mutant which displays a severe Complex I deficiency (Figure 4.2 B). Analysis via BN-PAGE showed that the \textit{amc11} mutant accumulates a 700 kDa subcomplex, similar to the \textit{amc5} mutant (Figure 4.4). This is a feature typically observed in Complex I mutants, which are unable to form a 950 kDa mature holoenzyme, because the assembly of the membrane arm is impeded \cite{56,130,131}. This 700 kDa subcomplex is highly labile in the \textit{amc11} mutant, and is not always detected in independent extractions. In one instance alone, a very faint mature complex could also be detected, in addition to the 700 kDa subcomplex (Figure 4.4 C).

To determine the nature of the \textit{amc11} mutation with respect to the wild-type allele, we performed genetic analyses in Chapter 2. The \textit{amc11}/+ diploids were restored for growth in the dark, thereby confirming that the \textit{amc11} mutation is recessive with respect to the wild-type allele. To test if the \textit{amc11} mutation is linked to the insertional cassette, segregation of the \textit{sid} phenotype, with respect to the \textit{HyB} \textsuperscript{R} phenotype, was monitored in the meiotic progeny obtained from two independent crosses of \textit{amc11} x WT (Table 4.5). All the \textit{HyB} \textsuperscript{R} spores (total: 159) displayed a \textit{sid} phenotype while the heterotrophic growth of all the \textit{Hyg} \textsuperscript{S} was wild-type like, indicating that the \textit{amc11} mutation is linked to the insertional cassette.
We reasoned that the co-segregation of the *sid* phenotype with HyB^R^ could be explained by insertion of the cassette in a gene that controls Complex I biogenesis. To identify the gene disrupted in the *amc11* mutant, we employed TAIL-PCR analysis to recover the genomic sequence flanking the insertion site of the cassette, as described in Section 4.2.4. A truncated form of the insertional cassette, lacking the first 123 bps of the iHyg3 cassette (constituting part of the promoter sequence), was mapped to exon 2 of a novel gene, designated as Cre16.g688900. Sequencing revealed that DNA, which is neither part of the *Chlamydomonas* genome nor the cassette, also co-integrated at the 5'- and 3'-end of the site of insertion (see Fig. 4.1A). Similarity searches, using NCBI BLAST, indicated that the sequence integrated at the 5'-end displays 94% identity to an Arctic cod fish gene (*Boreogadus saida*). This raises the possibility that the co-integrated sequences could be derived from the herring sperm DNA (genome sequence unavailable), used as carrier DNA during the transformation procedure (154). The insertional event is also accompanied by a deletion of 42 bp of the Cre16.g688900 gene at the site of insertion (Figure 4.1 A). We shall henceforth refer to this novel gene as *AMC11*.

To confirm the location of the insertional cassette in *AMC11*, we performed diagnostic PCRs with the *amc11* mutant (Figure 4.1 B). The presence of the cassette was confirmed, with iHyg3- and *AMC11*-specific primers, by amplifying across the *AMC11/iHyg3* junctions. As expected, bands of the predicted size (988 bp using primer pair 2F3/APH7R6 and 592 bp using primer pair APH7F8 / 2) were
only observed in the *amc11* mutant, but not in the wild-type strain (Figure 4.1 B). On the other hand, the amplification of the region, spanning the insertion site, resulted in a band of expected size (400 bp) in the wild-type strain but not in the *amc11* mutant. The above results, together with additional diagnostic PCRs (data not shown), have confirmed that the *AMC11* gene carries no other molecular lesions than the insertional cassette in the second exon of the coding sequence.

### 4.3.2. The *AMC11* gene restores Complex I activity and assembly in the *amc11* mutant

To test if the disrupted gene is responsible for the Complex I defect in the *amc11* mutant, we transformed the 10G11 strain with genomic DNA containing the *AMC11* gene (see Section 4.2.8). The *amc11*(10G11) strain was transformed by biolistics with an *ARG7*-containing cosmid (Cosmid A) carrying the *AMC11* gene (Figure 4.2 A). The transformant, referred to as *amc11* (*AMC11*)-A, was restored for Complex I (rotenone-sensitive NADH: duroquinone oxidoreductase) activity (Figure 4.2 B). This transforming cosmid A, spans two additional genes, in addition to *AMC11* (Figure 4.2 A). Hence, to confirm that the *AMC11* gene is solely responsible for the restoration of Complex I proficiency, a truncated form of the cosmid (referred to as Cosmid B) was generated (Figure 4.2 A). Cosmid B, containing only one full-sized gene – *AMC11*, was introduced into the *amc11* strain. The resulting transformant, referred to as *amc11*(*AMC11*)-B, also displays wild-type levels of Complex I activity (Figure 4.2 B), thereby confirming that
disruption of the \textit{AMC11} gene is responsible for the Complex I defect in the \textit{amc11} mutant.

In accord with the \textit{sid} phenotype, the \textit{amc11} mutant requires a generation time of 45 h in the dark as opposed to the wild-type strains that double within 28 h in the dark. As shown in Figure 4.3 A and B, the \textit{amc11(AMC11)} – A and -B strains, have a generation time of only 24 h (Figure 4.3 A and B), an indication that the defect in heterotrophic growth is fully complemented. We also observed a similar rescue of the growth phenotype on solid medium (Figure 4.3 C).

To assess the restoration of Complex I assembly upon complementation, we conducted BN-PAGE analysis followed by \textit{in-gel} staining for Complex I activity and immunoblotting experiments (Figure 4.4). We observed that the \textit{amc11} (\textit{AMC11})- A and –B strains assemble a mature Complex I (950 kDa), similar to the wild-type strain. Steady-state levels of the Complex I subunits, 49 kDa, 51 kDa and TYKY, were also restored by the presence of the wild-type \textit{AMC11} gene. From these results, we concluded that \textit{AMC11} is a novel gene required for Complex I assembly.
Table 4.5. The amc11 mutation is linked to the insertional cassette.

Two independent crosses were conducted and haploid spores were obtained by bulk germination. Only the arginine auxotrophic (arg+) and HyB resistant (HyB^R) haploid spores from cross I were selected. Meiotic progeny from cross II were plated on TARG medium to obtain haploid spores of all phenotypes. The relevant phenotypes were tested by replica-plating. The sid phenotype segregated with the HyB^R trait.


<table>
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<th>Phenotype</th>
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Figure 4.1. The AMC11 locus is defined by a novel gene Cre16.g688900.

A. The position of the insertional cassette in the amc11 mutant is shown here. The cassette is inserted in the gene Cre16.g688900. The gray rectangles indicate the 5' and 3' UTRs of the gene. The orange rectangles indicate regions of the exons corresponding to the coding sequence of AMC11, as predicted by the Chlamydomonas genome database JGI v5.5. The thin black lines correspond to introns. The symbol “X” marks the location of the deleted nucleotides in the amc11 mutant. The purple rectangles in the inserted sequence represent the 568 bp and 42 bp sequences that co-integrated at the 5'- and 3'-ends of the cassette, respectively. The brown and black arrows represent the primers specific for the iHyg3 cassette and the AMC11 gene, respectively. The yellow and green arrows indicate the extent of the genomic sequence obtained by TAIL-PCR and conventional PCR, respectively. F3, F5 and F8 represent the position of the aph7"-specific primers used in TAIL-PCR to recover the sequence flanking the 3'-end of the cassette. APH7R5 and 2F3 are the primers used to obtain the sequence flanking the 5'-end of the cassette by conventional PCR. B. The molecular lesion in the amc11 mutant was confirmed by diagnostic PCR with AMC11- and aph7"-specific primers as shown in A. The primer sequences are provided in Table 4.2.
Figure 4.2. The AMC11 gene restores Complex I proficiency to the amc11 mutant.

The wild-type (WT and amc11 strains used here are 4C- and amc11 (10G11), respectively. The amc11 (10G11) strain was transformed with a cosmid containing the wild-type AMC11 gene by biolistics. The transformants containing the full 1H10 cosmid (Cosmid A) and the truncated 1H10 cosmid (Cosmid B) are referred to as amc11 (AMC11) – A and – B, respectively A. Simplified map of the insert contained in cosmid 1H10 (Cosmid A) carrying the truncated HPT2 gene (Cre16.g688850), the AMC11 gene, the CYG56 gene (Cre16.g688901) and Cre16.g688950. Cosmid 1H10 was digested by BamHI restriction enzyme to obtain a truncated cosmid (Cosmid B) that only retains one full-length gene, namely AMC11. Both cosmids were used to transform the amc11 mutant. B. Complex I (rotenone-sensitive NADH: duroquinone oxidoreductase) activity was determined with partially purified membranes. The activities are represented as an average of four biological replicates with the error bars indicating standard deviation of the mean. The significance of Complex I activity restoration was determined by two-tailed unequal variances t-test. The amc11 mutant has significantly lower Complex I activity compared to the WT (p-value = 0.00122). There is no significant difference between the activities measured for the amc11 (AMC11) and WT strains. However, there is a significant difference between: i) amc11 and amc11 (AMC11) – A (p-value = 0.00039), and ii) amc11 and amc11 (AMC11)–B (p-value = 0.002545). The WT and amc11 strains used here are 4C- and amc11 (10G11), respectively.
Figure 4.3. The sid phenotype of the amc11 mutant is rescued by the wild-type AMC11 gene.

In sections A, B and C, the WT and the amc11 strain used were 4C- and amc11 (10G11), respectively. The growth of WT, amc11, amc11 (AMC11) - A and amc11 (AMC11) - B was documented by measuring optical density at $A_{750}$, in the light or in the dark. The average of three biological replicates is reported here, with error bars indicating standard deviation of the mean. B. The average generation time for each strain calculated from the growth curves in A is indicated here. The error bars represent standard deviation of the mean. The two-tailed unequal variances $t$-test revealed a significant increase in the generation time of amc11 with respect to wild-type in the light ($p$-value = 0.0168) and in the dark ($p$-value=0.0085). In addition, the rescue of generation time in the dark is also significant for the complemented strains with respect to the amc11 mutant: amc11 (AMC11)-A ($p$-value = 0.00752) and amc11(AMC11)-B ($p$-value = 0.00356). C. Restoration of the growth phenotype in the two amc11 (AMC11) strains was confirmed by ten-fold dilution series, plated on acetate containing medium, and incubated in the light and in the dark for 12 days.
Figure 4.4. Complex I assembly is restored in the amc11 mutant upon introduction of the AMC11 gene.

A. Blue-Native PAGE was conducted on 200 µg of partially purified membrane fraction. In-gel Complex I activity was detected by NBT staining. The amc11 mutant accumulates a labile 700 kDa subcomplex, similar to the amc5 mutant. B and C. Immunoblotting using anti-51 kDa antibody, of complexes separated by BN-PAGE to detect the assembled soluble arm of Complex I. Two hundred µg of protein was loaded per lane. D. SDS-PAGE immunoblotting was conducted on 10 µg of partially purified membranes using polyclonal antibodies to detect soluble arm Complex I subunits: α- 49 kDa, α- 51 kDa, α- TYKY. α– cyt f was used to confirm equal loading. In A, B, C and D, the WT strain was 4C− and, the amc5 and amc11 were the original amc5 (87D3) and amc11 (10G11) strains, respectively.
4.3.3. The *amc1* and the *amc11* mutations are allelic.

To establish whether the *amc11* mutation was allelic to the *amc1* – to -13 mutations, *amc11/amcx* diploids were generated (as described in Table 4.1). The resultant diploids were tested for growth in the dark by ten-fold dilution series (Figure 4.5). All the *amc11/amcx* diploids were restored for growth in the dark, except for *amc11/amc1*, *amc11/amc2* and *amc11/amc13*. Further genetic and molecular analyses need to be conducted to determine the cause of non-complementation of the *AMC11* locus with the *AMC2* and the *AMC13* loci. It must be noted that *amc11/amc4* diploids could not be generated and the *amc11/amc12* diploids shall be discussed in Chapter 5.

In the case of the *amc11/amc1* diploids, we generated three additional diploids and confirmed the *sid* phenotype (Figure 4.6). In order to determine whether the *amc11* and *amc1* mutations are allelic, we obtained meiotic progeny by crossing an *amc1* strain with an *amc11* strain as described in Section 4.2.2. All the haploid spores (98 meiotic spores: ratio of HyB$^S$/HyB$^R$=45/53) displayed a *sid* phenotype, further substantiating that the *AMC1* and *AMC11* loci define the same gene.

If the *amc1* mutation is allelic to *amc11*, it is expected that the *AMC11* gene carries a molecular lesion in the *amc1* strain. To determine if the *amc1* mutant carries a mutation in the *AMC11* gene, we performed diagnostic PCRs. In the *amc1* mutant, PCR analyses revealed a molecular lesion in exon 2 of the *AMC11* gene, upstream of the insertional site found in *amc11* (Figure 4.7). The primer pair
(1 / 2), amplifying a region spanning the *amc11* insertion site, failed to produce the expected PCR product (881 bp) in both the *amc1* and the *amc11* mutants. On the other hand, the primer pair (au5.g6830 exon 2F3 / 2) could amplify a band of expected size (468 bp) in the *amc1* mutant and not in the *amc11* mutant. Also, PCR products 626 bp upstream of the *amc11* mutation, could be amplified in both the mutants, with the primer pair (Cre16.g688900 E2F33 / E2R9). These results suggest that the molecular lesion in the *amc1* mutant must be present within a 340 bp region (between primers E2R9 and 2F3), upstream of the *amc11* mutation. Additional diagnostic PCR analyses, confirmed that there were no other major rearrangements present in the *AMC11* gene of the *amc1* mutant.

One possible cause for the failure to amplify this 340 bp region of the *AMC11* gene in the *amc1* mutant, is an insertional mutation interrupting this site. To test this hypothesis, we performed TAIL-PCR analysis with *AMC11*-specific primers (as described in Section 4.2.4), and identified an inserted sequence of 1.572 kb corresponding to an intergenic region from Chromosome 17 (Figure 4.7 A). This intergenic region interrupted the *AMC11* gene at a location 25 bp downstream of the E2R9 primer binding site, thereby validating the diagnostic PCR results (Figure 4.7 B). Further PCR analyses conducted with *AMC11* - and Chr17- specific primers (1/amc1 insert R2) amplified bands of expected size only in the *amc1* mutants. From these results, we conclude that the *amc1* and *amc11* mutations map to the same gene. To confirm the molecular data, we are currently working to complement the *amc1* mutant with the *AMC11* gene.
Figure 4.5. Analysis of amc11/amc11 diploids.

The amc11/amc11 diploids were generated according to Table 4.1. Diploids were tested for the growth phenotype by ten-fold dilution series that were plated on acetate containing medium and incubated in the light or in the dark for 16 days. Only one representative diploid (out of two diploids that were tested) is depicted here. Two dilutions are shown here. The growth of the following diploids are depicted in panels a) to k) wherein WT (4C-) and the respective parental strains were used as controls: a) amc1xamc11, b) amc2xamc11, c) amc3xamc11, d) amc6xamc11, e) amc7xamc11 (the amc11 control strain used here is amc11(65), f) amc8xamc11, g) amc10xamc11 (the amc10 control strain shown here is amc10 (12C) and, h) amc13xamc11.
Figure 4.6. The *amc1* and *amc11* mutations are allelic.

Diploids were obtained by mating the arginine prototrophic strain *amc1* (2) (*mt*+, *amc1*) with the original *amc11* (10G11) (*mt*, *amc11::aph7″, arg7-8) strain (Table 4.1). The *sid* phenotype of three independent diploids was tested by ten-fold dilution series. The dilutions were plated on acetate containing medium and incubated, in the light and in the dark, for 16 days. The parental strains used for generating the diploids were used as controls. The WT strain used here was 4C+. 

![Figure 4.6: Differently dilutions for WT, amc11, amc1, amc11 x amc1, and amc11 (AMC11) - A strains in light and dark conditions.](image-url)
Figure 4.7. The AMC11 gene is disrupted in the amc1 mutant.

A. The position of the insertions in the AMC11 gene in the amc1 and the amc11 mutants is represented here. In the amc1 strain, the insertional sequence is a 1.572 kb intergenic region from Chromosome 17. The gray rectangles indicate UTRs, the orange rectangles indicate the regions of the exons corresponding to the coding sequence and the black lines correspond to the introns of the AMC11 gene, as predicted by the Chlamydomonas genome database JGI v5.5. The AMC11-specific primers E2F32, E2F33, 2F2 and 1 were used in TAIL-PCR to determine the insertional sequence in the amc1 mutant. The amc1 insert-specific primer (amc1 insert F1) and AMC11-specific primer (amc11 del-1R) were used in conventional PCR to obtain the complete insertional sequence disrupting the AMC11 gene in the amc1 mutant. This molecular lesion in the AMC11 gene was confirmed from two independent amc1 strains: amc1 (4C10) and amc1 (27). The position of the insertional cassette in the amc11 mutant is also provided for comparison.

B. The molecular lesions in the AMC11 gene were analyzed using AMC11-specific primers as represented in A. Two amc1 strains, the original amc1 (4C10) and a haploid spore amc1 (27), were used to confirm the presence of the molecular lesion in the AMC11 gene. The amc11 (10G11) original strain, with its isogenic wild-type (4C-) strain and the corresponding complemented strains amc11 (AMC11) – A and –B are shown here as controls.
4.3.4. The N-terminus of AMC11 carries a mitochondrial targeting signal

According to the *Chlamydomonas* genome database JGI v5.5, the *AMC11* gene specifies a hypothetical protein that is 2566 amino acids in length and does not possess any recognizable domains indicating a possible function. No cDNA clones were available for *AMC11* to corroborate the predicted coding sequence (172). To confirm the predicted coding sequence, we performed RT-PCR on total RNA isolated from wild-type strain. Amplification of the *AMC11* cDNA was extremely challenging due to low abundance and high GC content, and we were successful in sequencing 49% of the cDNA (brown lines in Figure 4.8 A). For instance, the majority of the exon 2, constituting 40% of the cDNA, could not be successfully amplified. The high GC content of this region (72% GC content), might have been a contributing factor to this difficulty. Parts of exon 3 and exon 4 (373 bp and 404 bp, respectively) were also difficult to amplify. However, the retrieved sequences confirmed all the exon-exon junctions, except for the exon4-exon5 junction that could not be amplified. The *Chlamydomonas* RNA-Seq database (Oct.2007 Assembly) (128,221,222), corroborates the exon4-exon5 junction. From these results, we conclude that coding sequence predicted by the JGI v5.5 is experimentally corroborated. The above RT-PCR experiments were useful in retrieving the cDNA sequence spanning the 5’ UTR, exon 1 and the beginning of exon 2, which encode for the first 59 amino acids of the AMC11 protein. This cloned amplicon was used in plasmid construction for N-terminal targeting experiments (described in Section 4.2.7 and represented in Figure 4.9).
As the *amc11* mutant is interrupted in the coding sequence of the *AMC11* gene, we do not expect a full-length *AMC11* transcript to be produced. From RT-PCR analysis of the *AMC11* transcript, we observed low abundance of a chimeric transcript in the *amc11* mutant. The transcripts were restored to wild-type levels upon introduction of the *AMC11* gene (Figure 4.8 B), further confirming the molecular complementation in the *amc11 (AMC11)* strains.

If AMC11 plays a role in mitochondrial Complex I biogenesis, it is possible its mitochondrial localization is required for its function. Mitochondrial targeting to the matrix is usually facilitated by an N-terminal leader sequence forming an amphiphilic $\alpha$-helix. (223). Algorithms such as PredAlgo, Target P, MitoProtII and Predotar, have been designed to predict N-terminal mitochondrial targeting peptides from protein sequences (Table 4.6). Mitochondrial targeting of AMC11 is predicted only by the Target P and PredAlgo (Mscore >1 is indicative of specific localization) algorithms. However, these scores are not as definitive for AMC11 as for other canonical mitochondrial proteins, such as the Complex I subunit NUO5 (Table 4.6). To experimentally determine whether the N-terminus of AMC11 can act as a mitochondrial targeting sequence, we decided to use a simple heterologous expression system with the UbiG reporter protein.

The *E. coli* UbiG and the *S. cerevisiae* Coq3 are proteins involved in ubiquinone biosynthesis. In *S. cerevisiae*, mitochondrial ubiquinone biosynthesis, is essential for respiratory growth. It has been shown that *E. coli* UbiG can functionally complement the Δcoq3 yeast mutant, if UbiG is specifically targeted to
the mitochondria with an appropriate targeting signal (213). We decided to utilize this heterologous system to test whether the AMC11 N-terminus can target *E. coli* UbiG to yeast mitochondria and restore respiration. For this purpose, a construct (pAHG59) containing the sequence encoding the 1-59 amino acids of AMC11, fused to the coding sequence of the *ubiG* gene, was created. Also, constructs made by Hsu *et al*, 1996, (pAH3, pAHG, pQMG) were used as controls (213) (Figure 4.9A). The *ubiG* gene expressed without a mitochondrial targeting sequence fails to restore respiration, or respires inefficiently if multiple copies are present (213). Accordingly, we observed very inefficient growth of the Δcoq3 strain transformed with the pAHG construct that expressed *ubiG*. On the contrary, UbiG fused with the first 59 amino acids of the AMC11 N-terminal sequence restored respiratory growth (pAHG59 construct) (Figure 4.9 B). From these results, we can conclude that the AMC11 N-terminal sequence is capable of targeting a reporter protein to the yeast mitochondria. Therefore, we inferred that in *Chlamydomonas*, AMC11 localizes to the mitochondria by virtue of its N-terminal sequence.
Figure 4.8. Transcript levels in the amc11 mutant are restored by AMC11 genomic DNA.

A. The current Chlamydomonas genome database JGI v5.5 predicts the model for the AMC11 gene as represented in the top panel. This gene model was experimentally corroborated by PCR amplification using AMC11-specific primers from total cDNA, prepared from WT (4C-), as described in Section 4.2.5. The brown lines, in the bottom panel, represent the AMC11-specific cDNA that was successfully amplified and confirmed by sequencing. B. Transcript abundance of AMC11 was analyzed by RT-PCR in the wild-type (4C-), amc11 (10G11) strain and its corresponding complemented strain amc11(AMC11)-A. The AMC11-specific primers (6F2/8R), used for amplification, are represented in A with black arrows. CCDA, a gene involved in photosynthesis, was used as control for constitutive expression. Three independent biological replicates were performed, and one representative is shown in this figure.
Table 4.6. Target prediction softwares predict a possible mitochondrial localization for AMC11.

<table>
<thead>
<tr>
<th>Target Prediction Softwares</th>
<th>Scoring Method</th>
<th>Min-Max value</th>
<th>NUO5</th>
<th>AMC11</th>
</tr>
</thead>
<tbody>
<tr>
<td>TargetP</td>
<td>Probability</td>
<td>0-1</td>
<td>0.953</td>
<td>0.826</td>
</tr>
<tr>
<td>MitoProt II</td>
<td>Probability</td>
<td>0-1</td>
<td>0.8852</td>
<td>0.2313</td>
</tr>
<tr>
<td>Predotar</td>
<td>Probability</td>
<td>0-1</td>
<td>0.81</td>
<td>0.26</td>
</tr>
<tr>
<td>PredAlgo</td>
<td>Mscore</td>
<td>0-5</td>
<td>3.9227</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Algorithms such as TargetP (192), MitoProt II (193), Predotar (194) and Predalgo (195) were used to predict the mitochondrial localization of AMC11. The results are compared to NUO5, a Complex I subunit known to be localized to the mitochondria.
Figure 4.9. The N-terminus of AMC11 targets the reporter protein UbiG to the yeast mitochondria.

A. Simplified representation of the inserts carried by the different plasmids used. The construction of the plasmids is described in Section 4.2.7 and (213).

B. The respiratory deficient Δcoq3 mutant was transformed with constructs producing the UbiG reporter as a translational fusion with the 1-59 aa N-terminus of AMC11. The Δcoq3 mutant transformed with the wild-type yeast COQ3 gene (pAH3), the ubiG gene (pAHG) and the ubiG gene with the COQ3 mitochondrial targeting sequence (pQMG) were used as controls. Ten-fold serial dilution was conducted on one representative transformant and plated on solid fermentable glucose medium lacking uracil (SD-ura) or non-fermentable respiratory medium containing ethanol (YPEtOH) and incubated at 28°C for 7 days.
4.3.5. **AMC11 contributes to post-transcriptional regulation of a mitochondrial gene.**

The mitochondrial genome of *Chlamydomonas* is a 15.8 kb linear chromosome containing terminal repeats with 3’ single stranded extensions (145,224) (144). This small genome contains thirteen intronless genes, of which eight encode for proteins (Figure 4.10): *cob* (apocytochrome *b*, subunit of Complex III), *cox1* (subunit 1 of Complex IV), *nd1, nd2, nd4, nd5, nd6* (subunits of Complex I) and *rtl* (reverse-transcriptase like protein), three tRNAs (*trnW, trnQ, trnM*) and rRNAs (139). The mitochondrial genome is transcribed as two bi-directional mRNAs, which are further processed to yield the 13 individual transcripts (225).

Five subunits that are localized in the membrane arm of Complex I are encoded in the mitochondrial genome (Figure 4.10 A). To determine whether the Complex I dysfunction caused by the loss of AMC11 is due to defects in the mitochondrial expression of Complex I subunits, abundance of corresponding mitochondrial transcripts was assessed. RT-PCR analyses showed that the *nd4* transcript levels are reduced in the amc11 mutant, and restored in the presence of the wild-type AMC11 gene (Figure 4.10 C). To corroborate these preliminary results, we conducted real-time qPCR analysis and confirmed the decrease in the *nd4* transcript levels (63% reduction) in the amc11 mutant compared to the wild-type (Figure 4.11). We also recorded decrease in the levels of additional Complex I-encoding transcripts, such as *nd2, nd6* and *nd1* transcripts (70%, 31% and 65% decrease with respect to wild-type levels, respectively). It is interesting to note that
compared to the qPCR analysis, the preliminary RT-PCR analysis revealed decrease of only the \textit{nd4} transcript. It is possible that end-point analysis of RT-PCR was not as sensitive as the qPCR method, in detecting mitochondrial transcript levels. Upon complementation with the wild-type \textit{AMC11} gene, the \textit{nd4}, \textit{nd2} and \textit{nd6} transcripts were restored to wild-type levels, but \textit{nd1} transcript abundance was only partially restored. Additionally, \textit{cob} and \textit{cox1} transcript levels were also reduced by 47\% in the \textit{amc11} mutant and restored upon complementation. Due to unknown reasons, the \textit{rtl} transcript, which has a 2-fold decrease in the \textit{amc11} mutant, is not restored upon complementation with the wild-type gene (Figure 4.11).

To further corroborate that loss of AMC11 results in reduced levels of mitochondrial transcripts encoding Complex I subunits, we decided to test the \textit{amc1} mutant, which also harbors a molecular lesion in the \textit{AMC11} gene (Figure 4.13 A). The reduced abundance of transcripts encoding the Complex I subunits \textit{nd4}, \textit{nd2}, \textit{nd6} and \textit{nd1} was also observed in the \textit{amc1} mutant, thereby validating the results observed for the \textit{amc11} mutant. However, the \textit{cob}, \textit{cox1} and \textit{rtl} transcripts accumulate to wild-type levels in the \textit{amc1} mutant, as opposed to the reduced accumulation evidenced in the \textit{amc11} mutant. The \textit{nd5} transcript accumulates to wild-type levels in both the \textit{amc1} and the \textit{amc11} mutants. From these results, we can conclude that loss of AMC11 affects the abundance of the mitochondrial transcripts \textit{nd4}, \textit{nd2}, \textit{nd6} and \textit{nd1}, encoding Complex I subunits.
One possible reason for the observed reduction in transcript levels is a decrease in the copy number of the mitochondrial DNA (mtDNA) in the amc1 and the amc11 mutants. However, qPCR analysis demonstrated there is no significant change in the mtDNA content of the amc1 and amc11 mutants, compared to wild-type (Figure 4.12 A and 4.13 B).

It is intriguing that only the amc11 mutant, and not the amc1 mutant, displays a two-fold decrease in cob and cox1 transcripts, which encode for Complex III and Complex IV subunits, respectively. However, it is clear that down-accumulation of these mitochondrial transcripts does not result in a defect in Complex III or IV activity, because amc1 and amc11 do not exhibit the typical dark dier phenotype of complex III or IV deficient strains (141). Furthermore, Complex II+III activities on amc11 showed the characteristic compensatory increase in activity (Figure 4.12 B) as observed in other amc mutants, underscoring that the primary respiratory defect in amc11 is Complex I deficiency. Previous biochemical analyses also confirmed wild-type levels of Complex III and Complex IV activity in the amc1 mutant (131).

The mitochondrial genome is transcribed in a bi-directional manner, generating the left and right transcriptional units (LTU and RTU, respectively) (225). The LTU is produced by the co-transcription of the nd5, nd4 and cob genes (225,226), which are later processed to individual transcripts. In both the amc1 and the amc11 mutants, the nd5 transcript accumulate to wild-type levels (Figures 4.10 and 4.11), ruling out a defect in the transcription of the LTU. In the amc1 mutant,
the cob transcript accumulates to wild-type levels as well (Figure 4.13). In the amc11 mutant, a decrease in cob transcript levels (Figure 4.11) does not seem to affect the Complex II+III activity (Figure 4.12 B), indicating that the COB subunit is successfully expressed and incorporated to form a functional Complex III enzyme. The only other transcript, affected for accumulation in the LTU, is nd4. Previous studies on mitochondrial mutants of Chlamydomonas have revealed that, loss of ND4 results in the accumulation of a 700 kDa subcomplex (130,146), similar to the phenotype observed in the amc11 and amc1 mutants. On the other hand, loss of ND6 or ND1 subunits, transcripts of which are also reduced in the amc1 and the amc11 mutants, would result in the failure to assemble even the 700 kDa subcomplex (145,146). Hence, we hypothesize that the assembly defect, resulting in the formation of the 700 kDa subcomplex in the ammc11 and the amc1 mutants, could be due to a specific loss of ND4 expression. One approach to test this hypothesis would be to detect protein levels by immunoblotting experiments. However, an antibody that specifically recognizes the Chlamydomonas ND4 subunit is not currently available. Hence, we designed a reporter system to monitor the expression at the nd4 locus.

The reporter protein T-urf13 was utilized for this purpose. T-urf13 is a 13 kDa channel-forming protein localized to the inner mitochondrial membrane. It is produced from a chimeric ORF that arose from the recombination of maize mitochondrial DNA (227). This chimeric protein was first observed in cytoplasmic male sterile maize, that were susceptible to fungal toxins and methomyl, an
insecticide commonly used for crops (228,229). It has also been shown that expression of T-urf13 in the mitochondria of S. cerevisiae (230), P. pastoris (231) and tobacco (232) confers methomyl sensitivity. (233). Upon binding of methomyl or fungal toxins to T-urf13, the mitochondrial membranes are permeabilized, which results in the loss of membrane potential and mitochondrial function (227). Using biolistics, we engineered a Chlamydomonas strain (nd4::T-urf13) by replacing the mitochondrial nd4 coding sequence with the t-urf13 ORF, codon optimized for expression in the Chlamydomonas mitochondria (130) (Figure 4.14). This strain carries the T-urf13 ORF under the control of the 5' and 3' UTRs (58 bp and 22 bp, respectively) of the nd4 gene (Figure 4.15 A). Chlamydomonas strains carrying the mitochondrial T-urf13 gene did not display the expected methomyl sensitivity (data not shown). To assess the impact of the amc11 mutation, we generated the amc11 (nd4::T-urf13) strain by genetic crosses. In the amc11 mutant, the full-length T-urf13 transcript (normalized to cob levels) accumulates to 85% of wild-type (quantified by GelQuant software) (Figure 4.15 B and C). Interestingly, immunoblotting analysis revealed that the T-urf13 reporter protein is detected only in the presence of the wild-type AMC11 gene but not in the presence of the amc11 mutation. From these results, we concluded that AMC11 plays a role in the post-transcriptional regulation of the nd4 locus.
Figure 4.10. AMC11 is required for the accumulation of the mitochondrial nd4 transcript.

A. Simplified map of the 15.8 kb mitochondrial genome of C. reinhardtii adapted from (3). The rectangles represent the eight protein coding genes. The yellow arrows represent the terminal inverted repeats. The blue arrows indicate the direction of transcription. The left transcriptional unit (LTU) contains the cob cistron encoding apocytochrome b, a subunit of Complex III; nd4 and nd5 cistrons encoding the Complex I subunits ND4 and ND5, respectively. The right transcriptional unit (RTU) contains cox1, cistron encoding subunit I of Complex IV; nd2, nd6 and nd1 cistrons encoding the corresponding Complex I subunits; rtl, cistron encoding a reverse-transcriptase like protein. The genes, transcripts and corresponding proteins are color coded in A, B and C. B. Simplified representation of L-shaped mitochondrial Complex I, embedded in the inner mitochondrial membrane. The FMN molecule is indicated in green and the Fe-S clusters are represented in yellow. The putative location of the mitochondrially-encoded subunits of the membrane arm of Complex I are also represented here. C. RT-PCR of the eight protein-coding transcripts was conducted on the WT (4C), amc11 (10G11) and amc11 (AMC11)-A strains and the PCR products were separated by electrophoresis. The results of one representative, from three independent replicates, is shown here.
Figure 4.11. Loss of AMC11 results in the decreased accumulation of multiple mitochondrial transcripts.

Real-time quantitative PCR (qPCR) was used to assess the relative abundance of the mitochondrial transcripts, *cob*, *nd4*, *nd5*, *cox1*, *nd2*, *nd6*, *nd1* and *rtl*. The strains tested are WT (4C), *amc11* (10G11), *amc11* (AMC11)-A and −B. The relative abundance of the mitochondrial transcripts was determined with respect to the geometric mean of transcript levels of *UBI* and *CBLP*. The average is represented from three biological replicates, except for *amc11* (AMC11)-B with two biological replicates, each analyzed in three technical replicates. The error bars represent standard deviation of the mean. The results are represented as fold change relative to WT (WT set to 1.0).
Figure 4.12. The *amc11* mutant contains wild-type levels of mitochondrial DNA.

**A.** Real-time quantitative PCR (qPCR) was used to assess the relative quantity of mtDNA. The mitochondrial *nd4* gene was used as a target gene and the nuclear gene *TUA2*, encoding the alpha tubulin 2 protein, was used as the reference gene. The strains tested here are the same as in Figure 4.11. The average was obtained from two biological replicates, each including three technical replicates, and the error bars represent standard deviation of the mean. The results are represented as fold change relative to WT (WT set to 1.0). The mtDNA content in the *amc11* mutant complemented with the full cosmid 1H10 – *amc11 (AMC11)*-A, is 1.2 times more than WT. Two-tailed unequal variances *t*-test indicates a significant *p*-value = 0.03 for this apparent increase.

**B.** Complex II+III combined activities (succinate: cytochrome c oxidoreductase) was determined from four independent biological replicates. The error bars indicate standard deviation of the mean. The WT and *amc11* strains tested here are 4C and *amc11 (10G11)*, respectively.
Figure 4.13. The *amc1* mutant displays reduced levels of mitochondrial transcripts similar to the *amc11* mutant.

Real-time quantitative PCR (qPCR) was used to assess the relative abundance of the mitochondrial transcripts, *cob*, *nd4*, *nd5*, *cox1*, *nd2*, *nd6*, *nd1* and *rtl*. The strains tested are the WT (3A⁺) and *amc1* (4C10). The relative abundance of the mitochondrial transcripts was determined with respect to the geometric mean of the transcript levels of *UBI* and *CBLP*. The average is represented from three biological replicates, each analyzed in three technical replicates. The error bars represent standard deviation of the mean. The results are represented as fold change relative to WT (WT set to 1.0).
Figure 4.14. Introduction of the reporter gene T-urf13 at the mitochondrial nd4 locus.

Simplified maps of the mitochondrial genome for the wild-type, recipient strain and the resultant transformants are provided here. Biolistic transformation of the mitochondria was achieved via established protocols (130). The recipient strain (dum11) lacks the left telomere and part of the cob gene. As a result, the recipient strain is a Complex III mutant, showing the characteristic dark dier phenotype (141). The transforming DNA pVU2, contained the telomere, cob, T-urf13 ORF replacing the nd4 ORF, and partial nd5 gene, was introduced into the recipient strain by biolistics. The transformants were selected for their ability to grow in the dark. Transformants containing the reporter gene were screened via their sid phenotype, conferred by the replacement of the nd4 gene with the reporter T-urf13.
Figure 4.15. AMC11 is required for the post-transcriptional regulation of the nd4 transcript.

A. The strains containing either the wild-type AMC11 gene or the amc11 nuclear mutation, in the presence of the mitochondrial T-urf13 gene, were analyzed. The mitochondrial nd4 ORF was replaced with the T-urf13 ORF (in blue), while retaining the 5' and 3' UTRs of nd4 (in red). B. Levels of T-urf13 transcript was analyzed by RT-PCR. The cob transcript levels were used as reference. C. Accumulation of the T-URF13 reporter protein was tested by SDS-PAGE immunoblotting. Total cellular protein was extracted and 50 µg of protein was loaded per lane. Proteins were separated by a 12.5% acrylamide gel and transferred to a PVDF membrane after electrophoresis. Immunodetection was carried out using antibodies against T-urf13 and cytochrome f. The strains tested in B and C were: i) CC124 with AMC11 and nd4 genes, ii) AMC11 and nd4::T-urf13 genes, iii) amc11 mutation in the presence of nd4::T-urf13 mitochondrial reporter.
4.4. Discussion

Here we report the identification of AMC11, a novel protein involved in Complex I biogenesis. We show that 1) two insertional mutations disrupting the AMC11 gene yield a Complex I assembly defect, characterized by the accumulation of a 700 kDa subcomplex, 2) the AMC11 gene encodes a novel protein with a N-terminal extension that functions as a mitochondrial targeting sequence, 3) loss of AMC11 function results in down-accumulation of mitochondrial transcripts encoding the ND1, ND2, ND4 and ND6 subunits. Moreover, using a mitochondrial reporter system, we also show that the synthesis of the ND4 subunit is under the control of the AMC11 protein.

4.4.1. AMC11 controls the assembly of the distal end of Complex I membrane arm

Loss of function of AMC11 in the insertional mutants, amc1 and amc11, is characterized by the presence of a labile 700 kDa subcomplex, which is diagnostic of a defect in the assembly of the membrane arm of Complex I (2). Such a subcomplex is also observed upon loss of membrane arm subunits such as the nuclear-encoded NUOB10 subunit (1) and the mitochondrially-encoded ND4 and ND5 subunits (2,3). NUOB10, ND4 and ND5 are subunits are localized to the subcomplex Iβ forming the distal part of the membrane arm (Figure 1.1 B) (4). The 700 kDa subcomplex is loosely anchored to the inner mitochondrial membrane via the proximal end of the membrane arm (2). From mass spectrometry analysis, this subcomplex was shown to contain at least 16 different Complex I subunits that are part of the subcomplex Iα (corresponding to the soluble arm with NADH
dehydrogenase activity), the subcomplex Iγ (forming the proximal membrane arm) and additional plant-specific non-core subunits (2). However, subunits belonging to the subcomplex Iβ were absent, further underscoring the loss of the distal membrane arm in the 700 kDa subcomplex. Since the amc11 and amc1 strains also accumulate a labile 700 kDa subcomplex (Figure 4.4 and (1)), it is reasonable to postulate that loss of AMC11 also impairs the formation of the distal membrane arm of Complex I.

4.4.2. AMC11 is required for post-transcriptional regulation of the nd4 transcript:

We showed that the nd4, nd2, nd6 and nd1 transcripts display decrease in accumulation in the amc11 and the amc1 mutants (Figures 4.10, 4.11 and 4.13). This pleiotropic effect is caused by loss of function of AMC11 as the transcript levels are restored upon complementation of the amc11 strain. One possibility is that loss of AMC11 yields a defect in the transcription of the left and right units. However, the nd5 transcript, which originates from a primary polycistronic mRNA also containing nd4, accumulates to wild-type levels in both the amc11 and the amc1 mutants (Figures 4.10, 4.11 and 4.13) (19). This rules out the possibility that reduced levels of the nd4 transcript is due to decrease in the transcription of the left transcriptional unit.
The secondary structure of AMC11 is predicted to contain a majority of helical structures. The structure of the AMC11 protein was predicted using the online tool PredictProtein (234). The amino acid position of AMC11 is represented by the numbered axis at the top. The AMC11 sequence is represented by the thin blue line. The possible protein binding regions are predicted using the ISIS algorithm for identifying interaction sites (235) and indicated as red diamonds. A polynucleotide binding region (shown as a yellow circle) is predicted at the 1375 amino acid position (236). The secondary structure predictions, using ReProfSec algorithm, are indicated in red (helix) and blue (strand) rectangles, respectively. Further, prediction of exposed vs buried regions (blue / yellow rectangles, respectively) were determined by predicting solvent accessibility using the Profacc algorithm. Five transmembrane helices (shown as pink rectangles) are predicted for AMC11 (237). Multiple disordered regions (shown as green rectangles), that indicate regions with possible flexibility of the three-dimensional structure, are predicted for AMC11 (238).
Loss of the ND4 and the ND5 subunits in mitochondrial mutants results in impaired assembly of the distal arm and accumulation of a 700 kDa subcomplex (20-22), a trait also observed in the amc11 and amc1 mutants. In contrast, loss of ND2, ND6 and ND1 subunits yields a defect in the proximal arm of Complex I (14,20), which prevents the accumulation of the 700 kDa subcomplex. While the down-accumulation of the nd4, nd2, nd6 and nd1 mRNAs implies a defect in the formation of both the proximal and distal membrane arms of Complex I, the biochemical phenotype indicates a block in the assembly of only the distal arm due to loss of AMC11 function.

Hence, a simple hypothesis to account for the assembly defect in the distal arm in the amc11 mutant is that there is a loss of ND4 and/or ND5. Based on the fact that the abundance of the nd4 transcript is severely reduced but not that of nd5, we postulate that the AMC11-dependent assembly defect is probably due to loss of ND4. To test if the ND4 subunit was still produced in the amc11 mutant, we substituted the nd4 ORF with the reporter T-urf13 protein (Figure 4.14), and monitored its steady-state accumulation (Figure 4.15). We observed that the reporter protein was not detected in the amc11 mutant, indicating that synthesis of the ND4 protein is dependent upon AMC11. We cannot exclude the possibility that the synthesis of the ND5 protein is also under the control of AMC11.

4.4.3 AMC11, an RNA binding protein?

Our current view is that AMC11 interacts with the nd4 transcript in the mitochondria. A site of action in the mitochondrial matrix for AMC11 is supported
by the fact that the N-terminal sequence can target a matrix-localized reporter protein (Figure 4.9). While AMC11 does not display known RNA binding motifs found in proteins interacting with organellar transcripts, such as PPR (Pentatrico Peptide Repeat) or OPR (Octotrico Peptide Repeat), other features in the protein suggest a possible RNA binding activity (239), (240). First, AMC11 contains low-complexity regions (LCR), which are regions of biased amino acid composition, a feature present in several nuclear-encoded factors controlling organellar gene expression. In AMC11, the predominant amino acids are Ala (24.2%), Gly (11.3%), Leu (9.9%), and Pro (7.1%). Additional features, noted in several RNA-interacting proteins in organelles are: the propensity to form α-helical repeats (Figure 4.16) and the large size of the protein (2,566 residues). The latter seems to be a common trait in several nuclear-encoded proteins controlling chloroplast transcript stability and/or translation in Chlamydomonas, where the size ranges from 1116 amino acids to 1802 amino acids (240). In addition, a structure prediction tool PredictProtein also predicts the presence of a polynucleotide binding region in AMC11 (Figure 4.16). This further raises the possibility of a role in RNA-binding for AMC11.

4.4.4. Proposed mode of action for AMC11

We have reported that T-urf13 expression, at the mitochondrial nd4 locus, is AMC11-dependent. The T-urf13 ORF was still under the control of the 5’ and 3’ UTRs of nd4. Hence, we can infer that AMC11 controls the nd4 transcript via interaction with its UTRs. At the present time, the mode of action of AMC11 is
speculative but based on our results, we postulate that AMC11 stabilizes the \( nd4 \) transcript and also controls the synthesis of ND4. Because the \( T\)-\textit{urf13} transcript level was not as reduced as the \( nd4 \) transcript levels in the \textit{amc11} mutant (Figure 4.15), we hypothesized that elements within the coding sequence of \( nd4 \) are necessary for AMC11-dependent stabilization of the \( nd4 \) mRNA. In addition, it is also possible that AMC11-dependent stabilization is required for the \( nd2, \) \( nd6 \) and \( nd1 \) transcripts. It must be noted that, since we observe the formation of a 700 kDa subcomplex, which can happen only in the presence of the ND2, ND6 and ND1 subunits, it is possible that there is still a basal level of expression of these subunits in the \textit{amc11} and \textit{amc1} mutants, in spite of the reduction in transcript levels.

In \textit{Chlamydomonas}, two isoforms of the \( nd4 \) transcript exist in the mitochondria (226). The most abundant (90\%) was found to contain a 5’ UTR (60 nucleotides) while the other isoform (10\%) started at the initiation codon of \( nd4 \) (226). It is not known if one or possibly both transcript isoforms are competent for translation. It is conceivable that the isoform starting at AUG is the only one active in translation. This scenario is supported by the finding that 1) other protein-encoding mitochondrial transcripts (\textit{cox1}, \( nd2, \) \( nd6, \) \textit{rtl}) start directly at the initiation codon in \textit{Chlamydomonas} (241) and in several organisms like humans (242) and fission yeast (243,244) and, 2) mammalian ribosomes display a clear preference for translation initiation on 5’ UTR-less transcripts (245). It is also likely that the isoform starting at the AUG results from the processing of the 5’ UTR containing transcript but this has not been experimentally determined. While multiple models
for AMC11 action can be envisioned, we favor the following scenario where AMC11 controls translation by promoting the processing of the 5’ UTR containing isoform (5’ UTR-nd4) to the AUG starting isoform (AUG-nd4) of the nd4 transcript. Loss of ND4 synthesis in amc11 can be simply explained by the failure to generate the 5’ UTR-less nd4 transcript. If AUG-nd4 is the only isoform competent for translation, it is not obvious why mitochondria produce also the 5’ UTR-nd4. One attractive possibility is that the 5’ UTR-nd4 form acts as storage of untranslated transcripts. In such a model, we envision that “activation” of translation is dependent upon binding of AMC11 to the 5’ UTR and subsequent conversion to the AUG-nd4 isoform. Additional experiments are required to elaborate the mechanism of action of AMC11.
CHAPTER 5

CHARACTERIZATION OF AMC MUTANTS ASSEMBLING A MATURE ENZYME

5.1. Introduction

The mitochondrial genome of *Chlamydomonas* is a 15.8 kb sequence, which is predominantly linear (139,224). Each copy encodes eight protein coding genes (*nd4, nd5, nd2, nd6, nd1* – encoding corresponding Complex I subunits; *cob* – encoding Complex III apocytochrome *b*; *cox1* – encoding Complex IV subunit 1; *rtl* – encoding reverse transcriptase-like protein) (144) (Figure 4.10). These protein-coding genes are interspersed with genes encoding for three tRNAs and the large and small subunits of rRNA (144). The mitochondrial genome is co-transcribed in a bi-directional manner, with the left transcriptional unit encoding three genes (*nd5, nd4, cob*) and the right transcriptional unit consisting of five protein-coding genes (*cox1, nd2, nd6, nd1, rtl*), *tRNAW, tRNAQ, tRNAM* and modules of *rRNAs* (226,241,246,247). The long polycistronic transcripts are then further processed to yield the mature transcripts (226,241,246,247). In Chapter 4,
we described the discovery of AMC11, a novel factor involved in mitochondrial gene expression. Specifically, we found that loss of AMC11 resulted in low abundance of mitochondrial transcripts encoding Complex I subunits, including \textit{nd4}. Furthermore, we also showed that the expression of a reporter gene, from the \textit{nd4} locus, is AMC11-dependent, indicating that AMC11 is involved in post-transcriptional regulation at the \textit{nd4} locus.

In this chapter, we have extended the analysis of mitochondrial transcript abundance to additional Complex I mutants isolated from the forward genetic screen described in Chapter 2. Since Chapters 3 and 4 describe the characterization of the \textit{amc9} and the \textit{amc11} mutants, respectively, in this chapter, we shall focus on the remaining mutants \textit{amc8, amc10, amc12} and \textit{amc13}. We have previously described that the \textit{amc8} mutant exhibits drastically decreased levels of Complex I activity (21\% activity with respect to the wild-type). On the other hand, the \textit{amc10, amc12} and \textit{amc13} mutants display only partial deficiency (57\%, 40\% and 59\% activity, respectively, compared to wild-type) (Table 2.2). Additionally, analysis via BN-PAGE revealed the presence of a mature Complex I in these mutants, although at lower levels compared to wild-type. It is interesting to note that the assembly phenotype of these mutants is similar to that of the \textit{amc3} and the \textit{amc4} mutants, isolated in our laboratory from the first genetic screen (131). We describe the genetic analysis of the \textit{amc12} and the \textit{amc8} mutants and document the levels of mitochondrial transcripts in these mutants.
5.2. Experimental Procedures

5.2.1. Strains and culture conditions

Strains and culture conditions are same as described in Chapters 2, 3, and 4. Specifically, this chapter utilizes the strains amc8 (1H5) ($mt$, amc8, aph7" , arg7-8) and haploid spores derived from 1H5: amc8(2), amc8(35) and amc8(42); amc10 haploid spores derived from the original strain: amc10 (12C) ($mt$, amc10, aph7" , arg7-8) and amc10 (12D) ($mt$, amc10, aph7" , arg7-8); amc12 (6E9) ($mt$, aph7" , arg7-8) haploid spores derived from 6E9: amc12m1, amc12m2, amc12m3 and amc12m4; amc13 original strain (4C3) ($mt$, amc13, aph7" , arg7-8) or its haploid spore amc13(16) ($mt$, amc13, aph7" , arg7-8).

5.2.2. Genetic analyses

Genetic crosses were performed as described previously in Sections 2.2.8 and 3.2.2. The amc8/amcx and amc12/amcx diploids were constructed according to Tables 5.1 and 5.2, respectively.

5.2.3. DNA and RNA extractions and PCR analyses

Genomic DNA and RNA extractions were conducted as described in Sections 3.2.3 and 4.2.3. Diagnostic PCRs and TAIL-PCR analyses were performed as detailed in Sections 3.2.4 and 4.2.4. The sequence flanking the 5′-end of the insertional cassette in the gene Cre07.g329861 was retrieved by TAIL-PCR using the partially degenerate primer AD1 and iHyg3-specific primers APH7R3, APH7R4 and APH7R5 (Table 5.3). Real-time qPCR analysis, to assess mitochondrial DNA content and relative mitochondrial transcript levels, was
performed as explained in Section 4.2.5. The transcripts for three housekeeping genes *CBLP, TUA2* and *UBI* were assessed for each mutant. Two housekeeping genes, whose relative transcript levels in the mutants were comparable to wild-type, were chosen as the reference genes.

**5.2.4. Biochemical and growth analyses**

Respiratory activity assays were conducted as described in Sections 2.2.5 and 4.2.10. Blue-Native PAGE and *in-gel* activity staining for Complexes I and V were performed as described in Section 2.2.6. Ten-fold serial dilutions were performed according to Section 2.2.4.

**5.2.5. PCR-based screening of the Chlamydomonas genomic library and Biolistics.**

Screening for cosmids carrying the genes of interest, and subsequent nuclear transformations by biolistics were performed according to Sections 3.2.7 and Section 3.2.8, respectively. The cosmid 64B9, carrying the gene Cre07.g329861, was identified by PCR using the primer pair (Cre07.g329861 exon 1F / exon 1R). The cosmid 81H1, carrying the gene Cre07.g329850, was identified by amplification with the primer pair (au5.g14035 5′ UTR F1 / au5.g14035 exon 2R1). It must be noted that cosmid 81H1 does not carry a functional *ARG7* marker. Transformants were selected by co-transforming 81H1 with the pCB412 plasmid carrying the *ARG7* selectable marker. The cosmid 34C2 was identified using the primer pairs (Cre07.g330100 1F/1R) and (Cre07.g330000 1F/1R). The cosmids 64B9 and 34C2 together span wild-type genes 50 kb downstream of the insertion.
site. However, the gene Cre07.g329900 is only partially present in both cosmids, and we were unable to isolate a cosmid that carries the full sequence of this gene.

<table>
<thead>
<tr>
<th>Diploid</th>
<th>mt- Strain</th>
<th>Phenotype</th>
<th>mt- Strain</th>
<th>Phenotype</th>
<th>Diploid Phenotype</th>
</tr>
</thead>
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<tr>
<td>amc8xamc1</td>
<td>amc1 (4C10)</td>
<td>arg-, HyB^R</td>
<td>amc8 (42)</td>
<td>arg+, HyB^B</td>
<td>arg+, HyB^R</td>
</tr>
<tr>
<td>amc8xamc2</td>
<td>amc2 (169dn26)</td>
<td>arg-, HyB^S</td>
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<td>arg+, HyB^R</td>
<td>arg+, HyB^R</td>
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<tr>
<td>amc8xamc3</td>
<td>amc8 (35)</td>
<td>arg+, HyB^B</td>
<td>amc3 (17G7)</td>
<td>arg-, HyB^R</td>
<td>arg+, HyB^R</td>
</tr>
<tr>
<td>amc8xamc4</td>
<td>amc8 (35)</td>
<td>arg+, HyB^B</td>
<td>amc4 (A4G7)</td>
<td>arg-, HyB^R</td>
<td>arg+, HyB^R</td>
</tr>
<tr>
<td>amc8xamc6</td>
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<td>arg+, Pm^R</td>
<td>amc8 (1H5)</td>
<td>arg-, HyB^R</td>
<td>arg+, Pm^R, HyB^R</td>
</tr>
<tr>
<td>amc8xamc7</td>
<td>amc7 (111A4)</td>
<td>arg+, Pm^R</td>
<td>amc8 (1H5)</td>
<td>arg-, HyB^R</td>
<td>arg-, Pm^R, HyB^R</td>
</tr>
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<td>amc8xamc8</td>
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<td>arg+, HyB^B</td>
<td>amc8 (1H5)</td>
<td>arg-, HyB^R</td>
<td>arg+, HyB^R</td>
</tr>
<tr>
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<td>amc8 (35)</td>
<td>arg+, HyB^B</td>
<td>amc9 (41D9)</td>
<td>arg-, HyB^R</td>
<td>arg+, HyB^R</td>
</tr>
<tr>
<td>amc8xamc10</td>
<td>amc10 (12C)</td>
<td>arg-, HyB^R</td>
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<td>amc8xamc13</td>
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<td>arg-, HyB^R</td>
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</tbody>
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Table 5.1. Construction of amc8/amcx diploids.
The mating type minus (mt-) strains were crossed with mating type plus (mt+) strains to obtain diploids with specific phenotypes for selection. The amc8/amcx diploids were generated by using either the original amc8 (1H5) strain or haploid spores derived from the original strain, such as amc8(2), amc8(35) and amc8(42).

Key: arg+ : arginine prototrophic strain; arg- : arginine auxotrophic strain; HyB^R : hygromycin B resistant; HyB^S: hygromycin B sensitive; Pm^R : paromomycin resistant. All arginine auxotrophic strains carry the arg7-8 mutation.
The mating type minus (mt) strains were crossed with mating type plus (mt+) strains to obtain diploids with specific phenotypes for selection. The amc12/amcx diploids were generated by using either the original amc12 (6E9) strain or haploid spores derived from the original strain, such as amc12m2, amc12m1 or amc12m4. The cross highlighted in gray was not successful in generating diploids.

### Key:
- arg+: arginine prototrophic strain
- arg-: arginine auxotrophic strain
- HyB\(^R\): hygromycin B resistant
- HyB\(^S\): hygromycin B sensitive
- Pm\(^R\): paromomycin resistant

### All arginine auxotrophic strains carry the arg7-8 mutation.

#### Table 5.2. Generation of \textit{amc12/amcx} and \textit{amc12m2/amcx} diploids.

The mating type minus (mt) strains were crossed with mating type plus (mt+) strains to obtain diploids with specific phenotypes for selection. The \textit{amc12/amcx} diploids were generated by using either the original \textit{amc12} (6E9) strain or haploid spores derived from the original strain, such as \textit{amc12m2}, \textit{amc12m1} or \textit{amc12m4}. The cross highlighted in gray was not successful in generating diploids.

- arg+: arginine prototrophic strain
- arg-: arginine auxotrophic strain
- HyB\(^R\): hygromycin B resistant
- HyB\(^S\): hygromycin B sensitive
- Pm\(^R\): paromomycin resistant
### Table 5.3. Sequence of primers

List of primers used for diagnostic PCR and TAIL-PCR experiments described in Chapter 5.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’ to 3’)</th>
<th>Target</th>
</tr>
</thead>
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<td>APH7F10</td>
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<td>iHyg3</td>
</tr>
<tr>
<td>APH7F8</td>
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<td></td>
</tr>
<tr>
<td>APH7R3</td>
<td>AGAATTCCCTGCTGTTCCGCAAGTAGAATCATCGGAATGACAGG</td>
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</tr>
<tr>
<td>APH7R4</td>
<td>TAGGATCCATCCGAATCAATACGCGTCGAGAGTGAAACAGG</td>
<td></td>
</tr>
<tr>
<td>APH7R5</td>
<td>CCGGTCGAGAGTGAAACAGG</td>
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</tr>
<tr>
<td>au5.g14035 5'UTR F1</td>
<td>AACGTAACGTGGAATTGGCGTCACCAGTCATCCATAC</td>
<td>Cre07.g329850</td>
</tr>
<tr>
<td>au5.g14035 exon2R1</td>
<td>CGTCAACGTGTAATCCATC</td>
<td></td>
</tr>
<tr>
<td>Cre07.g329861 exon1F</td>
<td>AGACCTAGCTCTGTCGGTTTGAGGTAAAGGACACCGGAAAGCACTGACTGCTGAGAAGAGAACACCCGCCCAACAGG</td>
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<tr>
<td>Cre07.g329861 exon1R</td>
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</tr>
<tr>
<td>Cre07.g329861 exon1R2</td>
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<td>Cre07.g329861 exon3R1</td>
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</tr>
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<td>Cre07.g330000 1F</td>
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<td>Cre07.g330000 1R</td>
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<td>Cre07.g330100</td>
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<tr>
<td>Cre07.g3301001R</td>
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5.3. Results

5.3.1. The amc12 mutation co-segregates with the iHyg3 cassette.

The *amc12* original mutant (6E9) displays partial Complex I deficiency and accumulates lower levels of mature Complex I (Chapter 2). In addition, we established that the mutation in the *amc12* (6E9) strain is recessive with respect to the wild-type allele. To test if the *amc12* mutation is linked to the insertional cassette, we monitored the segregation of the *sid* phenotype in the meiotic progeny obtained from cross I (Figure 5.1 A). Only the recombinant haploid spores were selected (total: 85), and all displayed a *sid* phenotype. From these results, we initially concluded that the *amc12* mutation is linked to the insertional cassette. We reasoned that co-segregation of the *sid* phenotype with HyBR, could be due to the insertion of the cassette in a gene required for Complex I function. To identify the gene interrupted by the cassette, we performed TAIL-PCR analysis and retrieved the genomic sequence flanking the cassette in the *amc12* (6E9) mutant. The iHyg3 cassette was mapped to the fourth exon of a novel gene, designated as Cre07.g329861 by the Chlamydomonas genome database JGI v5.5 (Figure 5.1 B). Additionally, we identified the presence of a 202 bp DNA sequence, which is neither part of the *Chlamydomonas* genome nor the insertional cassette, but which had co-integrated at the 5'-end of the cassette. Similarity searches revealed that this sequence is 75% identical to Zebrafish DNA. We had previously reported a similar co-integration of sequences in the *amc11* mutant (Chapter 4). These results further corroborate the possibility that the herring sperm DNA, used as carrier DNA
during transformation (154), co-integrates into the genome of the mutants. In the
amc12 mutant, this co-integration is accompanied by the deletion of the first 4 bp
of the iHyg3 cassette (Figure 5.1 B).

To test if the disrupted gene, Cre07.g329861, is responsible for the Complex I defect in the amc12 mutant, we transformed the 6E9 strain with the wild-type gene by biolistics (Cosmid 64B9, see Section 5.2.5) and examined the transformants for complementation of the sid phenotype. Among the 127 transformants we tested, none of them were restored for growth in the dark (data not shown). We also tested selected transformants and measured rotenone-sensitive NADH: duroquinone oxidoreductase activity, but did not observe restoration of Complex I activity either (data not shown). Transformation of the 6E9 strain with cosmids, 81H1 and 34C2, carrying the genes (Cre07.g329650, Cre07.g329700, Cre07.g329750, g7672, g7673, Cre07.g329800, Cre07.g329850, Cre07.g329950, Cre07.g330000, Cre07.g330050), neighboring the Cre07.g329861 gene, also failed to yield phenotypically rescued transformants (data not shown). Because the three cosmids 64B9, 81H1 and 34C2 cover 33 kb upstream and 50 kb downstream of the site of insertion, we concluded that 1) the original amc12 mutant phenotype is not caused only by the cassette, and 2) the phenotype is not caused by a molecular lesion immediately adjacent to the inserted cassette.
5.3.2. The amc12 (6E9) mutant carries two unlinked amc mutations, each contributing to Complex I deficiency.

To determine the reason for the inability to complement the amc12 mutant with the wild-type Cre07.g329861 gene, we decided to conduct detailed genetic analysis of the amc12 (6E9) mutant. Although our first preference was to conduct tetrad analysis of amc12xWT meiotic zygo- tes, these studies were unsuccessful due to poor germination of the zygotes. Hence, we obtained meiotic progeny from a cross of amc12(6E9) x WT (Figure 5.2 A) and monitored the segregation of the sid phenotype. All the HyB\(^R\) spores (total: 79) displayed sid phenotype, corroborating our previous observation that the sid phenotype co-segregates with the iHyg3 cassette.

Intriguingly, we observed that 35% of the HyB\(^S\) spores also displayed a sid phenotype and chose one representative HyB\(^S\) spore amc12m2 and HyB\(^R\) spore amc12m3 (mt\(^+\)) for further analysis. While silencing of the aph7\(^+\) gene could account for the HyB-sensitivity, diagnostic PCR in the amc12m2 and m3 spores ruled out this possibility, as the HyB\(^S\) amc12m2 spore failed to amplify the iHyg3 cassette (Figure 5.2 B).

Additionally, we measured rotenone-sensitive NADH: duroquinone oxidoreductase activity (Figure 5.2 D) and confirmed that amc12m2 displays Complex I deficiency. Analysis via BN-PAGE and subsequent \textit{in-gel} Complex I staining (Figure 5.2 E) showed that the amc12m2 spore assembles a mature complex (950 kDa), similar to original amc12 (6E9) strain. Although the 6E9 and
m2 strains grow significantly slower in the dark compared to the wild-type strain, we noted that the amc12m2 has a less pronounced sid phenotype than the amc12 (6E9) strain and accordingly exhibits higher Complex I activity compared to the original strain (Figure 5.2 C).

Taken together, our results indicate that the sid phenotype co-segregates with the iHyg3 cassette in all HyBR spores but is also observed independent of the insertional cassette in a fraction of the HyBS spores. Hence, we postulate that the original amc12 mutant contains two amc mutations, one tightly linked to the cassette and the second unlinked to the cassette.
A. Genetic crosses were conducted by mating wild-type strain CC125 (mt*) with the original amc12 (6E9) strain (mt, amc12, aph7, arg7-8). The meiotic progeny was obtained by bulk germination and plated on selective TAP+HyB solid media to select haploid spores with recombinant genotypes. The spores were replica-plated on TARG solid medium, incubated in the light or in the dark, and scored for the sid phenotype after 10 days of incubation.

B. The position of the insertional cassette in the gene Cre07.g329861 in the amc12 mutant is indicated. The purple rectangle in the inserted sequenced represents the 202 bp of sequence that co-integrated with the cassette at this location. The gray rectangles indicate the 5' and 3' UTRs of the gene. The yellow rectangles indicate regions of the exons corresponding to the coding sequence, and the thin lines represent introns, as predicted by the Chlamydomonas genome database JGI v5.5. The green arrow represents the extent of the genomic sequence, flanking the cassette insertion site, retrieved by TAIL-PCR and conventional PCR. The brown arrows indicate the iHyg3 specific primers (APH7R5 and APH7F10) that were used for retrieving the genomic sequence flanking the cassette insertion site. The black arrows represent the gene-specific primers (Cre07.g329861 exon1-F, Cre07.g329861 exon1R3, Cre07.g329861 exon1R2, Cre07.g329861 exon3R1) used for diagnostic PCR amplification in Figure 5.2 B. The sequence of the primers are provided in Table 5.3.
In this figure, the WT, *amc12*, *amc12m2* and *amc12m3* strains used were 4C−, *amc12 (6E9)* strain (*mt*, *aph7"*, *arg7-8*), *amc12m2 (mt*) and *amc12m3 (mt*, *aph7"*), respectively.

A. Genetic crosses were conducted by mating WT strain 1′ (*mt*) with the original *amc12 (6E9)* strain (*mt*, *aph7"*, *arg7-8*). The meiotic progeny, generated by bulk germination, was plated on TARG to obtain spores with both parental and recombinant genotypes. The growth phenotype of the spores was scored by replica-plating on TARG medium and incubating in the light and in the dark. One HyB spore *amc12m2 (mt*) and another HyB spore *amc12m3 (mt*, *aph7")*, both displaying the sid phenotype were chosen for further analysis. B. Diagnostic PCR was performed to confirm the absence of the insertional cassette in the HyB *amc12m2* spore. PCR amplification was conducted using primers depicted in Figure 5.1 B. C. The sid phenotype of the *amc12m2* spore was confirmed by ten-fold dilution series. Dilutions were plated on acetate containing medium and incubated in the light and in the dark for 10 days. D. Complex I (rotenone-sensitive NADH: dehydrogenase) activity was determined with partially purified membranes. The activities are represented as the average of six biological replicates with the error bars indicating standard deviation of the mean. Both *amc12* and *amc12m2* strains display significant decrease in Complex I activity with respect to WT as determined by two-tailed unequal variances t-test. *** indicates p-value < 0.001. E. Blue-Native PAGE was conducted on 150 µg of partially purified membranes. In-gel Complex I activity was detected by NBT staining. In-gel ATPase staining to detect Complex V was conducted to confirm equal protein loading.

**Figure 5.2.** The *amc12* mutant contains a second *amc* mutation, unlinked to the cassette.
5.3.3. Analysis of amc12/amcx diploids.

To determine if the amc12 mutations are allelic to the amc1-to-13 mutations, we performed a test of allelism test by studying amc12/amcx diploids (as described in Table 5.2). Diploids were generated with two amc12 strains: amc12 (6E9) original strain (carrying two mutations) and amc12m2 haploid spore (carrying one mutation unlinked to the cassette) (Table 5.2). The resulting diploids were monitored for growth in the dark via ten-fold dilution series (Figure 5.3) and selected diploids were further analyzed for Complex I assembly (via BN-PAGE analysis, Figure 5.4 A) and activity (via rotenone-sensitive NADH: duroquinone oxidoreductase activity, Figure 5.4 B). A summary of conclusions drawn from all the diploid analyses conducted in this thesis is available in Table 5.4.

i) Analysis of amc12(6E9)/amcx diploids:

We observed that the amc12/amc1 and amc12/amc7 diploids were complemented for growth in the dark (Figure 5.3). Additionally, amc12/amc1 diploids also assemble a mature complex (Figure 5.4 A). On the other hand, the amc12/amc8 diploid did not display restoration of growth in the dark. However, analysis via BN-PAGE revealed that the amc12/amc8 diploid assembles wild-type levels of mature complex and exhibits wild-type levels of Complex I activity (Figure 5.4). From these results, we concluded that the AMC12 loci are distinct from the AMC1, AMC7 or AMC8 loci.

On the other hand, the amc12/amc13 diploids (D1, D2 and D3), obtained from three independent crosses, failed to restore the sid phenotype and Complex
I proficiency. These \textit{amc12/amc13} diploids display Complex I activities similar to that of the \textit{amc12/amc12} or the \textit{amc13/amc13} diploids, which are homozygous for the \textit{amc12} and \textit{amc13} mutations, respectively (Figure 5.4 B). Further genetic analysis of \textit{amc12/amc13} meiotic progeny need to be conducted to confirm allelism.

ii) Analysis of \textit{amc12m2/amcx} diploids

The \textit{amc12m2/amc2} and \textit{amc12m2/amc3} diploids were restored for growth in the dark and assembled wild-type levels of mature Complex I. However, the \textit{amc12m2/amc4} diploids were not rescued for the \textit{sid} phenotype. Furthermore, we could not detect any Complex I in the \textit{amc12m2/amc4} diploids, similar to the \textit{amc4} parental strain. Finally, the \textit{amc12m2/amc11} diploid displayed partial rescue of growth in the dark and Complex I activity and was able to accumulate wild-type levels of mature Complex I. From these results, we concluded that the locus defined by the \textit{m2} mutation is distinct from the \textit{AMC2, AMC3} and \textit{AMC11} loci. Further genetic analysis, by studying the segregation of the \textit{sid} phenotype in meiotic progeny, needs to be conducted to test for allelism between the \textit{m2} mutation and the \textit{amc4} mutation.

In conclusion, the \textit{AMC12} loci are distinct from the \textit{AMC11/1, AMC2, AMC3, AMC7} and \textit{AMC8} loci.

5.3.4. \textit{The \textit{amc12m2} strain exhibits low abundance of mitochondrial transcripts.}

The \textit{amc12} original strain is characterized by reduced accumulation of a mature Complex I (Figure 5.2 E). A mitochondrial \textit{nd5} mutant (\textit{dum5}) also displays
reduced abundance of mature complex due to a single nucleotide deletion in the 3’ UTR of the mitochondrial *nd5* gene, resulting in decreased levels of the mature *nd5* transcript (145). Therefore, it is possible that the diminished accumulation of mature Complex I observed in the *amc12* mutant is due to low abundance of nuclear- and/or mitochondrially-encoded Complex I subunits. We have already shown that the *amc12* mutant is characterized by reduced steady-state levels of three nuclear-encoded Complex I subunits (49 kDa, 51 kDa and TYKY) (Chapter 2). To test whether the expression of mitochondrial genes could also be affected, we determined the relative transcript levels of protein-coding genes by real-time qPCR (Figure 5.5) in the *amc12m2* strain, which is a monogenic mutant. All the protein-coding transcripts accumulated at least two-fold lower than wild-type. In particular, the *nd2* and *nd6* transcripts, encoding Complex I subunits, were reduced to 10% and 14% of wild-type levels, respectively, whereas other Complex I subunit encoding transcripts were present at 30-40% of wild-type levels. One possible explanation to account for such a pleiotropic down-accumulation of transcript levels is that the *amc12 m2* mutation causes depletion of mitochondrial DNA. However, we found that mtDNA content in this mutant was comparable to wild-type levels (Figure 5.5 B). From these results, we conclude that the mitochondrial transcript levels are affected in the *amc12m2* strain.
The amc12/amcx diploids were generated according to Table 5.2. Diploids were tested for the growth phenotype by ten-fold dilution series that were plated on acetate containing medium and incubated in the light or in the dark for 16 days. One or two representative diploids (out of two diploids that were tested) are depicted here. Two dilutions are shown here. The growth of the following diploids are depicted in panels a) to k) wherein WT (4C-) and the respective parental strains were used as controls. The top panels a) to e) depict the diploids constructed with the original amc12 strain (6E9), whereas the bottom panels f) to j) depict the diploids generated with the amc12m2 spore: a) amc1xamc12, b) amc7xamc12, c) amc8xamc12, d) amc12xamc12, amc13xamc13, amc12xamc13, e) amc7xamc13, f) amc2xamc12m2, g) amc3xamc12m2, h) amc4xamc12m2, i) amc10xamc12m2 (the amc10 control strain shown here is amc10 (12C) and, j) amc12xamc12m2.

Figure 5.3. Analysis of amc12/amcx diploids
Figure 5.4. Biochemical analyses of amc12/amcx diploids.

A. Blue-Native PAGE was conducted on 150 µg of partially purified membranes. In-gel Complex I activity was detected by NBT staining. The WT strain is 4C, and the amc strains are the parental strains used for generating the diploids (Table 5.2). One representative diploid from each cross is shown in panels a) to g). Panel a) amc1xamc12, b) amc2xamc12m2, c) amc3xamc12m2, d) amc4xamc12m2, e) amc8xamc12, f) amc10xamc12m2, g) amc11xamc12m2. B. Complex I (rotenone-sensitive NADH: dehydrogenase) activity was determined with partially purified membranes. The activities are represented as the average of three biological replicates with the error bars indicating standard deviation of the mean. A significant decrease in Complex I activity, with respect to WT, was determined by two-tailed unequal variances t-test. * indicates p-value < 0.05, and ** indicates p-value <0.01.
Figure 5.5. The *amc12 m2* nuclear mutation affects the accumulation of all the protein-coding mitochondrial transcripts.

**A.** Real-time quantitative PCR (qPCR) was performed to assess the relative abundance of the mitochondrial transcripts, *cob, nd4, nd5, cox1, nd2, nd6, nd1* and *rtl*. The strains tested are WT (4C⁻) and *amc12m2*. The relative abundance of the mitochondrial transcripts was determined with respect to the geometric mean of the of *UBI* and *TUA2* transcript levels. The average is represented from three biological replicates, each analyzed in three technical replicates. The error bars represent standard deviation of the mean. The results are represented as fold change relative to WT (WT set to 1.0).

**B.** Real-time quantitative PCR (qPCR) was used to assess the relative quantity of mtDNA. The mitochondrial *nd4* gene was used as a target gene and the nuclear gene *TUA2*, was used as the reference gene. The average was obtained from two biological replicates, each including three technical replicates, and the error bars represent standard deviation of the mean. The results are represented as fold change relative to WT (WT set to 1.0).
5.3.5. The amc8 mutant is characterized by reduced mitochondrial transcript levels.

We have previously observed that the amc8 mutant exhibits a diminished accumulation of Complex I with reduced levels of nuclear-encoded subunits (Chapter 2), similar to the amc12 mutant. In order to determine whether the amc8 mutant also behaves like the amc12 mutant with respect to mitochondrial expression, we analyzed the transcript levels of the mitochondrial protein-coding genes by real-time PCR. In the amc8 strain, all eight protein-coding mitochondrial transcripts were diminished, compared to wild-type (Figure 5.6 A). We also found that the amc8 mutant harbors wild-type levels of mtDNA (Figures 5.6 B), and we can therefore rule out the possibility that reduced transcript levels is due to decrease in mtDNA copy number. Furthermore, the amc8 mutant displays wild-type levels of Complex IV activity and increased levels of Complex II+III activity, a compensatory trait previously observed in Complex I mutants (131). Therefore, the decrease in transcript levels for cob and cox1, in the amc8 mutant, are not reflected in the Complex III or IV activity. From these results, we concluded that the amc8 mutant is characterized by reduced levels of mitochondrial transcripts encoding Complex I subunits.

From previous analyses, we know that the amc8 mutation is recessive, monogenic and unlinked to the insertional cassette. To determine if the amc8 mutation defines a novel locus or maps to the other genetically defined AMC loci, we constructed amc8/amcx diploids as described in Table 5.1. These diploids were
tested for growth in the dark by ten-fold dilution series (Figure 5.6). We observed that all the diploids were restored for growth in the dark, thereby indicating that the \textit{AMC8} locus is distinct from the \textit{AMC1-to-13} loci and defines the 8\textsuperscript{th} complementation group.
A. Real-time quantitative PCR (qPCR) was performed to assess the relative abundance of the mitochondrial transcripts, cob, nd4, nd5, cox1, nd2, nd6, nd1 and rtl. The strains tested are WT (4C⁻) and amc8 (1H5). The relative abundance of the mitochondrial transcripts was determined with respect to the geometric mean of the transcript levels of UBI and TUA2. The average is represented from three biological replicates, each analyzed in three technical replicates. The error bars represent standard deviation of the mean. The results are represented as fold change relative to WT (WT set to 1.0). B. Real-time quantitative PCR (qPCR) was used to assess the relative quantity of mtDNA. The mitochondrial nd4 gene was used as a target gene and the nuclear gene TUA2, encoding the alpha tubulin 2 protein, was used as the reference gene. The average was obtained from two biological replicates, each including three technical replicates, and the error bars represent standard deviation of the mean. The results are represented as fold change relative to WT (WT set to 1.0). C. Complex II+III combined activities (succinate: cytochrome c oxidoreductase) was determined from four independent biological replicates. The error bars indicate standard deviation of the mean. The dum11 strain is a Complex III mutant (214). D. Complex IV activity (cytochrome c oxidase) is represented as an average of three biological replicates. The error bars indicate standard deviation of the mean. dum18 is a Complex IV mutant (143). The WT and amc11 strains tested here are 4C⁻ and amc11 (10G11), respectively.
The amc8/amc8 diploids were generated by genetic crosses according to Table 5.1. Complementation was assessed by observing growth in the dark via ten-fold dilution series. Dilutions were plated on acetate containing medium and incubated in the light and in the dark for 12 days. Out of six dilutions, two dilutions are represented here. One representative diploid from each cross is shown here. The growth of the following diploids are shown in panels a) to l), wherein WT and amc8 strains shown are 4C- and amc8 (1H5), respectively. The other amc strains shown are the respective parental strains, used for constructing the diploids (Table 5.1). Panel a) amc1xamc8, b) amc2xamc8, c) amc3xamc8, d) amc4xamc8, e) amc6xamc8, f) amc7xamc8, g) amc8xamc8, h) amc9xamc8, i) amc10xamc8, j) amc11xamc8, k) amc12xamc8 and, l) amc13xamc8.

Figure 5.7. The AMC8 locus defines the 8th complementation group.
5.3.6. Characterization of the amc10 and amc13 mutants.

The amc10 and the amc13 mutants are partially deficient for Complex I and assemble lower levels of mature complexes that can be detected via in-gel Complex I staining. The amc8 and amc12m2 strains, which also assemble a mature complex, exhibited a general decrease in all protein-coding transcripts. We decided to test whether reduced levels of mitochondrial transcripts was a general phenotype of Complex I deficient strains that display decreased levels of mature Complex I. Using qPCR, we found that the amc10 (12D) strain displays 50-60% reduction in transcript levels only for nd2, nd1, and rtl transcripts (Figure 5.8 A). The remaining protein-coding transcripts accumulated to wild-type levels. This reduction in transcript levels is observed despite the fact that there is a 60% increase in mtDNA in the amc10 (12D) strain compared to the wild-type strain (Figure 5.8 B). On the other hand, the amc13 mutant, which contains wild-type levels of mtDNA, exhibits wild-type levels of transcripts encoding Complex I and Complex IV subunits and a five-fold increase in the cob transcript encoding the Complex III subunit (Figure 5.9). From these results, we concluded that a reduction in mitochondrial transcript levels is not a general phenotype of all amc mutants.

Since we observed a 1.6-fold increase in mtDNA content in the amc10 (12D) strain, we wanted to test whether the mtDNA content was affected in any other amc mutant. For this purpose, the relative mtDNA quantity was measured by real-time qPCR for amc1-to-13 (Figure 5.10). In the majority of the mutants, the relative mitochondrial DNA content is not affected. Another haploid spore amc10
(12C), derived from the *amc10* original strain, does not exhibit an increase in mtDNA content. The reason for the difference in mtDNA content between two haploid spores, both derived from the same original strain, is currently unknown but it might not be dependent on the *amc10* mutation. Intriguingly, we found that the *amc3* mutant contains only 16% of mtDNA compared to WT.
Figure 5.8. The *amc10* mutant accumulates reduced levels of *nd1* and *nd6* transcripts.

A. Real-time quantitative PCR (qPCR) was performed to assess the relative abundance of the mitochondrial transcripts, *cob*, *nd4*, *nd5*, *cox1*, *nd2*, *nd6*, *nd1* and *rtl*. The strains tested are WT (4C) and *amc10* (12D). The relative abundance of the mitochondrial transcripts was determined with respect to the geometric mean of the transcript levels of *UBI* and *CBLP*. The average is represented from three biological replicates, each analyzed in three technical replicates. The error bars represent standard deviation of the mean. The results are represented as fold change relative to WT (WT set to 1.0).

B. Real-time quantitative PCR (qPCR) was used to assess the relative quantity of mtDNA. The mitochondrial *nd4* gene was used as a target gene and the nuclear gene *TUA2*, encoding the alpha tubulin 2 protein, was used as the reference gene. The average was obtained from two biological replicates, each including three technical replicates, and the error bars represent standard deviation of the mean. The results are represented as fold change relative to WT (WT set to 1.0). *amc10* (12D) displays significantly higher levels of mtDNA as determined by two-tailed unequal variances t-test. ** indicates *p*=0.002.
Figure 5.9. The amc13 nuclear mutation does not result in the down-accumulation of mitochondrial transcripts.

A. Real-time quantitative PCR (qPCR) was performed to assess the relative abundance of the mitochondrial transcripts, cob, nd4, nd5, cox1, nd2, nd6, nd1 and rtl. The strains tested are WT (4C-) and amc13 (16). The relative abundance of the mitochondrial transcripts was determined with respect to the geometric mean of the transcript levels of CBLP and TUA2. The average is represented from three biological replicates, each analyzed in three technical replicates. The error bars represent standard deviation of the mean. The results are represented as fold change relative to WT (WT set to 1.0). B. Real-time quantitative PCR (qPCR) was used to assess the relative quantity of mtDNA. The mitochondrial nd4 gene was used as a target gene and the nuclear gene TUA2, was used as the reference gene. The average was obtained from two biological replicates, each including three technical replicates, and the error bars represent standard deviation of the mean. The results are represented as fold change relative to WT (WT set to 1.0).
Real-time quantitative PCR (qPCR) was used to assess the relative quantity of mtDNA. The mitochondrial \textit{nd4} gene was used as a target gene and the nuclear gene encoding α-tubulin 2 was used as reference gene. All \textit{amc} mutants tested here are the original strains except for \textit{amc10} and \textit{amc13}, which are \textit{amc10 (12C)} and \textit{amc13 (16)}, respectively. The average was obtained from two biological replicates, each including three technical replicates, and the error bars represent standard deviation of the mean. The results are represented as fold change relative to WT (WT set to 1.0). The \textit{amc3} strain displays significantly reduced levels of mtDNA compared to WT as determined by two-tailed unequal variances \textit{t}-test. ** indicates \( p=0.00173 \).

**Figure 5.10. Relative mitochondrial DNA content is reduced in \textit{amc3}.

Real-time quantitative PCR (qPCR) was used to assess the relative quantity of mtDNA. The mitochondrial \textit{nd4} gene was used as a target gene and the nuclear gene encoding α-tubulin 2 was used as reference gene. All \textit{amc} mutants tested here are the original strains except for \textit{amc10} and \textit{amc13}, which are \textit{amc10 (12C)} and \textit{amc13 (16)}, respectively. The average was obtained from two biological replicates, each including three technical replicates, and the error bars represent standard deviation of the mean. The results are represented as fold change relative to WT (WT set to 1.0). The \textit{amc3} strain displays significantly reduced levels of mtDNA compared to WT as determined by two-tailed unequal variances \textit{t}-test. ** indicates \( p=0.00173 \).
Table 5.4. Summary of diploid complementation studies.

Tables A) and B) provide a summary of the genetic complementation studies described in this thesis. The cells highlighted in gray indicate diploid combinations that could not be generated. The symbol (+) indicates complementation, the symbol (−) indicates non-complementation and the symbol (+/−) indicates partial complementation, as analyzed by studying the growth phenotype of the diploids. The symbol (*) indicates that the diploid’s phenotype has been tested via BN-PAGE analysis or rotenone-sensitive NADH: duroquinone oxidoreductase activities. The symbol (**) indicates that the diploid’s phenotype has been tested by both BN-PAGE and rotenone-sensitive NADH: duroquinone oxidoreductase activity assays.
5.4. Discussion

In this chapter, we described the genetic and molecular analysis of the Complex I deficient mutants, \textit{amc8}, \textit{amc10}, \textit{amc12} and \textit{amc13}, which do assemble a mature complex I, albeit a reduced amount.

\textit{5.4.1. Genetic analysis of the amc mutants.}

Genetic analysis established that the \textit{amc12} original (6E9) strain contains two \textit{amc} mutations, one of which is tightly linked to the insertional cassette. The cassette was mapped to a novel gene Cre07.g329861, encoding a large hypothetical protein (3253 amino acids) with low-complexity regions and no easily recognizable motifs to predict function, similar to AMC11 studied in Chapter 4. Interestingly, a mitochondrial targeting sequence is predicted at the N-terminus, by online target prediction algorithms, an indication that the protein might reside inside the mitochondria. The role of this protein in Complex I activity needs to be confirmed by complementation of a monogenic haploid spore carrying only the insertional mutation. Further genetic analysis has now confirmed the \textit{amc12m2} mutation is monogenic and genetically unlinked to the insertional cassette (A. Castonguay, personal communication).

The \textit{amc13/amc12}, \textit{amc13/amc11} and \textit{amc13/amc9} diploids are not complemented for their \textit{sid} phenotype (Table 5.4). We already know that the \textit{AMC9} locus, which corresponds to the \textit{NUO5} gene encoding Complex I subunit (Chapter 3), is distinct from the \textit{AMC11/1} locus, which encodes for a novel protein (Chapter 4). Furthermore, the \textit{amc9/amc11} diploids were restored for growth in the dark,
confirming that they are not allelic. Hence, we conclude that it is a case of non-allelic non-complementation (248) involving the amc13 mutant allele.

5.4.2. **Effect of nuclear amc mutations on mitochondrial DNA and transcript levels.**

All amc mutants analyzed in this chapter, with the exception of amc13, display a low abundance of mitochondrial transcripts. However, the transcripts that are affected vary based on the amc mutation. While amc8 and amc12 mutants display reduced levels of all protein-coding transcripts, amc10 exhibits reduced levels of specific transcripts only. Since amc13 is not associated with low abundance of the tested transcripts, we can conclude that mitochondrial transcript reduction is not a general trait of the amc mutations. Interestingly, the mutants displaying low abundance of mitochondrial transcripts are still capable of assembling a mature Complex I, which implies that reduction in transcript levels does not result in a complete loss of synthesis of the corresponding subunits (3,56,130,145,146). However, *in-organello* translation experiments (249) will help us determine whether the translation of the mitochondrially-encoded Complex I subunits is affected in these mutants, thereby resulting in lower accumulation of a mature complex.

So far, the identity of the AMC3, AMC8, AMC10, AMC12 and AMC13 genes has not been determined. Hence the function of their gene products can only been speculated upon. One possibility is that these mutants harbor mutations in genes encoding Complex I subunits. Examples of such mutations, that result in Complex I deficiency, where a mature complex is detected, have been previously reported.
in *Chlamydomonas*. For instance, an L157P substitution in the *Chlamydomonas* mitochondrial ND4 subunit results in the formation of the mature 950 kDa enzyme that is still severely affected for NADH: duroquinone oxidoreductase activity (148). In another instance, a single nucleotide deletion in the 3′ UTR of the *nd5* gene causes reduction in transcript levels and lower abundance of the mature enzyme and consequently, reduced Complex I activity (145). Similarly, insertional mutations in the promoter and 3′ UTR of nuclear genes encoding ND9 and NUOP4 subunits, respectively, also resulted in the accumulation of reduced levels of the mature enzyme, due to partial inactivation of the gene expression (3,150,180).

Another possibility is that these strains carry mutations in genes encoding assembly factors involved in a post-translational step of assembly or factors required for mitochondrial gene expression. For instance, the *amc8, amc10* and *amc12* mutants display reduction in transcript levels, which could be due to i) reduced transcription of the primary polycistronic transcripts, ii) defects in processing of the polycistronic transcripts to produce mature mRNAs, or iii) reduced stability of the transcripts. Although factors required for transcription, RNA processing and stability play essential roles in mitochondrial gene expression, their identity in plant mitochondria is only now emerging. So far, in *Chlamydomonas*, only MOC1, a mitochondrial transcription termination factor (mTERF)-like protein has been identified to play a role in mitochondrial gene expression (218). MOC1, specifically binds to the gene encoding rRNA module S3 and acts as a transcription terminator at this site (218). Loss of MOC1 results in 30% reduction of mtDNA.
content, increased RNA processing and 10-30% reduction in the level of protein-coding mRNAs. MOC1 is only one mTERF-like protein that has been characterized in *Chlamydomonas*. There are three other uncharacterized mTERF-like proteins that are predicted to be mitochondrially targeted (218). It would be interesting to determine whether AMC8, AMC10, AMC12 or AMC13 loci map to genes encoding these predicted mTERF-like proteins. Alternatively, they may also encode for novel proteins involved in mitochondrial gene expression.

We also reported that the *amc3* mutant, which is also capable of assembling a mature Complex I, displays severe depletion of mtDNA content. This is intriguing, as mtDNA depletion does not seem to be a general phenotype of all Complex I mutants (Figure 5.10). mtDNA depletion has been observed in human patients with mutations in nuclear genes required for mtDNA and cytosolic dNTP synthesis (250) and mitochondrial DNA replication (251,252). However, these patients also tend to exhibit different degrees of deficiencies in multiple respiratory complexes in various combinations (251,253,254). It is interesting to note that the *amc3* mutant, in spite of the reduction in mtDNA content, is only partially deficient for Complex I activity and is not affected for Complex II, Complex III or Complex IV activities (131). Although the *Chlamydomonas* mitochondrial genome is present in 50-100 copies per cell (139), reduction in mtDNA content may affect mitochondrial transcription and translation. Determining the mitochondrial transcript and protein levels in the *amc3* mutant, may provide insight into the contribution of the observed
mtDNA depletion to mitochondrial gene expression in the *amc3* mutant, and thereby understand the role of the *AMC3* locus in Complex I function.

Ultimately, identification of the *amc* mutations would help us understand their role in Complex I assembly and activity.
The objective of this study was to identify novel assembly factors involved in Complex I biogenesis. For this purpose, we chose to utilize Chlamydomonas reinhardtii, a unicellular photosynthetic alga, as a model system. The study of Complex I in Chlamydomonas provides a distinct advantage because Complex I mutants are viable and display a unique phenotype—slow growth in the dark (sid). We exploited this phenotype to conduct a forward genetic screen and isolate Complex I mutants via insertional mutagenesis. The first screen, described in Barbieri et al, 2011, led to the isolation of seven Complex I mutants, amc1-to-7 (assembly of mitochondrial complex I), which defined six distinct AMC loci (131). Notably, the amc5 and amc7 mutations are allelic and correspond to molecular lesions in the NUOB10 gene, encoding a bona fide Complex I subunit (131). In this study, we have extended this screen and isolated six additional amc mutants, named amc8-to-13.
Figure 6.11. The role of the AMC proteins in Complex I assembly process in *Chlamydomonas reinhardtii.*

A schematic representation of the recruitment of the AMC gene-products in Complex I assembly. In a simplified model, a hydrophilic module, consisting of soluble subunits including NUO5, is generated in the mitochondrial matrix. Simultaneously, hydrophobic subunits, including mitochondrially-encoded ND1 and ND6, are assembled at the inner mitochondrial membrane. The hydrophobic and hydrophilic modules are then assembled together to form the 700 kDa subcomplex. This order of assembly is proposed on the observation that loss of the soluble subunit NUO5 in the amc9 mutant (Chapter 3) and loss of ND1 and ND6 (145,146), results in a failure to detect mature Complex I and subcomplexes. The 700 kDa subcomplex, to which a hydrophobic module needs to be added to form the mature holoenzyme, is loosely-bound to the inner mitochondrial membrane. Loss of NUOB10 (in the amc5/7 mutants) and mitochondrially-encoded subunits ND4 and ND5, results in the accumulation of this subcomplex and a failure to assemble the mature enzyme (56,130,131,145). AMC11 is required for the synthesis of mitochondrial ND4 (Chapter 4). The amc2 and amc6 mutants, whose loci are yet to be identified, also accumulate the 700 kDa subcomplex. Hence, we expect that the AMC2 and AMC6 gene-products are also involved in assembling the distal membrane arm of Complex I, similar to AMC11.
6.1. The nuo5 mutant – a promising platform for studying the relevance of Complex I deficiency to human health.

We have been able to map the amc9 mutation to the NUO5 gene which encodes the 24 kDa core subunit of Complex I. This soluble subunit is localized to the peripheral arm of Complex I and harbors a 2Fe-2S cluster (11,12). In Chlamydomonas, proteomic analysis and mass spectrometry has revealed the presence of the NUO5 subunit in the 700 kDa subcomplex (56). In addition, our methods failed to detect any mature complexes or subcomplexes in the amc9 mutant (Chapter 3). From our current results, we propose that NUO5 is involved in the early stages of Complex I assembly (Figure 6.1). Thus, the identification of bona fide genes encoding subunits, is a proof of concept that our forward genetic screen is successful in isolating Complex I mutants.

Roles for NUO5 in minimizing ROS production, promoting assembly and maintaining the stability of the enzyme have been postulated (29,255). In addition, the NUO5 human ortholog has been categorized as a candidate gene in various diseases such as Parkinson’s disease, bipolar disorder, schizophrenia and Leigh syndrome (184,185,187,190,256). Chlamydomonas offers many advantages as a platform for testing the role of mutations in human Complex I deficiency. For instance, complete lack of Complex I activity is viable in Chlamydomonas. Furthermore, it is possible to manipulate both the mitochondrial and nuclear genomes, which encode Complex I subunits (2). However, most of these mutations are considered ‘provisional’ because their actual contribution to Complex I
deficiency and disease is not experimentally confirmed. The Remacle group has
described the successful reconstruction of a human mutation found in the
mitochondrial \textit{nd4} gene, that was deemed ‘provisional’, into the \textit{Chlamydomonas}
mitochondria (139). The analysis of this mitochondrial mutation in \textit{Chlamydomonas}, was instrumental in confirming its contribution to Complex I
deficiency.

Although, the mitochondrial genome of \textit{Chlamydomonas} is easily amenable
to targeted manipulation through homologous recombination (139), the nuclear
genome is transformed mostly by random non-homologous recombination (126).
Methods for targeted manipulation of the nuclear genome are still in their infancy.
Hence, it was previously not possible to easily reconstruct human Complex I-
related nuclear mutations in \textit{Chlamydomonas} at the endogenous locus. This
limitation is alleviated by our study, where we described the isolation of the \textit{amc9}
strain carrying a loss-of-function mutation in the nuclear-encoded \textit{NUO5} gene.
Since, the \textit{Chlamydomonas} and human NUO5 orthologs exhibit 51% identity, we
could utilize this null mutant in \textit{Chlamydomonas} as a basis for reconstructing and
assessing provisional human mutations in conserved residues. Our study makes
this application possible because we have optimized biolistics for successful
transformation of Complex I mutants (Chapter 3), which was not previously
possible (131). Hence, \textit{NUO5} constructs carrying the desired mutation could be
incorporated into the nuclear genome of the \textit{amc9} mutant, by random insertional
mutagenesis. The resulting transformants, carrying the mutant NUO5, can be
tested for rotenone-sensitive NADH: duroquinone oxidoreductase activity. Further, we could also determine if the mutation impedes Complex I assembly/accumulation through in-gel activity assays and immunoblotting. Hence, this study now enables us to use *Chlamydomonas* as a platform to assess the effect of human nuclear mutations on Complex I activity and thereby elucidate the molecular basis of the associated diseases.

In humans, mutations in at least 15 nuclear-encoded Complex I subunits were found to be associated to diseases (35,88). It is conceivable to reconstruct pathogenic mutations in subunit-encoding genes other than *NUO5*. This could be achieved if: i) additional loss-of-function mutants for Complex I subunits are isolated through an extensive forward genetic screen, or ii) if targeted manipulations of the endogenous genes are developed. Nuclease-based targeted manipulations, using Zinc-fingered nucleases (ZFNs), TALEs, and Crispr/Cas9 systems, popular in other model systems, have been applied to *Chlamydomonas* (126).

So far, one instance of engineered ZFNs for targeted manipulation of the endogenous *COP3* gene has been successful (137). However, this method demonstrated the use of ZFNs for creating deletion / insertion at only this single target locus (137). Optimization of this method for introducing point mutations and extending the versatility of this tool to manipulate multiple genes is required for establishing ZFNs as a reliable system.
An alternative method is the popular Crispr/Cas9 tool (257). Transient expression of Cas9 and single guide RNA yielded targeted gene disruption in *Chlamydomonas* (138). However, stable transformants could not be obtained due to putative toxicity of constitutively expressed Cas9 (138). This challenge could be circumvented with modifications to prevent the accumulation of Cas9 in stable transformants. In conclusion, establishing reproducible methods for stable, targeted nuclear manipulation would pave way to exciting new applications in translational research.

6.2. Identification of AMC11 as a novel factor for Complex I biogenesis.

The potential for identifying novel Complex I biogenesis factors by using *Chlamydomonas reinhardtii* is illustrated by the *amc11* and *amc1* mutants (Chapter 4). The *amc11* and *amc1* strains display the same defect in the assembly of the distal membrane arm, characterized by the accumulation of a labile 700 kDa subcomplex (Figure 6.1). Genetic analysis revealed that the mutations in *amc11* and *amc1* are allelic and mapped to a novel gene, termed as *AMC11*, in Chromosome 16. The involvement of the *AMC11* gene in Complex I assembly was confirmed through complementation studies. The *AMC11* gene encodes a large 2566 amino acid protein with no conserved domains. So far, the identification of AMC11, a novel factor required for Complex I biogenesis, shows that we have been successful in our initial objective of our forward genetic screen.
6.3. Role for AMC11 in mitochondrial gene expression.

Although the AMC11 protein does not carry motifs that could suggest a function, we have identified that the N-terminus of AMC11 is capable of targeting a reporter protein to the yeast mitochondria. These results implicate that the site of action of AMC11 is in the mitochondria. Most of the biogenesis factors, identified to date, function in the mitochondria (Chapter 1). They are localized to the mitochondrial matrix (for example: IND1), inner mitochondrial membrane (for instance: NDUFAF3, NDUFAF4, C3orf1) or the intermembrane space (for example: AIF) (97,101,106,109,116,118,258,259). Detection of AMC11, using a specific antibody, in sub-mitochondrial fractions would define the mitochondrial localization of AMC11 and help to refine its mechanism of action.

Further characterization of the amc11 and amc1 mutants revealed that loss of AMC11 results in decreased abundance of the mitochondrial transcripts encoding Complex I subunits: nd4, nd2, nd6 and nd1. A problem in transcription can be ruled out as the nd5, cox1 and rtl transcripts, co-transcribed with the affected nd transcripts, accumulate to wild-type levels in the amc1 mutant. Hence, in a simplified model, we hypothesize that AMC11 is required for the stability of these transcripts.

We also reported that substitution of the mitochondrial nd4 coding sequence with a reporter, in the amc11 mutant (while retaining nd4’s 5’ UTR and 3’ UTR), results in a failure to detect the reporter protein. From these results, we extrapolate that the expression of the nd4 transcript is AMC11-dependent. Also, accumulation
of the 700 kDa subcomplex, upon loss of AMC11, is similar to the assembly defect observed by the loss of ND4 in *Chlamydomonas* (130,145). Hence, we hypothesize that AMC11 is required for synthesis of ND4.

We propose that the function of AMC11 in mitochondrial gene expression could occur at various stages: i) maintaining stability of the mature transcripts, ii) processing of the primary transcript, and iii) translation.

Is AMC11 required for the accumulation of mitochondrial translational products?

In addition to the *nd4* transcript levels, the accumulation of the *nd2*, *nd6* and *nd1* transcripts-encoding Complex I subunits is also affected by loss of AMC11. Hence, it is possible that AMC11 controls the synthesis of multiple Complex I subunits in the mitochondria. Synthesis of multiple mitochondrial proteins is affected in *Arabidopsis* by loss of INDH, the Fe-S protein required for Complex I biogenesis (118). However, the formation of the 700 kDa subcomplex in *Chlamydomonas* is dependent on the presence of the ND2, ND6 and ND1 subunits (145,146). Although we do not exclude the possibility that AMC11 may be required for regulating the synthesis of these subunits, we expect that there is some level of synthesis of these subunits to enable the formation of the 700 kDa subcomplex. Low transcript levels, that still allows the synthesis the protein has been previously reported for the mitochondrial *dum5* mutant of *Chlamydomonas*, carrying a single nucleotide deletion in the 3′ UTR of *nd5* (145). This mutant was characterized by severe reduction in the *nd5* transcript that nevertheless allowed the accumulation
of a mature Complex I, indicating that ND5 is synthesized in spite of the low abundance of its transcript (145).

To test the hypothesis that AMC11 plays a role in expression of the mitochondrially-encoded subunits, we need to assess the synthesis of translation products by \textit{in-organello} translation (249) in the \textit{amc1} and \textit{amc11} mitochondria. Such an analysis would provide information regarding the synthesis of the ND subunits in the absence of AMC11. If AMC11 is required for the synthesis of ND4, we expect to observe a lack of ND4. It is also possible that we may observe low levels of ND2, ND6 and ND1, if AMC11 also regulates their synthesis. Alternatively, AMC11 could be involved in protecting the ND subunits from post-translational proteolytic degradation. Pulse-chase experiments could be performed to determine the rate of degradation of the translated products in the absence of AMC11.

\textbf{Is AMC11 required for transcript stability?}

Accumulation of the polycistronic and mature transcripts in the \textit{amc1} and \textit{amc11} mutants needs to be determined by RNA hybridization studies. We expect to observe lower abundance of the full-length mature transcript if there is a problem in endonucleolytic cleavage of the poly-cistronic transcript and / or increased degradation of the mature transcript. If the degradation intermediates are stable, they could also be detected by RNA hybridization.
Is AMC11 involved in post-transcriptional processing of the \textit{nd} transcripts?

In particular, the \textit{nd4} transcript is known to exist in two forms: the 5' UTR – \textit{nd4} and AUG-\textit{nd4} forms (226). It is conceivable that the AUG-\textit{nd4} form is preferred for translation because, other transcripts of \textit{Chlamydomonas} and animal mitochondria also begin at the start codon (225,226,241,243,260). Hence, it is conceivable that AMC11 is required for processing of the 5' UTR of the \textit{nd4} transcript to yield the 'translatable' AUG-\textit{nd4} form. Primer extension studies on the \textit{nd4} transcript, in the \textit{amc1} and \textit{amc11} mutants, will reveal the distribution of the two isoforms in the absence of the AMC11 protein. If AMC11 is required for processing of the \textit{nd4} transcript, we expect to observe a decrease in abundance of the AUG-\textit{nd4} form in the \textit{amc1} and \textit{amc11} mutants.

\textit{6.4. Mode of action of AMC11.}

In this study, we have reported that AMC11 is required for mitochondrial gene expression. The protein sequence of AMC11 does not contain conserved motifs. However, it does contain features of low-complexity regions and helical secondary structures (Figure 4.16) which are found in RNA-binding proteins involved in organellar gene expression (261,262).

Such mitochondrial RNA binding proteins (RBPs) have been identified in humans. For example, LRPPRC and SLIRP form a complex involved in processing of mature mitochondrial transcripts, maintaining poly(A) tails and improving mRNA stability (263-265). Mutations in LRPPRC, detected in patients with French Canadian Leigh Syndrome, causes tissue-specific defects in different respiratory
complexes (266). Mitochondrial GRSF1 is another RBP that is required for the stability of several mitochondrial mRNA, ribosomal biogenesis and subsequent translation (267).

PPR proteins (containing degenerated 35 amino acid tandem repeats) are now emerging as important regulators of mitochondrial gene expression (261,268). The LRPPRC protein described above is one such example (269). In mosses and vascular plants, PPR proteins are required for 5' end processing, mitochondrial RNA editing and intron splicing (269-280). However, *Chlamydomonas* mitochondrial genome lacks introns and the transcripts do not undergo RNA editing (1,225). Although the PPR family consists more than 400 members in land plants (281), only 14 PPR proteins have been identified in *Chlamydomonas* (239). On the other hand, *Chlamydomonas* contains more than 100 members of a family of proteins called as OPR (Octotricopeptide repeats) proteins, which have established roles in chloroplast transcript maturation, stability and translation (262,282-285).

So far, any role for *Chlamydomonas* PPR or OPR proteins in mitochondrial gene expression has not been established. It is possible that AMC11 belongs to a novel class of RNA-binding proteins that does not contain easily recognizable motifs. Furthermore, the PredictProtein tool predicts the presence of a polynucleotide binding region for AMC11 (Figure 4.16). In a simple scenario, AMC11 may control gene expression by directly binding to the *nd* transcripts. Co-immunoprecipitation using an AMC11-specific antibody could help us isolate
associated RNA(s). Further studies by gel-shift assays would enable us to test the direct interaction of AMC11 with the isolated RNA targets. If AMC11 directly binds to the \textit{nd} transcripts for its action, we expect to find a \textit{cis} element of the \textit{nd} transcripts to be recognized by AMC11.

In particular, the reporter T-urf13 protein at the \textit{nd4} locus was still under the control of the endogenous 5' and 3' UTRs of \textit{nd4}. Since the reporter protein was not expressed when AMC11 function is lost, it is reasonable to hypothesize that AMC11 mediates its action through the UTRs of \textit{nd4}. Analysis of specific interaction between AMC11 and 5' UTR and/or 3' UTR of \textit{nd4} could further clarify the mode of action of AMC11. As an alternative, we could follow the expression of a reporter at the \textit{nd4} locus after replacing the endogenous UTRs with those of the \textit{nd5} transcript, whose levels are unaffected by loss of AMC11. These experiments would help in delineating the elements of the \textit{nd4} transcript required for AMC11-dependent regulation.

In the scenario that AMC11 directly binds to the mitochondrial transcripts, we expect that AMC11 localizes to the mitochondrial matrix. However, an interesting feature for AMC11 is the presence of five transmembrane helices (Figure 4.16), suggesting an inner mitochondrial membrane localization for the protein. It is also possible that AMC11 has a dual function in mediating synthesis of the ND subunits and their insertion into the membrane. Such an occurrence has been reported with Mba1, a protein associated to the yeast inner mitochondrial membrane, which is required for co-translational membrane insertion of Cox1.
(286). Furthermore, translation of mitochondrial-encoded proteins has been observed at close proximity to the inner membrane, with a tight association of mitochondrial ribosomes with the inner mitochondrial membrane (287). Future experiments need to be performed to determine the sub-mitochondrial localization of AMC11 to test the hypothesis that it is membrane-associated (6).

Another possibility is that AMC11 interacts with other regulatory proteins to control mitochondrial gene expression. The PredictProtein tool predicts multiple protein-binding regions for AMC11 (Figure 4.16). Potential interacting partners could be identified by employing yeast two-hybrid assays and/or co-immunoprecipitation techniques, to further delineate the mode of action of AMC11.

The identity of factors involved in post-transcriptional regulation of mitochondrial transcripts-encoding Complex I subunits is only now emerging. It is possible that nuclear-encoded factors regulating mitochondrial gene expression co-evolve with the corresponding mRNA sequence. For instance, orthologs of mitochondrial translational activators, with the same function in different species of budding yeasts, display a high sequence divergence (288). These differences correlate to the sequence changes in their corresponding RNA targets, indicating a co-evolution of the proteins with their RNA targets (288). Hence, it is conceivable that AMC11 is specific for the mitochondrial transcripts of Chlamydomonas. Although AMC11 does not appear to be conserved in other organisms, understanding its function will be instrumental in providing insights into processes required for synthesis and subsequent assembly of mitochondrially-encoded
Complex I subunits. In addition, these studies may also provide clues to identify functional counterparts in other organisms, including humans.

6.5. Other promising AMC loci?

Other amc mutants, such as amc8, amc10 and amc12m2 also display reduced abundance of mitochondrial transcripts, suggesting that proteins controlling the expression of the mitochondrial genome might play crucial roles in Complex I biogenesis. Factors coordinating mitochondrial gene expression remain unexplored in *Chlamydomonas*, and this study may mark the beginning towards understanding the mechanisms involved. In addition to the mutations in the amc8, amc10, amc12 and amc13 strains, the identity of the mutations in the amc2, amc3, amc4 and amc6 strains, isolated from the first genetic screen, still remain undeciphered. Future experiments shall be directed towards identifying the remaining AMC loci through linkage analysis or genome sequencing, a technology now available in *Chlamydomonas* (128,289,290). These AMC loci could potentially encode yet-to-be discovered factors required for biogenesis of Complex I and provide insights into this intricate process.
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