The assembly of mitochondrial complex I

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The assembly of mitochondrial complex I - a product of nuclear-mitochondrial synergy
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Thesis Radboud University Nijmegen with a summary in Dutch

ISBN: 978-90-9022057-4

Cover: The assembly of mitochondrial complex I depicted as a Lego system. The combination of the two bricks that together form the L-shaped complex, representing mitochondrial and nuclear DNA-encoded building blocks, respectively, requires tight cooperation between nucleus and mitochondrion.

Printed by: Print Partners Ipskamp, Enschede, The Netherlands

The assembly of mitochondrial complex I

A product of nuclear-mitochondrial synergy

Een wetenschappelijke proeve op het gebied van de
Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus **prof. mr. S.C.J.J. Kortmann**,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op **woensdag 14 november 2007**
om **13.30 uur** precies

door

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geboren op **14 augustus 1978**
te **Hardinxveld Giessendam**

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Publication of this thesis was made possible with financial support from sigma-tau B.V.

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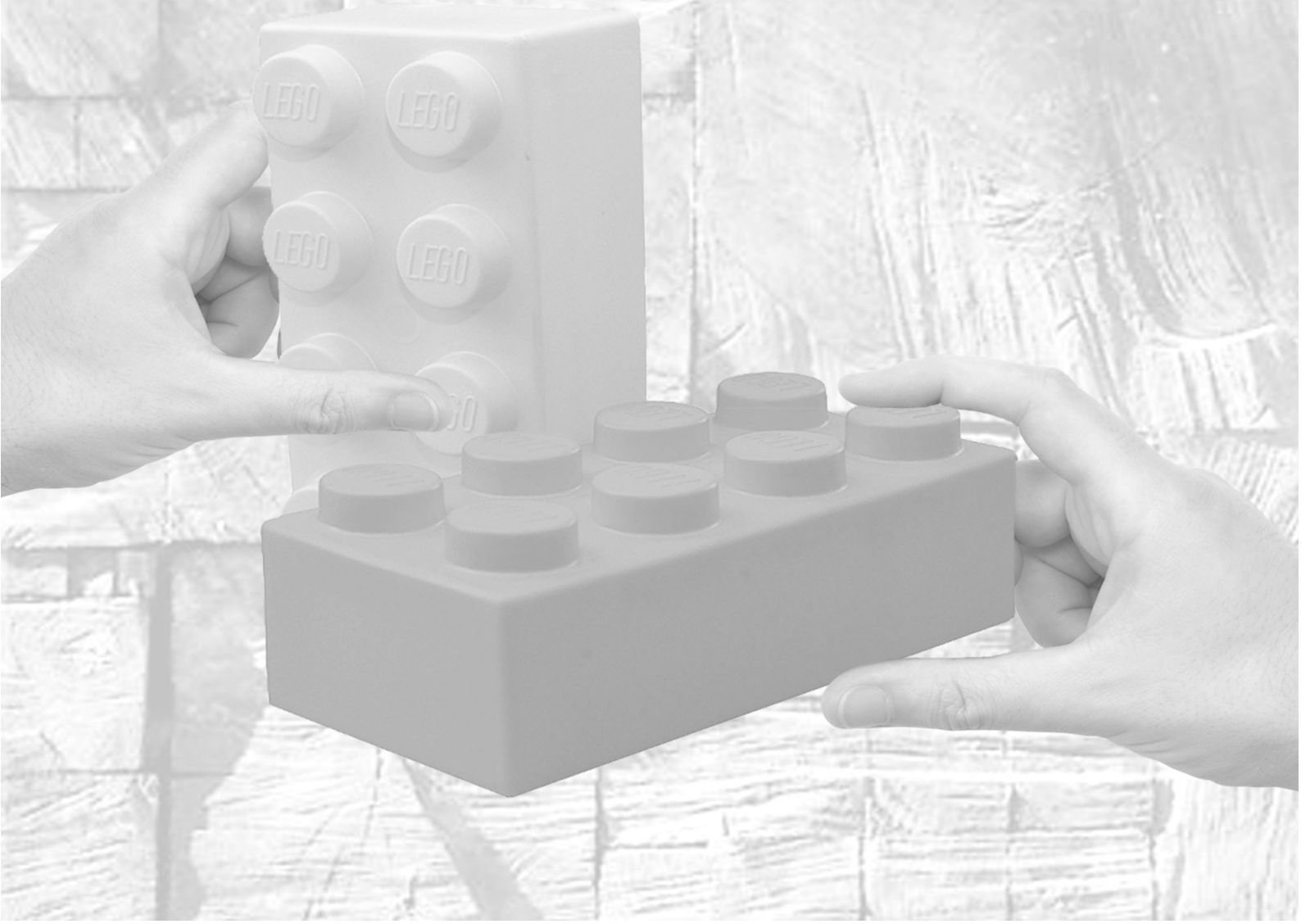
List of frequently used abbreviations

1D	One-dimensional
2D	Two-dimensional
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BN	Blue native
CI-CV	Complex I-V
DDM	n-Dodecyl β-D-maltoside
DNA	Deoxyribo nucleic acid
dsRNA	Double stranded RNA
ECL	Enhanced chemiluminescence
FeS	Iron-sulfur
FMN	Flavin mono-nucleotide
GFP	Green fluorescent protein
HEK	Human embryonic kidney
kDa	Kilo Dalton
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
NADH/NAD+	Nicotinamide adenine dinucleotide (reduced/oxidized)
NADPH/NADP+	Nicotinamide adenine dinucleotide phosphate (reduced/oxidized)
ND	NADH dehydrogenase
NDUF	NADH dehydrogenase (ubiquinone) fraction
OMIM	Online mendelian inheritance in man
OXPHOS	Oxidative Phosphorylation
PAGE	Polyacrylamide gel electrophoresis
Q	Ubiquinone
RNA	Ribo nucleic acid
RNAi	RNA interference
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA
WB	Western blot

Chapter 1

General introduction, objective and outline of this thesis

The assembly of complex I (NADH:ubiquinone oxidoreductase, EC 1.6.5.3), the largest multi-protein complex of the oxidative phosphorylation (OXPHOS) system, is a formidable cellular achievement. The process encompasses the combination of 38 nuclear DNA-encoded and seven mitochondrial DNA-encoded constituents, eight iron-sulfur clusters and a noncovalently bound flavine mononucleotide, resulting in one of the most complex structures in the mitochondrion. This chapter discusses the function, structure and assembly of complex I, in both health and disease.



Complex I: An introduction

Mitochondria and oxidative phosphorylation

Every heartbeat, breath, movement and thought requires energy to occur; energy that predominantly originates from a tiny organelle called the mitochondrion. Its central compartment harbours a broad spectrum of enzymes involved in different pathways and its own genome, the 16,569 base pair mitochondrial DNA (mtDNA). This so-called matrix compartment is enveloped by the mitochondrial inner and outer membranes, across which communication takes place with the rest of the cell. The inner membrane is in direct contact with the mitochondrial matrix, and it is here where important metabolic pathways, such as the Krebs cycle, beta-oxidation and the oxidative phosphorylation (OXPHOS) system, are connected to produce the energy carrier adenosine triphosphate (ATP).

Mitochondria are highly energetic, as illustrated by the potential difference across the inner membrane: conversion of the 150 mV per 5 nm equals an astonishing 300 kV per cm, the equivalent of the amount of electricity produced by a power plant! The membrane potential is generated through the action of the OXPHOS system: a set of five enzymatic complexes (termed complexes I, II, III, IV and V, abbreviated as CI-CV) which oxidize the substrates NADH and $FADH_2$ produced during glycolysis, the Krebs cycle and beta-oxidation. Upon substrate oxidation, electrons are ultimately transferred via the electron transport chain to CIV, at which molecular oxygen is hydrolyzed to water (see figure 1). This process is accompanied by proton transport across the mitochondrial inner membrane by complexes I, III and IV. Thus, a charge difference is created between the matrix and inter membrane space compartments (the membrane potential) used by complex V, an ATP synthase, to drive ATP synthesis from ADP and inorganic phosphate (Nijtmans et al., 2004). Hence the name OXPHOS: substrates are oxidized and ADP is phosphorylated to form ATP.

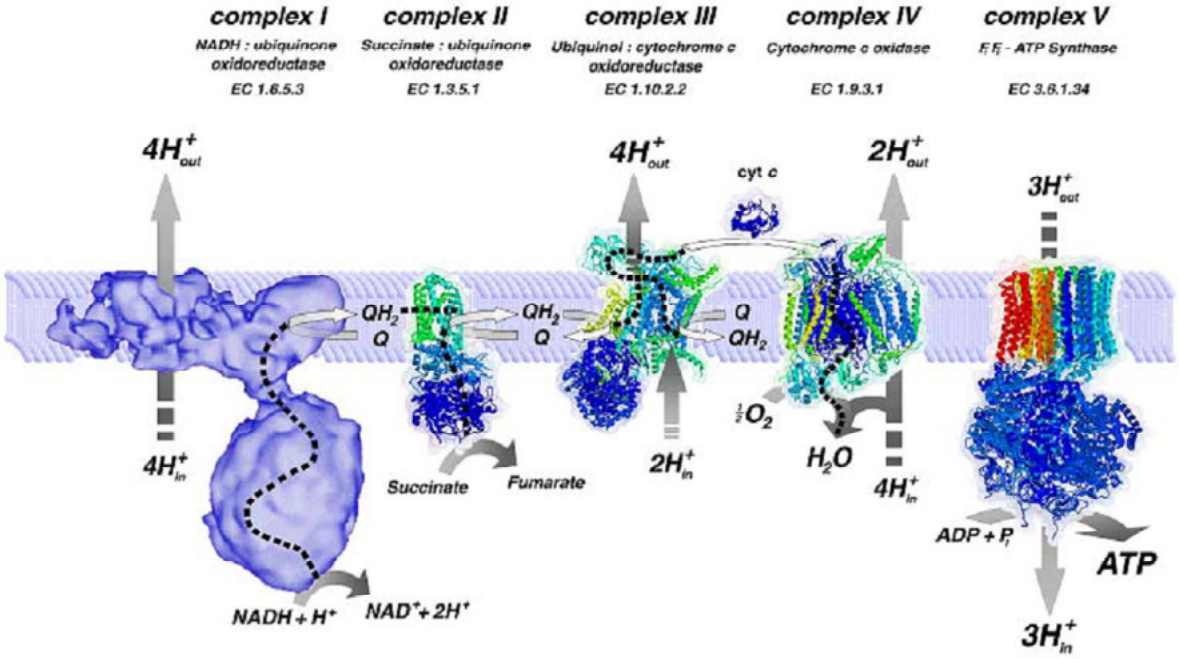


Figure 1: A scheme of the five OXPHOS enzyme complexes: complexes I-V
NADH (at CI) and succinate (at CII) are oxidized to obtain electrons, which are transferred through the system via electron carriers ubiquinone (Q) and cytochrome c (cyt c). The electrons lose energy during this transfer, which is used by complexes I, III and IV to translocate protons across the mitochondrial inner membrane. Ultimately, CIV uses the electrons to produce water from molecular oxygen and protons. In addition, CV, an ATP synthase, produces ATP driven by the proton gradient across the membrane.

Complex I (NADH:ubiquinone oxidoreductase, EC 1.6.5.3) is the largest and most complex of the five OXPHOS enzymes (Brandt, 2006; Janssen et al., 2006). The main focus of this thesis is the study of its assembly process. In order to understand the complexity and relevance of this topic, this chapter provides an overview of what is known about CI function, structure, deficiency, and ultimately assembly.

Complex I function and structure

Complex I binds and oxidizes NADH to NAD^+ to free electrons, which are transferred via a cascade of eight (or nine in bacterial CI) iron-sulfur (FeS) clusters to the electron acceptor ubiquinone (Q), which subsequently transfers electrons to CIII. The energy released during this electron transfer is used to translocate protons across the mitochondrial inner membrane, resulting in the following overall reaction (Galkin et al., 1999; Galkin et al., 2006):

$NADH + H^+ + Q + 4H^+_{matrix} \rightarrow NAD^+ + QH_2 + 4H^+_{intermembrane\ space}$

How electron transfer is coupled to proton translocation is subject to debate (Vinogradov, 2001; Brandt, 2006). It could be directly coupled via close proximity of the Q binding site to proton translocation, or indirectly via conformational changes of the enzyme complex. Recent data obtained for bacterial and *Yarrowia lipolytica* CI provide support for the latter option (Bottcher et al., 2002; Mamedova et al., 2004; Brandt et al., 2005; Hinchliffe and Sazanov, 2005; Baranova et al., 2007). In addition, CI is observed to undergo slow active/de-active transitions in a range of eukaryotes depending on temperature, pH and the presence of bivalent cations (Maklashina et al., 1994; Vinogradov, 1998; Grivennikova et al., 2001; Grivennikova et al., 2003; Maklashina et al., 2003).

Electron microscopy of CI reveals an L-shaped complex consisting of two perpendicular arms: a hydrophobic membrane arm residing in the mitochondrial inner membrane and a hydrophilic peripheral or matrix arm which protrudes into the mitochondrial matrix (Leonard et al., 1987; Hofhaus et al., 1991; Guenebaut et al., 1997) (see also figure 2). Three-dimensional electron microscopy of *Yarrowia lipolytica* CI recently demonstrated detailed contours and protrusions of the complex, underlining that CI is a complicated and possibly highly dynamic structure (Radermacher et al., 2006). At a higher resolution, the crystal structure of the peripiheral arm of *Thermus thermophylus* CI recently shed light onto the exact arrangement of the iron-sulfur clusters within the complex (Hinchliffe and Sazanov, 2005; Sazanov and Hinchliffe, 2006; Sazanov, 2007). Hopefully, these studies will stimulate the elucidation of structural details of CI in higher eukaryotes.

Bacterial CI consists of the 14 most conserved subunits and is considered to be the 'mimimal' structure required for functionality of the enzyme. A typical example of bacterial CI is *Escherichia coli* NDH-1 (Leif et al., 1993; Friedrich et al., 1993). By analogy to its core structure, three functional modules can be distinguished for human CI. The first is the dehydrogenase module, which is responsible for the oxidation of NADH and consists of at least the NDUFV2, NDUFV1 and NDUFS1 subunits (homologues of the nuoE, F and G subunits of bacterial NDH-1). The second is the hydrogenase module, which guides the released electrons to electron acceptor ubiquinone and consists of at least the NDUFS2, NDUFS3, NDUFS7 and NDUFS8 subunits (homologues of the nuoD, C, B and I subunits of NDH-1). Finally, the third is the proton translocation module, which consists of at

least the ND1, ND2, ND3, ND4, ND4L, ND5 and ND6 subunits (homologues of the nuoH, N, A, M, K, L and J subunits of NDH-1) (see table 1 for an overview).

Table 1. CI subunit nomenclature
This table lists the nomenclature, distribution after fractionation (bovine CI, see also figure 1), and allocation in the three functional modules of the 14 most conserved CI subunits from Escherichia coli, Homo sapiens *and* Bos taurus.

CI subunit			Fraction	Module
E. coli	*H. sapiens*	*B. taurus*		
NuoA	ND3	ND3	Iγ	Membrane
NuoB	NDUFS7	PSST	Iλ	Hydrogenase
NuoC	NDUFS3	30 kDa	Iλ	Hydrogenase
NuoD	NDUFS2	49 kDa	Iλ	Hydrogenase
NuoE	NDUFV2	24 kDa	Iλ	NADH dehydrogenase
NuoF	NDUFV1	51 kDa	Iλ	NADH dehydrogenase
NuoG	NDUFS1	75 kDa	Iλ	NADH dehydrogenase
NuoH	ND1	ND1	Iγ	Membrane
NuoI	NDUFS8	TYKY	Iλ	Hydrogenase
NuoJ	ND6	ND6	Iα-λ	Membrane
NuoK	ND4L	ND4L	Iγ	Membrane
NuoL	ND5	ND5	Iβ	Membrane
NuoM	ND4	ND4	Iβ	Membrane
NuoN	ND2	ND2	Iγ	Membrane

Currently, 45 subunits have been described for human CI, an addition of 31 supernumerary subunits to the functional “core” structure of 14 subunits. Their topology was inferred by fractionation of bovine CI using chaotropic salts and the detergent *N,N*-dimethyldodecylamine *N*-oxide (Galante and Hatefi, 1978; Sazanov et al., 2000) (figure 2).

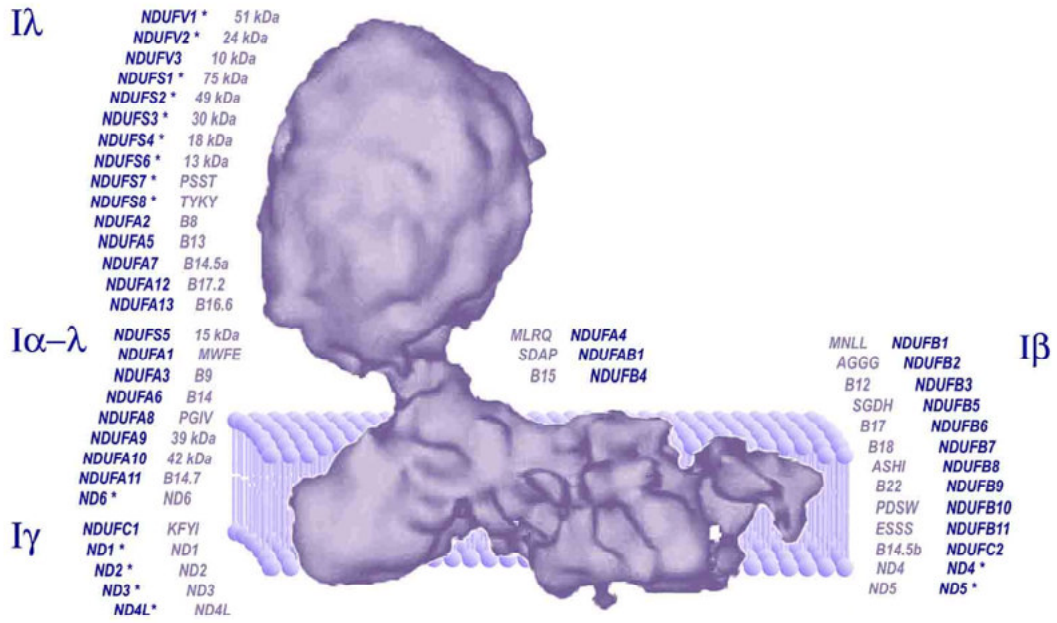


Figure 2. CI subunit topology (adapted from (Janssen et al., 2006))
CI is an L-shaped enzyme complex that can be dissected into several fragments, Iα, Iβ, Iλ, and Iγ. The composition of these fragments allows a basic arrangement of the 45 subunits that comprise CI.

The function of most of the supernumerary subunits is yet unclear. They are hypothesized to stabilize or to protect the complex from damage inflicted by reactive oxygen species (ROS). Furthermore, at least several of these subunits may have an additional function.

One example of such a function is in apoptosis. For example, the NDUFA13 (GRIM-19, or Gene associated with Retinoid-IFN induced Mortality in bovine CI) subunit is also a cell death regulatory protein induced by interferon-beta and retinoic acid and was demonstrated to be released from the mitochondrion upon apoptosis (Fearnley et al., 2001; Huang et al., 2004; Huang et al., 2007). Recently, GRIM-19 was shown to associate with the pro-apoptotic serine protease HtrA2 to promote cell death (Ma et al., 2007). An apoptotic function is also described for subunit NDUFS1, as caspase mediated cleavage of this subunit is a requirement for the mitochondrial changes associated with apoptosis (Ricci et al., 2004).

Another example is in fatty acid biosynthesis. The *Neurospora crassa* and bovine SDAP subunits (homologues of the human NDUFAB1 subunit) are closely related to the acyl-carrier proteins involved in bacterial fatty acid biosynthesis (Runswick et al., 1991; Sackmann et al., 1991; Zensen et al., 1992; Cronan et al., 2005). Interestingly, disruption of the gene in *N. crassa* resulted in a 4-fold increase in the amount of lysophospholipids in the mitochondrial membranes, suggestive of a function for this subunit in lysophospholipid recycling (Schneider et al., 1995; Schneider et al., 1997). Whether the *N. crassa* situation also applies to other organisms is subject to debate, as recent studies for bovine heart and *Arabidopsis thalania* mitochondria show that most of the of mitochondrial acyl-carrier protein is present in the mitochondrial matrix and not associated to CI (Cronan et al., 2005; Meyer et al., 2007).

For several other subunits some features are known, but the exact additional function is yet unclear. The NDUFA9 subunit is known to harbour a NADH/NADPH binding site (Yamaguchi et al., 1998; Schulte et al., 1999; Schulte, 2001; Yamaguchi et al., 2000). The importance of this binding site for CI stability was recently demonstrated by mutagenesis of the *Yarrowia lipolytica* homologue, which resulted in CI destabilization, presumably by destabilization of the structural fold of the subunit (Abdrakhmanova et al., 2006). The NDUFS4 subunit was described to be phosphorylated by a cAMP-dependent protein kinase (PKA), possibly indicating a function in regulation of CI activity (Papa, 2002; Papa et al., 2002a; Papa et al., 2002b). However, this later proved to be the ESSS subunit (NDUFB11 in humans)(Chen et al., 2004). Additional studies demonstrated phosphorylation of the bovine homologues of the NDUFC2, NDUFA1, NDUFA7, NDUFA10 and GRIM-19 subunits (Raha et al., 2002; Chen et al., 2004; Schilling et al., 2005; Palmisano et al., 2007). Based on the phylogenetic distribution of different CI subunit orthologs, the NDUFA2 and NDUFA10 subunits were shown to belong to a family of proteins including the mitochondrial ribosomal proteins L43 and S25, respectively (Gabaldon et al., 2005). Finally, the NDUFA11 subunit was found to be paralogous to the TIM17/22 family of proteins (Carroll et al., 2002; Gabaldon et al., 2005).

Complex I deficiency

Mutation in one of the structural subunits of CI can result in a misassembled and hence dysfunctional enzyme complex. In turn, disturbances in CI activity, stability and assembly can result in severe energy deficiencies. These deficiencies are a major cause of mitochondrial disorders and are characterized by a great variety of clinical manifestations, in many cases resulting in early childhood death (Smeitink et al., 2001). To the most frequently affected organs belong brain, eyes, skeletal and heart muscle, liver and kidneys. These organs are hypothesized to be the first affected because their tissues are the most energy demanding and the critical amount of ATP required for their functioning may be higher than that of the other tissues.

Complex I deficiency can be caused by mutations in either the mtDNA or the nuclear DNA. MtDNA mutations are normally inherited via the mother (maternally inherited) and are often characterized by heteroplasmy, which means that the percentage of mtDNA molecules harbouring the mutation varies per cell and sometimes per tissue, often resulting in tissue-specific defects. Although mtDNA mutations had already been associated with mitochondrial myopathy, they had not been linked to OXPHOS disorder and CI deficiency until 1988. In that year, one study related CI deficiency with multiple mtDNA deletions of up to 7 kb in size and another with a mutation in the ND4 gene, respectively (Holt et al., 1988; Wallace et al., 1988). Since then, mutations have been described for all mtDNA-encoded CI subunits (ND1-6 and ND4L. For an extensive review, see (Janssen et al., 2006)). The most often observed disorder caused by mtDNA mutations is MELAS (mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes; OMIM 540000). This syndrome is characterized by lactic acidosis, seizures and migraine, convulsions and psychomotor and mental retardation. Another frequently observed mitochondrial disorder is LHON (Leber hereditary optic neuropathy; OMIM 535000), which results in optic nerve damage and loss of central vision in late adolescence and young adults.

Nuclear DNA mutations can be inherited via the father and/or the mother. The first report of a nuclear DNA mutation resulting in respiratory chain deficiency was described in 1995, for the gene encoding the CII Fp subunit (Bourgeron et al., 1995). Only a few years later, Loeffen and colleagues described the first mutation in a nuclear CI gene (*NDUFS8*) (Loeffen et al., 1998). Since this discovery, mutations have been found in genes encoding subunits NDUFS1, NDUFS2,

NDUFS3, NDUFS4, NDUFS6, NDUFS7, NDUFV1, NDUFV2, NDUFA1 and NDUFA8 (for an extensive review, see (Janssen et al., 2006)). Nuclear DNA mutations in CI genes can result in a spectrum of disorders: fatal infantile lactic acidosis (FILA), Leigh syndrome, neonatal cardiomyopathy with lactic acidosis, leukodystrophy with macrocephaly and hepatopathy with renal tubulopathy (Pitkanen et al., 1996; Loeffen et al., 2000).

The recent description of patients harbouring a mutation in assembly chaperones B17.2L (Ogilvie et al., 2005) and NDUFAF1 (Sugiana et al., 2006) underlines that affected proteins other than structural constituents of CI can be the cause of an isolated CI deficiency, which broadens the scope of proteins putatively involved in the disease process beyond CI subunits.

Complex I assembly

The frequency and heterogeneous nature of CI deficiency illustrates the importance of an active, stable and properly assembled CI. Understandably, investigation of the CI assembly mechanism is a prerequisite for the elucidation and diagnosis of CI deficiencies. Furthermore, it will result in a better understanding of how mitochondrial protein complexes are made by combining nuclear DNA and mtDNA encoded proteins. The assembly of 38 nuclear DNA-encoded and seven mtDNA-encoded subunits into an approximately 1 MDa multi-subunit structure is an intricate process, and has for long remained an enigma. Fortunately, the topic has gained much recent scientific attention, which not only allowed further insights into the assembly process itself but also revealed links between CI assembly and processes that affect the entire cell, such as apoptosis (programmed cell death) and even immunity.

To put it simple, the assembly of a mitochondrial OXPHOS complex composed of both nuclear and mtDNA-encoded subunits requires delivery of nuclear DNA-encoded proteins to the mitochondrial surface, inner membrane insertion of the mtDNA-encoded proteins and the subsequent combination of preformed assembly intermediates (see figure 3).

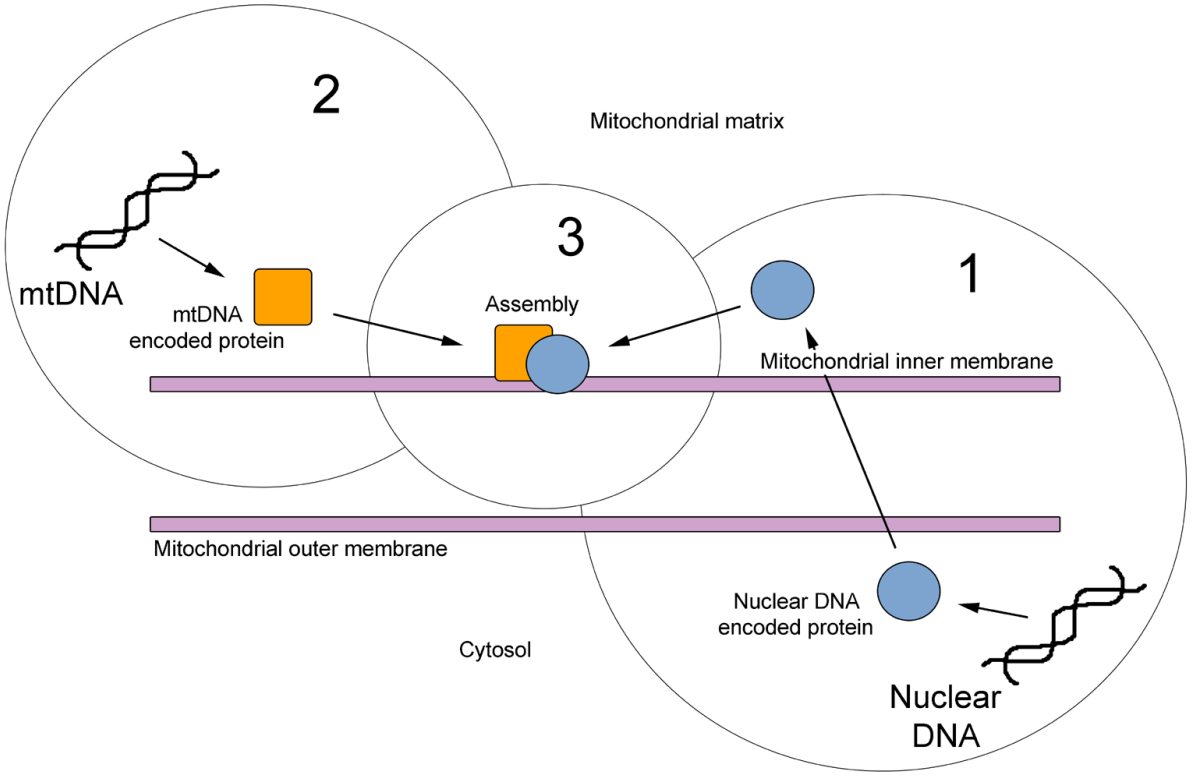


Figure 3. A schematic representation of OXPHOS assembly
This figure depicts the three basic processes required for OXPHOS complex assembly; (1) import and targeting of nuclear-DNA encoded subunits, (2) targeting of mtDNA-encoded subunits and (3) the combination of assembly intermediates resulting in fully assembled, membrane bound enzymes.

This paragraph will discuss each of the three processes in more detail. Most of the involved pathways were investigated in yeast mitochondria (*Saccharomyces cerevisiae*), which do not contain CI. However, processes such as import, targeting and quality control most likely are generic processes that also apply to CI assembly. The following section will first go into the general concept of import of nuclear DNA-encoded proteins into mitochondria and their targeting to submitochondrial compartments. Subsequently, the fate of mtDNA-encoded OXPHOS subunits will be discussed. Taken together, these sections will allow a better understanding of the context in which CI assembly takes place. The latter constitutes the third section and primary topic of this thesis: the combination of CI assembly intermediates and the involved assembly chaperones.

Nuclear DNA-encoded subunits

CI consists predominantly of subunits encoded by the nuclear DNA. Each of these 38 nuclear DNA-encoded CI subunits must be translated, transported to the mitochondrial outer surface, imported, processed and subsequently targeted to their final destination.

Cytoplasmic chaperones

Upon translation of nuclear mRNA, most precursors are chaperoned to the mitochondrial surface by cytoplasmic chaperones of the Hsp90 and Hsp70 classes, which maintain hydrophobic proteins in their unfolded conformation to prevent aggregation (Young et al., 2003). In addition, translation of nuclear DNA-encoded proteins has been described to occur in close proximity to the mitochondrial surface of yeast and mammalian mitochondria (Margeot et al., 2002; Mackenzie and Payne, 2004; Mukhopadhyay et al., 2004; Margeot et al., 2005). It is yet unknown whether translation in the vicinity of the mitochondrial outer membrane favours import and thus aids the assembly process of the OXPHOS complexes, although it has been argued that 'site-specific' translation of nuclear DNA-encoded proteins may coordinate the assembly of mitochondrial protein complexes in yeast (Garcia et al., 2007).

Mitochondrial recognition of nuclear DNA-encoded precursor proteins

Mitochondrial recognition of the cytosolic chaperone bound precursor takes place at the 450 kDa TOM complex (translocase of the outer mitochondrial membrane) (Rehling et al., 2004). This complex includes two receptor proteins, Tom20 and 70 (Sollner et al., 1989; Sollner et al., 1990). Tom20 recognizes N-terminal mitochondrial target sequences, whereas Tom70 preferentially binds precursor proteins with internal target sequences (Brix et al., 1997; Brix et al., 1999; Brix et al., 2000; Young et al., 2003). Upon recognition by the TOM complex, Tom22 directs precursor proteins towards a pore-forming core unit, the GIP (general-import pore) (Dekker et al., 1998; Kunkele et al., 1998; Rehling et al., 2004), of which the actual protein-conducting pore is formed by Tom40 (Hill et al., 1998). From there, different pathways can be ensued, depending on whether the precursor is targeted to the outer mitochondrial membrane, the inter membrane space, the inner mitochondrial membrane or the mitochondrial matrix.

Import of outer membrane and inter membrane space proteins

The assembly of several small inter membrane space proteins, such as Cox17, Cox19 and small Tim proteins, depends on a specific import machinery formed by Mia40 and Erv1 (Chacinska et al., 2004; Allen et al., 2005; Mesecke et al., 2005; Rissler et al., 2005). Outer membrane proteins, such as porin, are assembled by the SAM (sorting and assembly machinery) complex. These two pathways will not be discussed further here. Instead, the focus will be on the import of the inner membrane proteins and matrix proteins required for the assembly of OXPHOS complexes.

Import of inner membrane and matrix proteins

The import of inner membrane and matrix proteins is directed by the TIM (translocase of the inner mitochondrial membrane) complexes TIM22 and TIM23. Whether the TIM22 or TIM23 complex is used depends on whether the precursor protein has an internal or an N-terminal mitochondrial targeting sequence. In the case of internal targeting, after recognition of the internal targeting signal by Tom70, precursor proteins transverse the inter membrane space via the Tom40 pore to be directed to the TIM22 complex. In addition to Tim22, this complex consists of Tim9 and 10, which aid the transfer of precursors to Tim22 (Koehler et al., 1998). In brief, the 300 kDa TIM22 complex, consisting of two pores (hence its other name the 'twin-pore translocase'), inserts the inner membrane proteins aided by the small Tims that shuttle to and from the TOM complex to collect substrates (Rehling et al., 2003; Rehling et al., 2004). In the case of N-terminal targeting, presequence proteins are translocated across the outer membrane by electrostatic interactions between the positively charged presequence and acidic domains of Tom22 and Tom40 (Brix et al., 1999). From here, preproteins will be transferred to the TIM23 complex (also called the presequence translocase). This complex, consisting of Tim23, 17 and 50, accepts N-terminal targeted precursors for inner membrane or matrix and forms a pore across the membrane (Geissler et al., 2002; Yamamoto et al., 2002). Tim50 appears to interact with precursors destined for the mitochondrial matrix and transfers these proteins from TOM to TIM (Geissler et al., 2002; Yamamoto et al., 2002). This process is facilitated by small Tims (Tim8, Tim13), which are chaperones that pass precursors to the TIM23 complex (Leuenberger et al., 1999). The TIM23 complex can either insert proteins directly into the mitochondrial inner membrane or pass these to the mitochondrial matrix. For matrix import, the complex requires both the membrane potential and the ATP driven power of the PAM (presequence translocase associated import motor)

complex (Rehling et al., 2004; Rassow et al., 1994; Schneider et al., 1994; van der Laan et al., 2006). It has recently been proposed that the switch between TOM tethering (inner membrane sorting) and TIM23 complex-PAM binding (motor recruitment; matrix translocation) takes place via a reaction cycle involving Tim21 and Tim17 (Chacinska et al., 2005).

Protease processing and chaperone action

Upon arrival at the mitochondrial inner membrane, via different pathways, intermembrane space targeted and matrix translocated nuclear DNA-encoded precursor proteins are processed by proteases and chaperoned to their final destination. The events described below do not necessarily occur in a sequential order. Rather, assembly of the OXPHOS complexes is the result of their simultaneous, interdependent action.

Protease processing

For several intermembrane space targeted proteins, the sorting signal is cleaved at the inner membrane by the oligomeric IMP (inner membrane peptidase) complex, which consists of three subunits, Imp1, Imp2 and Som1 (Gakh et al., 2002). Examples of IMP complex substrates are Cytb_2 and Cytc_1 (Glick et al., 1992). As these are key components of the mitochondrial electron transport chain, neither Δimp1 mutants nor Δimp2 yeast mutants can grow on nonfermentable carbon sources. The human IMP homologues have recently been identified and shown to complement an IMP yeast knockout for the processing of the precursor of apoptotic protein DIABLO/Smac, which possesses a stop-transfer presequence that resembles that of Cytb_2 and Cytc_1 (Burri et al., 2005).

For matrix targeted proteins, the N-terminal targeting signal is cleaved off by MPP (mitochondrial processing peptidase) (Gakh et al., 2002). A single cleavage by MPP is usually sufficient for the maturation of most inner membrane and matrix precursors. However, some precursors (mostly those containing a specific N-terminal octapeptide domain) require additional processing catalysed by a second metalloprotease, the MIP (mitochondrial intermediate peptidase) (Isaya et al., 1992; Kalousek et al., 1992). The importance of MIPs for OXPHOS assembly was demonstrated by knockout of yeast MIP, which results in multiple electron transport chain enzyme deficits and loss of mtDNA (Isaya et al., 1994).

Matrix chaperones

Upon arrival in the mitochondrial matrix the imported protein is chaperoned by mtHsp70. MtHsp70 activity is regulated by cochaperones Mge1 and Mdj1 by substantially increasing the low intrinsic ATPase activity of mtHsp70 (Voos and Rottgers, 2002). Mge1 catalyzes nucleotide exchange and defects in its function lead to an insufficient interaction of mtHsp70 with protein substrates (Westermann et al., 1995). Mdj1 assists in substrate binding, and null mutations lead to respiratory defects and misfolding and aggregation of newly imported proteins (Prip-Buus et al., 1996). Besides nuclear DNA-encoded proteins, mtHsp70 also aids protein folding of mtDNA-encoded proteins (e.g. ATP-ase 6 of ATP synthase (Herrmann et al., 1994)). Furthermore, chaperone action of both mtHsp70 and Mdj1 was shown to be required for the solubilization of misfolded proteins, a prerequisite for their cleavage by the PIM1 protease (Wagner et al., 1994).

In addition to mtHsp70, a group of chaperonins termed 'type 1 chaperonins' consisting of the Hsp60 and Hsp10 proteins (homologues of the bacterial GroEL/GroES and cpn60/cpn10 systems) aids matrix protein folding and protection of stress-denatured proteins (Levy-Rimler et al., 2002). Hsp60 is one of the most important components of the protein folding system in the mitochondrial matrix (Martin, 1997). Exemplary for this, null mutants of Hsp60 in *S. cerevisiae* are not viable due to the severe defects in folding of mitochondrial proteins (Cheng et al., 1989). Furthermore, mutation in human Hsp60 has been associated with hereditary spastic paraplegia SPG13 (an autosomal dominant form of the disease) (Hansen et al., 2002). Hsp60 forms homo-oligomers of 14 subunits with seven subunits arranged in a ring (double doughnut structure) (Sigler et al., 1998). Proteins that enter the cavity are protected from interactions with other components of the surrounding environment. Hsp10 can form a lid at the top of the double ring system, closing the opening of the cavity (Fenton et al., 1996). ATP binding and hydrolysis plays a key role in binding and release of both substrate and Hsp10 to Hsp60 (Levy-Rimler et al., 2002). It is thought that newly imported mitochondrial preproteins interact with Hsp60 shortly after reaching the matrix (Ostermann et al., 1989; Heyrovska et al., 1998) and that Hsp70 and Hsp60 most likely cooperate in the folding reaction of imported proteins in a sequential order. Preproteins first encounter mtHsp70. Then, after being released from Hsp70, preproteins interact with the Hsp60 complex (Manning-Krieg et al., 1991). Cyclophilins assist protein folding by bringing prolyl bonds in a conformation suitable for further folding reactions. Cpr3 was shown to be important for efficient folding of newly imported

preproteins (Matouschek et al., 1995; Rassow et al., 1995). It acts in a cooperative manner with Hsp60 to obtain a high folding efficiency (von Ahsen et al., 1997).

Other than Hsp60 and Hsp70, bacterial members of the Hsp100 family (ClpA and ClpB) mediate protective reactions, preventing cellular damage caused by the accumulation of aggregated proteins (Goloubinoff et al., 1999; Zolkiewski, 1999). Hsp100 also assists the degradation of irreversible damaged polypeptides by proteolytic machineries. Hsp78 is the yeast homolog of the bacterial ClpB, which resolubilizes aggregated polypeptides in conjunction with Hsp70 (Leidhold et al., 2006). Interestingly, its presence is also required for activation of the mitochondrial protein synthesis machinery (Schmitt et al., 1996).

FeS cluster (and heme) assembly

Human CI houses eight iron-sulfur (FeS) clusters, required for electron transport from the NADH oxidation site to the electron acceptor ubiquinone (Q). These FeS clusters are assembled by the ISC (iron-sulfur cluster) assembly machinery, inherited from the bacterial ancestor of present day mitochondria. In yeast, the ISC assembly system comprises 14 known proteins of diverse functions (Lill et al., 2006). In brief, free cysteine provides the sulfur, released by Nfs1, which is transferred to Isu scaffold proteins via Isd11. Iron is imported into mitochondria and delivered to Isu proteins by Yfh1, the yeast homologue of frataxin. Ferredoxin proteins (Yah1p and Arh1p) are also essential to FeS cluster biosynthesis, as their depletion results in strong defects in maturation of mitochondrial FeS proteins (Manzella et al., 1998; Lange et al., 2000). FeS cluster assembly and chaperone activity of the Hsp70 system are connected as Hsp70 chaperone Ssq1, co-chaperone Jac1 and Mge1 are required for FeS cluster assembly (Voisine et al., 2000; Lutz et al., 2001; Lill et al., 2006), possibly by facilitating FeS cluster transfer. Maturation of Yfh1 is defective in deletion strains for Ssc2p, homologue of mtHsp70, again linking mtHsp70 function to FeS cluster assembly (Knight et al., 1998). A possible function for the Hsp70 cochaperones is the stabilization of the conformation of the scaffold proteins or to regenerate scaffold proteins in steps subsequent to the de novo synthesis of the Fe/S cluster on Isu1p (Muhlenhoff et al., 2003; Dutkiewicz et al., 2006).

In addition to their function in FeS cluster biosynthesis, frataxin, Yah1p (ferredoxin) and Arh1p (ferredoxin reductase) are also involved in biosynthesis of heme, by the delivery of iron, required for the assembly and function of CIV (Barros et al., 2002;

Lesuisse et al., 2003). CIV assembly is an intricate process known to involve many regulatory proteins, for stability, assembly and formation of heme and copper sites (for detailed descriptions see: (Khalimonchuk and Rodel, 2005; Barrientos et al., 2002)). Because the focus of this review is on CI assembly, this topic will not be discussed further here.

As an overview, figure 4 briefly summarizes the topics discussed so far.

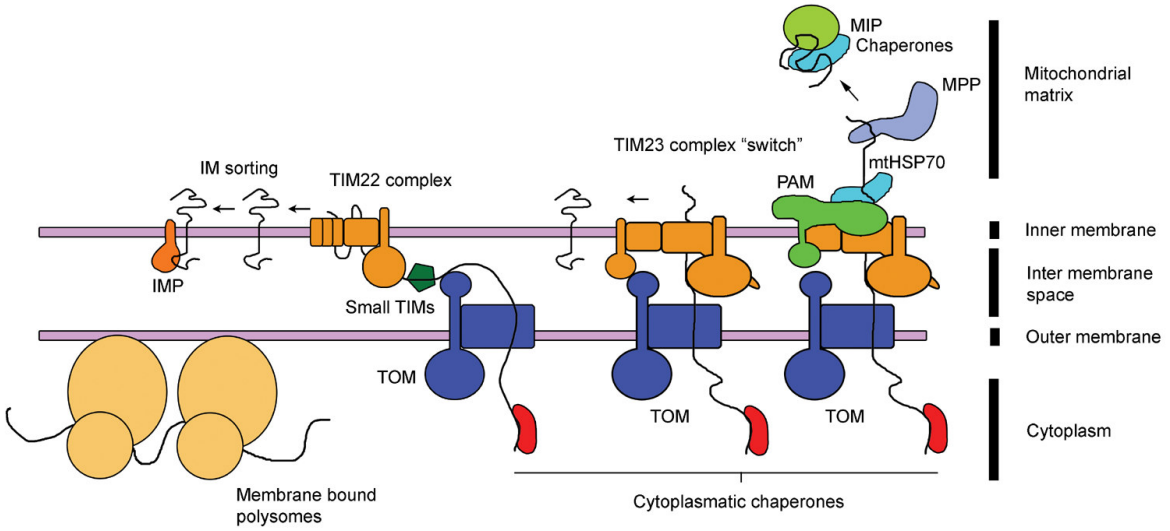


Figure 4. Import and targeting of nuclear DNA-encoded OXPHOS subunits
Upon translation, nuclear DNA-encoded OXPHOS proteins are targeted to the mitochondrial surface, at which they are imported by the TOM and TIM complexes and directed to either the mitochondrial matrix or the mitochondrial inner membrane.

Mitochondrial DNA-encoded subunits

Posttranslational membrane insertion of mtDNA-encoded precursors

All seven CI subunits encoded by the mitochondrial genome (ND1-6 and ND4L) are highly hydrophobic membrane proteins. Upon their translation, these hydrophobic mitochondrial translation products must be shielded to prevent their aggregation in the mitochondrial matrix (Herrmann et al., 1994). Therefore, an intimate relation between the inner membrane and the ribosome is essential. Evolution has come up with several solutions to this problem, mostly relying on tight coupling between ribosomal translation and membrane insertion via translocases.

For example, the bacterial TAT (twin arginine translocase) system translocates fully folded and often large proteins with the consensus sequence SRRxFLK ('twin arginine' motif) across the membrane (Palmer et al., 2005). The TAT system is thought to serve for membrane translocation of proteins that have incorporated a cofactor required for their activity already in the cytosol, and are hence no longer unfolded. Another pathway of membrane insertion is via the Sec (general secrection) complex, used in bacteria and the eukaryotic endoplasmic reticulum (de Keyzer et al., 2003). This complex receives unfolded hydrophobic membrane proteins either for membrane insertion or translocation, via the so-called 'dual-gating' mechanism. Prior to membrane insertion, the SRP (signal recognition particle) targets N-terminal precursors upon their translation (Poritz et al., 1990; Koch et al., 1999). For insertion, ATPase SecA receives the precursor bound SRP and engages the Sec complex. In *E. coli*, the SecY, E, and G proteins associate to form a protein-conducting channel (de Keyzer et al., 2003). YidC was found localized in the vicinity of the SecYEG complex, and shown to interact with transmembrane segments of nascent inner membrane proteins (Houben et al., 2000; Scotti et al., 2000). YidC acts as an insertase for several proteins in bacteria, and depletion of YidC results in strong defects in the assembly of CIV and CV (van der Laan et al., 2003). Recently, cooperation between YidC and the SecYEG complex was shown to be required for membrane insertion of the CyoA subunit of cytochrome bo3 oxidase (du Plessis et al., 2006), providing a possible novel insertion pathway.

Since present day mitochondria are believed to have evolved from endosymbiontic bacteria, one would expect to find at least some conservation of these membrane insertion pathways in mitochondria. However, mitochondria do not seem to use signal recognition particles and at least in *S. cerevisiae*, mitochondria lack a Sec complex counterpart (Glick and Von, 1996). A link between bacterial membrane insertion pathways and mitochondria was provided with the discovery of the Oxa1/YidC/Alb3 group of proteins (Kuhn et al., 2003). Oxa1 and Alb3 are the mitochondrial and chloroplast homologues of the bacterial YidC protein. Oxa1 was found to be essential for CIV and CV assembly in *S. cerevisiae* (Bonnefoy et al., 1994; Altamura et al., 1996; Kermorgant et al., 1997), and its reduction in *N. crassa* results in deficiency in CI and CIV (Nargang et al., 2002) (but somewhat surprisingly not in CIII (Altamura et al., 1996)). Analogous to YidC, Oxa1 mediates the insertion of membrane proteins (Hell et al., 2001) and ribosome binding to c-terminus of Oxa1 is required for co-translational insertion of several mtDNA-

encoded proteins (Jia et al., 2003; Szyrach et al., 2003). Recently, Mba1 was discovered to have a related role in ribosome tethering to the inner mitochondrial membrane (Preuss et al., 2001; Ott et al., 2006). Analogous to Oxa1, Mba1 is associated to the mitochondrial ribosome and its deletion leads to insertion defects (Jia et al., 2003; Szyrach et al., 2003; Rep and Grivell, 1996; Ott et al., 2006). Mba1 is suggested to serve as a membrane receptor for mitochondrial ribosomes that, in a concerted action with Oxa1, positions the ribosome to the protein insertion site at the inner membrane (Ott et al., 2006). Mba1 is most homologous to mitochondrial ribosome subunit Mrpl45 (Ott et al., 2006). This allows speculation about whether it is actually part of the ribosomal structure and could serve as some sort of signal recognition particle as described for bacteria. The most recent examples of the intimate relation between the ribosome and the inner membrane are Mdm38 and Ylh47, which also associate with ribosomes and aid the export of matrix proteins in the assembly of the respiratory chain complex (Frazier et al., 2006). Another distant YidC homologue is Oxa2/Cox18 (Funes et al., 2004). This protein is described to be essential for CIV assembly but in yeast Oxa1 and Cox18/Oxa2 do not complement each other (Saracco and Fox, 2002; Funes et al., 2004; Preuss et al., 2005). Finally, Cox11p, essential to Cu(B) site formation of CIV, was found associated to the ribosome (Khalimonchuk and Rodel, 2005).

Quality control of inner membrane proteins

Proteases are not only required for signal sequence cleavage, but also act as a quality control system for inner membrane proteins. AAA (triple A) proteases are main players of this system and have been demonstrated to degrade a large number of nonnative membrane proteins (Leonhard et al., 1996; Langer, 2000; Langer et al., 2001; Nolden et al., 2005). There are two types of AAA proteases: intermembrane space (i-AAA) and matrix (m-AAA) proteases. Yeast i-AAA protease, composed of Yme1, is active on the intermembrane side (Leonhard et al., 1996) and its deletion leads to respiratory deficiencies (Thorsness et al., 1993). The human m-AAA protease constitutes a hetero-oligomeric complex of Afg2l2 and paraplegin (Atorino et al., 2003). In the absence of m-AAA protease, yeast cells are respiratory deficient and lack assembled respiratory chain complexes (Arlt et al., 1998). Recently, the m-AAA protease is proposed not only to ensure the quality control of inner membrane proteins but also exert a regulatory function during mitochondrial biogenesis (Nolden et al., 2005), as it processes MRPL32 and thus affects mitochondrial ribosome assembly. MRPL32 becomes tightly associated with the mitochondrial inner membrane upon processing by the m-AAA protease and is recruited to ribosomes only at a late stage of their assembly. This supports that

ribosome assembly is completed at the inner membrane and that only membrane bound ribosomes are translationally active. Interestingly, overexpression of Oxa1 or Mba1 suppresses the defects of the yta10 and yta12 mutants (subunits of the yeast m-AAA protease), possibly because aberrant membrane tethering is compensated (Rep et al., 1996).

Another component of the quality control system is the prohibitin complex. This complex is assembled from subunits Phb1 and Phb2 and exists as diverse inner membrane bound ring-shaped structures in yeast mitochondria (Tatsuta et al., 2005). Prohibitins prevent misfolding of newly synthesized proteins, and as such serve as chaperones for respiratory chain complex processing (Nijtmans et al., 2000). Destabilization of mitochondrial polypeptides is observed upon disruption of the prohibitin genes, and further research demonstrated that the prohibitin complex can modulate the activity of the m-AAA protease (Steglich et al., 1999).

Figure 5 briefly summarizes the path of mtDNA-encoded subunits in OXPHOS complex assembly.

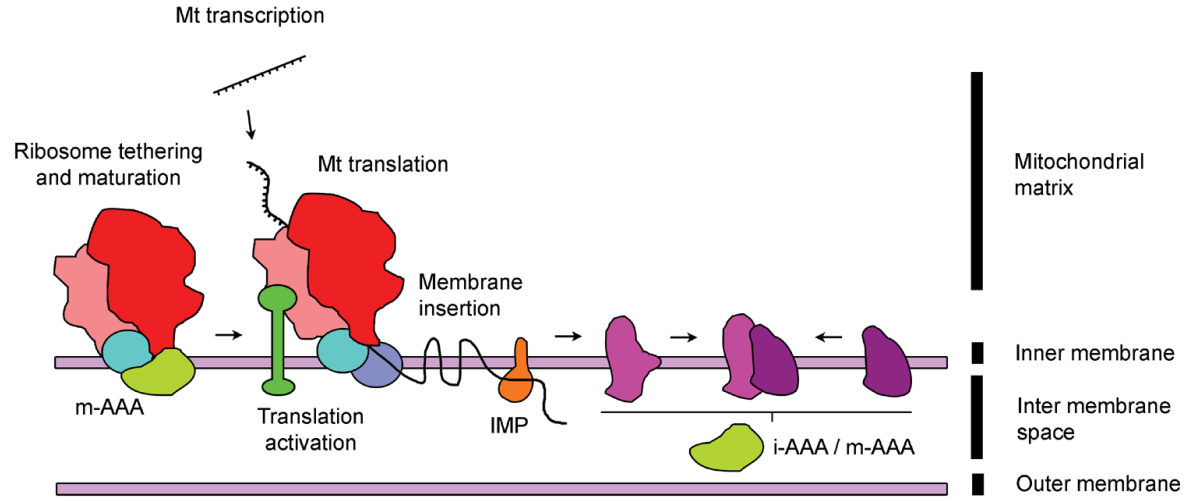


Figure 5. Membrane insertion of mtDNA-encoded OXPHOS subunits
Transcription and translation of mitochondrial gene products is coordinated by transcription factors and translational activators and depends on proper ribosome assembly. Upon translation, proteins targeted to the inner membrane are chaperoned and processed prior to combination with other membrane proteins.

Combination of assembly intermediates and chaperone action

The previous paragraphs have shown the many ways in which submitochondrial processes such as import, targeting and quality control are “indirectly” important for the assembly of the OXPHOS complexes. An obvious question is what happens next, when all the constituents that ultimately assemble into CI are present inside the mitochondrion?

This paragraph briefly discusses what is known about the combination of building blocks during CI assembly and which chaperones are required for the process (figure 6). It will cover the “state of the art” prior to the beginning of the research described in this thesis (in the year 2002), together with the relevant open questions at that time.

Complex I assembly occurs via intermediate complexes

Although about two decades ago CI structure and mechanism of function were still largely unknown, several ideas existed about the way in which human CI could be assembled, mainly based on studies performed in other organisms. It seemed conceivable that assembly takes place via substructures, termed assembly intermediates. These intermediates are combinations of subunits that are combined to form even larger structures, ultimately resulting in fully assembled CI. The first lines of evidence for this appeared in 1990, when Hall and Hare demonstrated that in rat hepatoma cells, nuclear DNA-encoded CI subunits are required to allow incorporation of mtDNA-encoded subunits into a presumably preformed scaffold (Hall and Hare, 1990). In the years to come, most if not all assembly studies in other organisms confirmed that a scaffold of nuclear DNA-encoded CI subunits can be formed in the absence of mtDNA-encoded subunits (Tuschen et al., 1990; Hall and Hare, 1990; Hofhaus and Attardi, 1993; Bai and Attardi, 1998; Bourges et al., 2004; Kirby et al., 2004; Potluri et al., 2004).

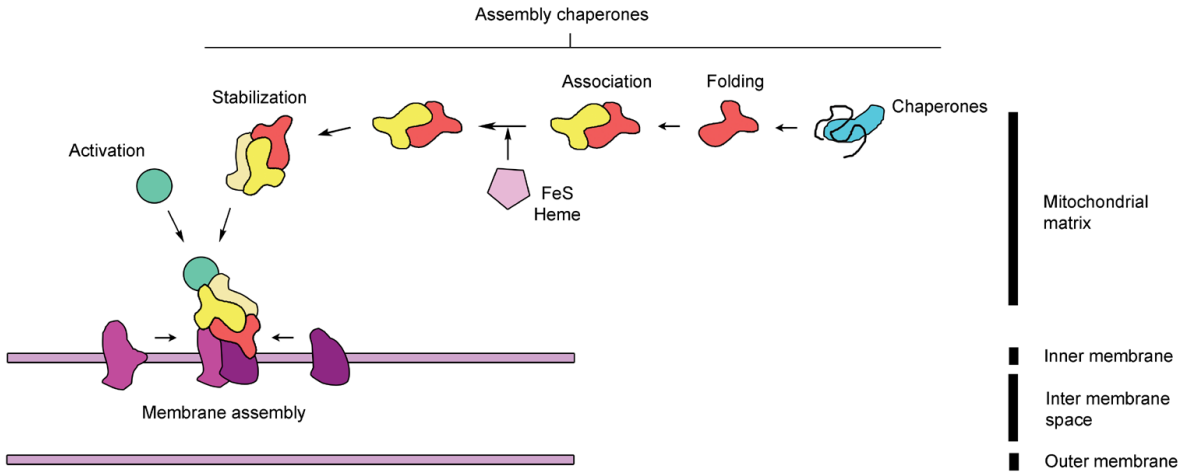


Figure 6. Combination of OXPHOS assembly intermediates
This figure shows the combination of chaperoned matrix proteins and the incorporation of prosthetic groups, formation of membrane complexes and the integration of both subassembled products into the final OXPHOS enzyme. An integral part of this process is FeS cluster assembly, which has been considered in the previous paragraph.

In the fungus *Neurospora crassa*, inhibition of either mitochondrial or nuclear translation demonstrated that the perhipheral arm of CI, consisting of nuclear DNA-encoded subunits, could be assembled independently from the membrane arm, consisting mainly of mtDNA-encoded subunits (Tuschen et al., 1990). In the following years, systematic mutation of *N. crassa* CI subunits delivered valuable information about which CI subunit is required for the assembly of each arm and it appeared that the membrane arm of *N. crassa* CI is assembled from a large and small intermediate (Weidner et al., 1992; Schulte et al., 1994; Videira, 1998; Videira and Duarte, 2001; Schulte, 2001; Videira and Duarte, 2002).

Until 2002, CI assembly was also studied in other organisms (Braun et al., 1998; Cardol et al., 2002; Yadava et al., 2002), but studies for the human CI assembly mechanism were still scarce. Most information about human CI assembly was to come from SDS-PAGE and blue-native PAGE studies for patients in which one of the subunits is altered or absent due to mutation (Van Coster et al., 2001; Triepels et al., 2001). In 2003, using two-dimensional blue-native PAGE, specific high-molecular weight CI intermediates were observed in a cohort of CI deficient patients which could represent intermediates of the assembly process (Antonicka et al., 2003). However, structurally altered CI subunits could disturb assembly or destabilize the holo-complex, therewith resulting in intermediates that do not primarily originate from the assembly process. In order to discriminate which

intermediates are assembly intermediates, detailed investigations in model systems of human CI assembly were required. These studies should then reveal whether specific assembly intermediates also exist for human CI, in which order they are combined, whether the assembly process reflects the structural build-up of the complex, and which steps in the assembly process are of crucial importance.

Complex I assembly chaperones

Analogous to CII-CV, the assembly process of CI requires the aid of assembly chaperones. These are proteins that aid the assembly process, e.g. via stabilization of assembly intermediates. Until 2002, only two putative CI chaperones were described, both in *N. crassa*: the complex I intermediate associated proteins CIA30 and CIA84. CIA30 and CIA84 were found associated with a large membrane arm CI intermediate in a *nuo21.3* mutant, and binding kinetics strongly suggested a chaperone function in CI assembly (Kuffner et al., 1998). In 2002, Janssen and colleagues identified the human homologue of this protein: NDUFAF1 (Janssen et al., 2002). Sequence analysis in 13 patients with an isolated CI deficiency did not deliver any mutation and although homologous, it was still uncertain whether NDUFAF1 was also orthologous to CIA30. Therefore, studies were required to demonstrate the requirement of NDUFAF1 in human CI assembly. Furthermore, its association with high-molecular weight protein complexes in healthy and CI deficient patient cell lines may deliver valuable information about its revelance for CI assembly. Finally, as many chaperones have been decribed to function in the assembly of the other OXPHOS complexes, one would expect more to exist than just NDUFAF1 for human CI assembly. A continuous search for new assembly chaperones will remain an essential aspect of CI assembly studies that aids the understanding of the process.

As a concluding overview of the processes required to assemble an OXPHOS complex, figure 7 provides a more detailed representation of figure 3 by combining figures 4, 5 and 6.

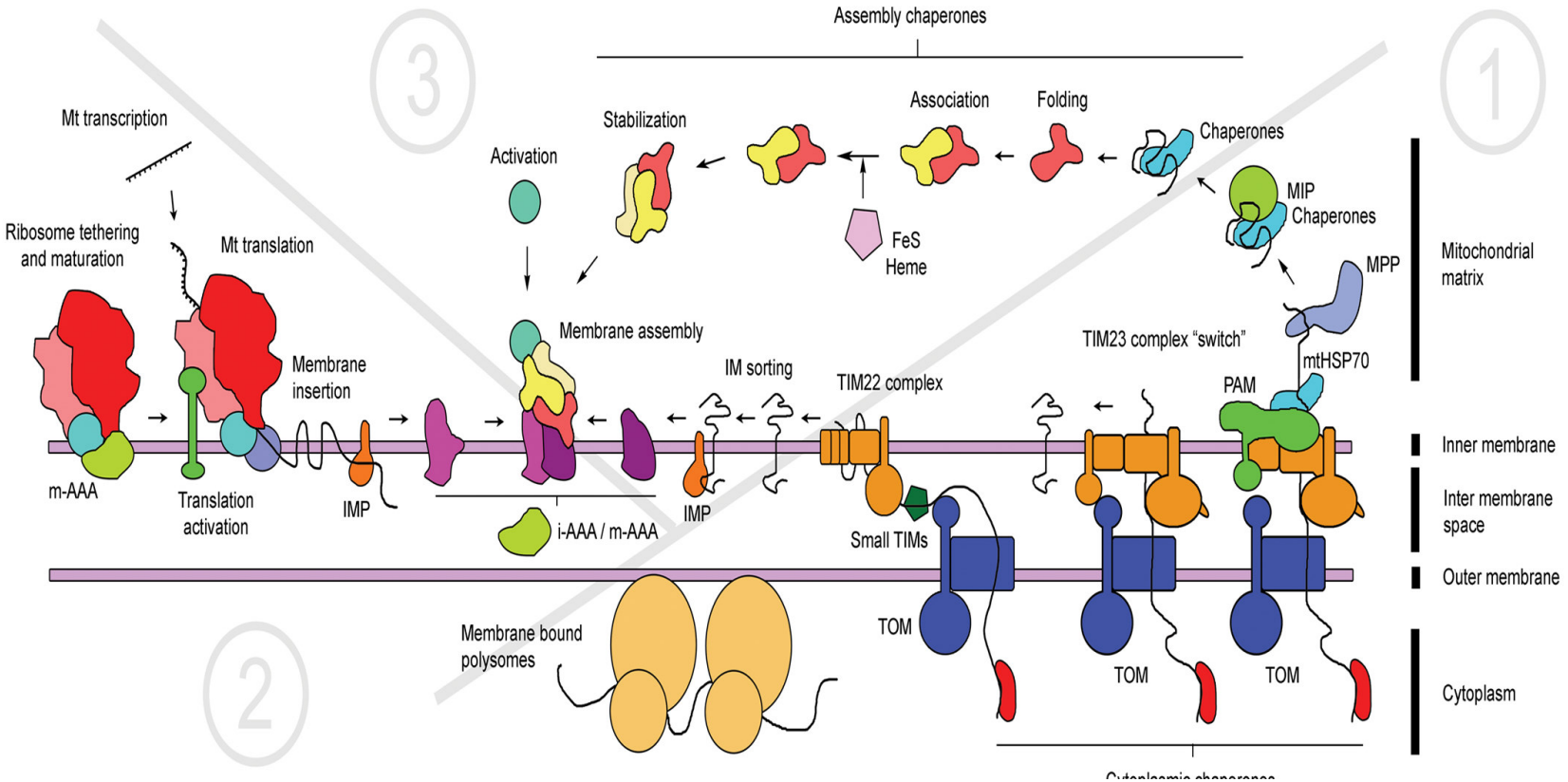


Figure 7. An overview of the different processes involved in the assembly of the OXPHOS complexes

OXPHOS complex assembly links import of nuclear DNA-encoded proteins to translation and membrane insertion of mtDNA-encodec proteins, to the processing, activation and combination of subcomplexes. After either post-translational or co-translational import, nuclear DNA-encoded precursors are targeted to the destined mitochondrial subcompartment. During and after transfer, the precursors are processec by proteases and chaperoned by chaperones such as heat shock proteins to be united with a putative binding partner. Mitochondrial subunits are membrane inserted, aided by quality control and chaperone mechanisms to ensure proper insertion without aggregation of the hydrophobic proteins. Ultimately, both nuclear DNA- and mtDNA-encoded subunits are assembled to form a membrane bound protein complex, which in the case of CI encompasses 45 subunits.

Closing remarks, aim, objectives and outline of this thesis

This chapter has illustrated the complicated and multi-faceted nature of the CI assembly process and serves as the context in which the content of this thesis should be read. Based on the future directions of research discussed in the previous paragraph, this final paragraph will present the aim, objectives and outline of this thesis.

Aim

- The primary aim is to elucidate the assembly process of human mitochondrial complex I.

Objectives

- Investigation of which subassemblies are formed and in which order these are combined to form fully assembled CI.
- Investigation of the requirement of assembly chaperones for CI assembly.

Outline

Part I describes three studies investigating which subassemblies occur during the course of CI assembly and in which order these are combined: chapter 2 describes a model for CI assembly based on the co-evolution of CI subunits and the structure of the complex, chapter 3 describes the development of our first model of CI assembly by using a conditional assembly system, and chapter 4 describes finding of the entry-point of mtDNA-encoded subunits in the assembly process and improvements to our first model.

Part II of this thesis describes three studies for the existence and function of CI specific assembly chaperones, which are hypothesized to play a vital role in CI assembly: chapter 5 demonstrates the importance of chaperone NDUFAF1 for CI assembly, chapter 6 describes the occurrence of chaperones NDUFAF1 and B17.2L in a cohort of CI deficient patients, and chapter 7 describes the mitochondrial function of the Ecsit protein, a signaling protein in the immune response and binding partner of NDUFAF1.

Chapter 8, the general discussion, will reflect on both parts of this thesis.

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Part I: The complex I assembly scheme

Chapter 2

Complex I assembly: a puzzling problem

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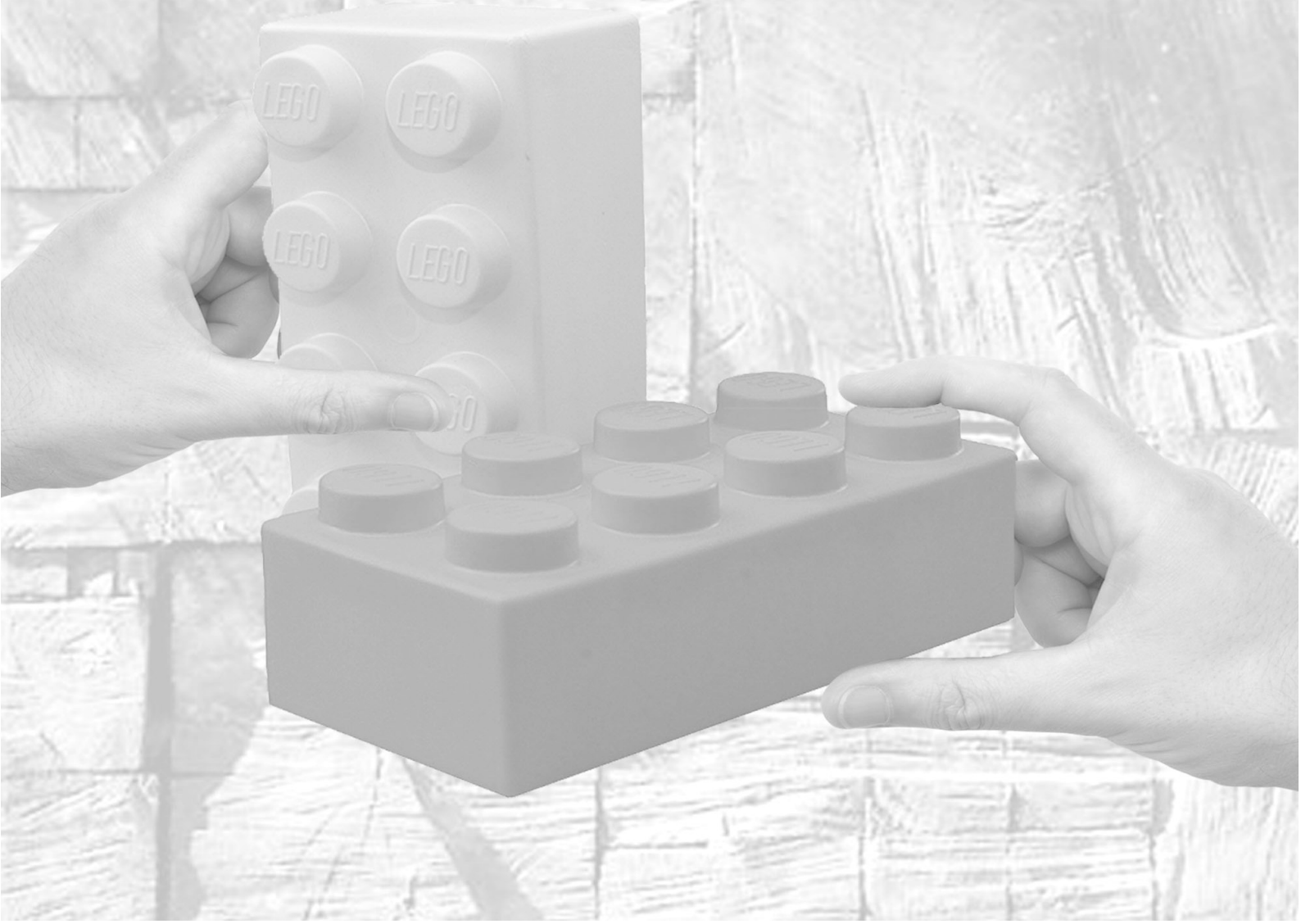
Current Opinion in Neurology 2004; 17(2): 179-186



Abstract

Disturbances in the mitochondrial oxidative phosphorylation pathway most often lead to devastating disorders with a fatal outcome. Of these, complex I deficiency is the most frequently encountered. Recent characterization of the mitochondrial and nuclear DNA encoded complex I subunits has allowed mutational analysis and reliable prenatal diagnosis. Nevertheless we are still confronted with complex I deficient patients without a mutation in any of the known subunits. It is assumed that these patients harbour defects in proteins involved in the assembly of this largest member of the oxidative phosphorylation complexes. This review describes our current understanding of complex I assembly, new developments and future perspectives. The first model of human complex I assembly has recently been proposed. There are new insights into supercomplex assembly and stability which may aid in explaining combined deficiencies. Recent functional characterisation of some of the 32 accessory subunits of the complex may link these subunits to complex I biogenesis and activity regulation. Research on complex I assembly is increasing rapidly. However, comparison between theoretical and experimental models of complex I assembly is still problematic. The growing understanding of complex I assembly at the subunit and supercomplex level will clarify the picture in the future. The elucidation of complex I assembly by combining patient data with new experimental methods will aid diagnosis and possibly therapy of many of the yet uncharacterised mitochondrial disorders.

Introduction

A person´s needs are hardly ever satisfied, but whatever the need one surely requires ATP. This energy carrier is mainly generated in mitochondria by the oxidative phosphorylation (OXPHOS) system via the concerted action of five protein complexes (I, II, III, IV and V) and two mobile electron carriers (cytochrome *c* and ubiquinone). The first four complexes transport electrons from NADH and $FADH_2$ to its final acceptor molecular oxygen. This is accompanied by proton transport across the inner mitochondrial membrane. The thus generated proton gradient is finally utilised by the fifth complex in the chain, ATP synthase, to generate ATP.

OXPHOS-system dysfunction is the cause of serious disorders mostly affecting tissues with a high-energy demand like brain, heart and skeletal muscle. They are observed approximately once every 10.000 births resulting in numerous multi-systemic disorders, often resulting in early childhood death (Smeitink et al., 2001).

The most frequent occurring enzymatic deficiencies are found in the first complex of the chain: complex I (NADH:ubiquinone oxidoreductase; OMIM 252010) (Triepels et al., 2001). Complex I is the largest of the five OXPHOS complexes and is the entry point for electrons donated from NADH. After sequencing the genes encoding its 45 subunits (identified in *H. sapiens*) in a group of 20 complex I deficient patients, about 40% of the deficiencies could be traced back to mutations in the structural building blocks (Triepels et al., 2000). It has been hypothesized that in the genetically unsolved cases the defect is in one of the intricate steps of complex I assembly of which, in contrast to e.g. complex III (Visapaa et al., 2002) and IV (Barrientos et al., 2002) of the OXPHOS-system, only scarce knowledge is available.

This review will focus on the development of the human complex I assembly model and combines current views on assembly from different perspectives.

Complex I: functional and structural aspects

In complex I, electrons provided by NADH are funnelled from the cofactor FMN via eight redox groups, the iron-sulfur (FeS) clusters, to the electron acceptor ubiquinone. Ubiquinone, in turn, transfers the electrons to the next complex in the chain (complex III). This electron transfer is coupled to proton translocation across the mitochondrial inner membrane. In summary, complex I pumps protons and transfers electrons in a stoichiometry of $4H^+/2e^-$.

Complex I is the least known enzyme-complex of the OXPHOS-system. Developments in proteomics have allowed the identification of several additional subunits bringing the total number of bovine structural proteins at 46 (Carroll et al., 2003; Hirst et al., 2003). These subunits are either encoded by the mitochondrial (7 subunits) or the nuclear genome (39 subunits). Single particle electron microscopy data depict the complex as an L-shaped ´boot´ (Hofhaus et al., 1991; Guenebaut et al., 1997; Guenebaut et al., 1998; Grigorieff, 1999; Djafarzadeh et al., 2000) with a hydrophobic membrane arm and a hydrophilic protruding arm. Electron micrographs in *Escherichia coli* show an additional “horse-shoe” conformation, which may reflect the active form of the complex (Bottcher et al., 2002).

In contrast to complex I in mammals, plants and fungi, certain bacteria harbour a much smaller proton-translocating NADH:ubiquinone oxidoreductase (called NDH-1) (Friedrich et al., 1995). This bacterial complex I is composed of only 14 subunits and can carry out the same functions as mitochondrial complex I. Due to its simplicity and functionality, the bacterial system is a good model system to study complex I function. When discussing the minimal complex I structure we will from now on refer to the 14 subunits in bacterial NDH-1.

In *E. coli*, the 13 complex I genes (two genes, *nuoC* and *D*, are fused), are organised in the so-called *nuo*-operon (NADH:ubiquinone oxidoreductase) (Friedrich et al., 1995). Six genes encode for peripheral hydrophilic proteins, including all proteins with binding motifs for NADH, FMN and all FeS clusters. The seven other genes encode hydrophobic membrane proteins. Several nomenclatures exist for the different subunits varying per organism of which the bovine nomenclature is most frequently used (Hirst et al., 2003). In this system, the peripheral subunits are named the 75, 51, 49 and 30 (fused), 24 kDa, PSST and

TYKY subunits. The membrane subunits are named ND1, ND2, ND3, ND4, ND4L, ND5 and ND6 (Figure 1).

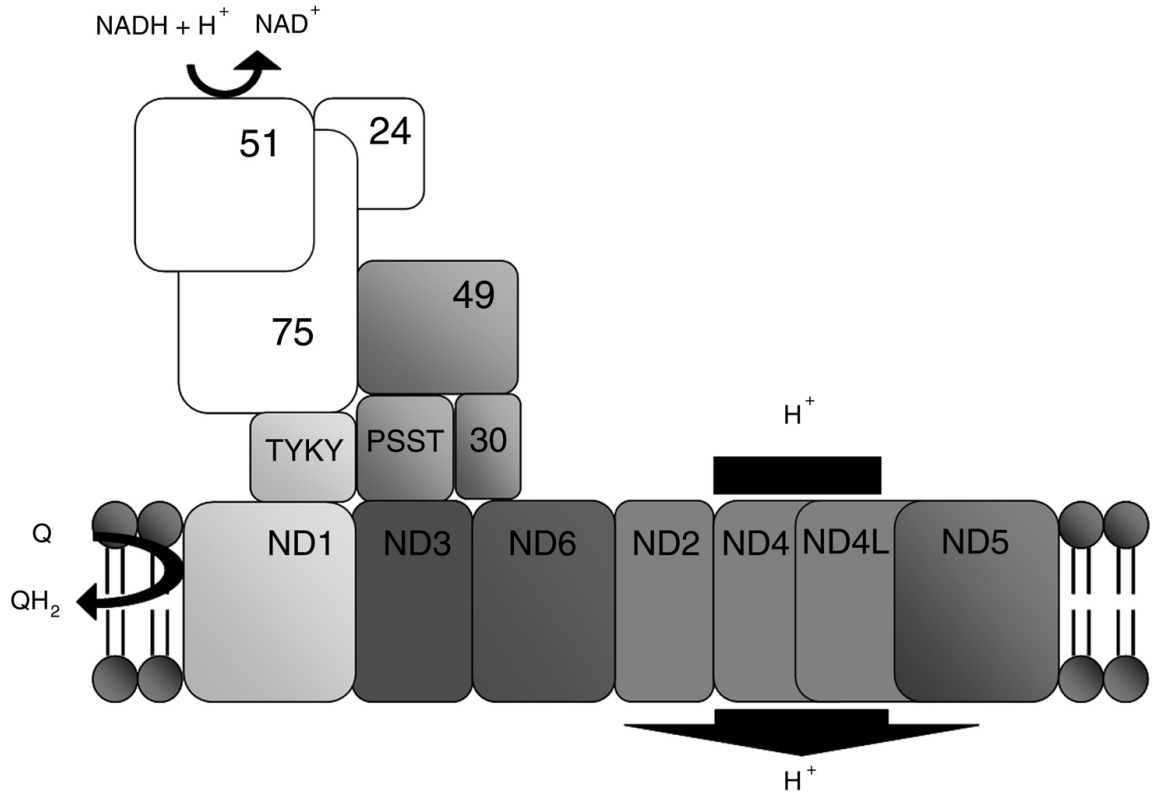


Figure 1: A schematic depiction of the subunit arrangement in bacterial complex I (NDH-1)
Numbers indicate the sizes of the individual subunits in kDa (bovine nomenclature).

Modular evolution of complex I

Bacterial complex I consists of three distinct functional modules. The first is the hydrophilic peripheral module containing the NADH dehydrogenase fragment, composed of the 24, 51 and 75 kDa subunits. The second module connects the peripheral and the membrane module and is called the connecting fragment. This fragment shows no reactivity towards NADH or quinones. It is composed of the TYKY, PSST and 30 and 49 kDa subunits. The third module is the membrane fragment. It consists of the seven hydrophobic ND subunits, of which several are related to K^+ or Na^+-H^+ antiporters. Therefore, it has been proposed that this fragment is involved in proton pumping (Friedrich, 1998).

Homology searches have revealed a great deal of conservation of certain modules of the complex between various organisms. Archaeal, cyanobacterial, bacterial and mammalian complex I share great resemblance in their complex I structure, although the electron input device (NADH dehydrogenase in mammalian complex I) varies. Based on sequence homology between functional modules of complex I in the various organisms, Friedrich and Weiss (1997) have proposed the so-called modular evolution scheme for complex I. The idea is that complex I most probably originated by fusion of pre-existing protein assemblies constituting modules for electron transfer and proton transport (Friedrich and Weiss, 1997). Starting point for the model is the ´minimal´ functional system of complex I observed in bacteria.

The proposed evolutionary modules described in the theory are a ferrodoxin (TYKY), a hydrogenase (PSST and 49 kDa), a transport protein (ND5), a protein with a quinone reduction site (ND1), a protein of unknown function (30 kDa) and a NADH dehydrogenase module (75, 51 and 24 kDa).

The hydrogenase module (also present in nowadays soluble [NiFe] hydrogenases) can be traced back to the oldest ancestor (Friedrich, 2001). This progenitor has lost its [NiFe] active site and its ability to react with molecular hydrogen. It has most probably gained a quinone-binding site (Friedrich and Scheide, 2000). Combining the soluble [NiFe] hydrogenase module with the ferrodoxin-type subunit TYKY, the ion-translocating ND5 and the quinone-binding ND1 resulted in the common ancestor of a family of membrane-bound multisubunit hydrogenases, including the hydrogenase 3 and 4 of the formate hydrogenlyase system of *E. coli*, the CO-induced hydrogenase of *Rhodospirillum rubrum* and the Ech hydrogenase of *Methanosarcina barkeri*. These hydrogenases seem to participate in the same reactions, namely oxidation of an electron donor and subsequent transfer of a positive charge. The combined subunits can be recognised in today's complex I as the hydrogenase module (Friedrich, 2001).

The membrane part of complex I is thought to have originated from triplication of the transporter subunit ND5 into ND2 and ND4 and acquisition of ND3, ND6 and ND4L. The membrane subunits ND4 and ND5 have shown to be related to a class of Na^+/H^+ antiporters in *Bacillus subtilis*. These antiporters come in two classes: MrpA and MrpD. It seems from recent work that ND5 is more like MrpA, and ND2 and ND4 are more like MrpD (Mathiesen and Hagerhall, 2003), arguing against the idea of triplication of the antiporter module. Acquisition of the NADH

dehydrogenase module resulted in today's mitochondrial complex I. Homologous to *Ralstonia eutropha,* the electron input domain of mammalian complex I is made up of the 24, the 51 kDa and the N-terminal segment of the 75 kDa subunit (Finel, 1998) (Figure 2).

The great number of gene fusions, the interspecies sequence homology of functional modules and the corresponding gene arrangements strongly support the idea of modular evolution. Furthermore, one could imagine that complex I assembly occurs in modules as well.

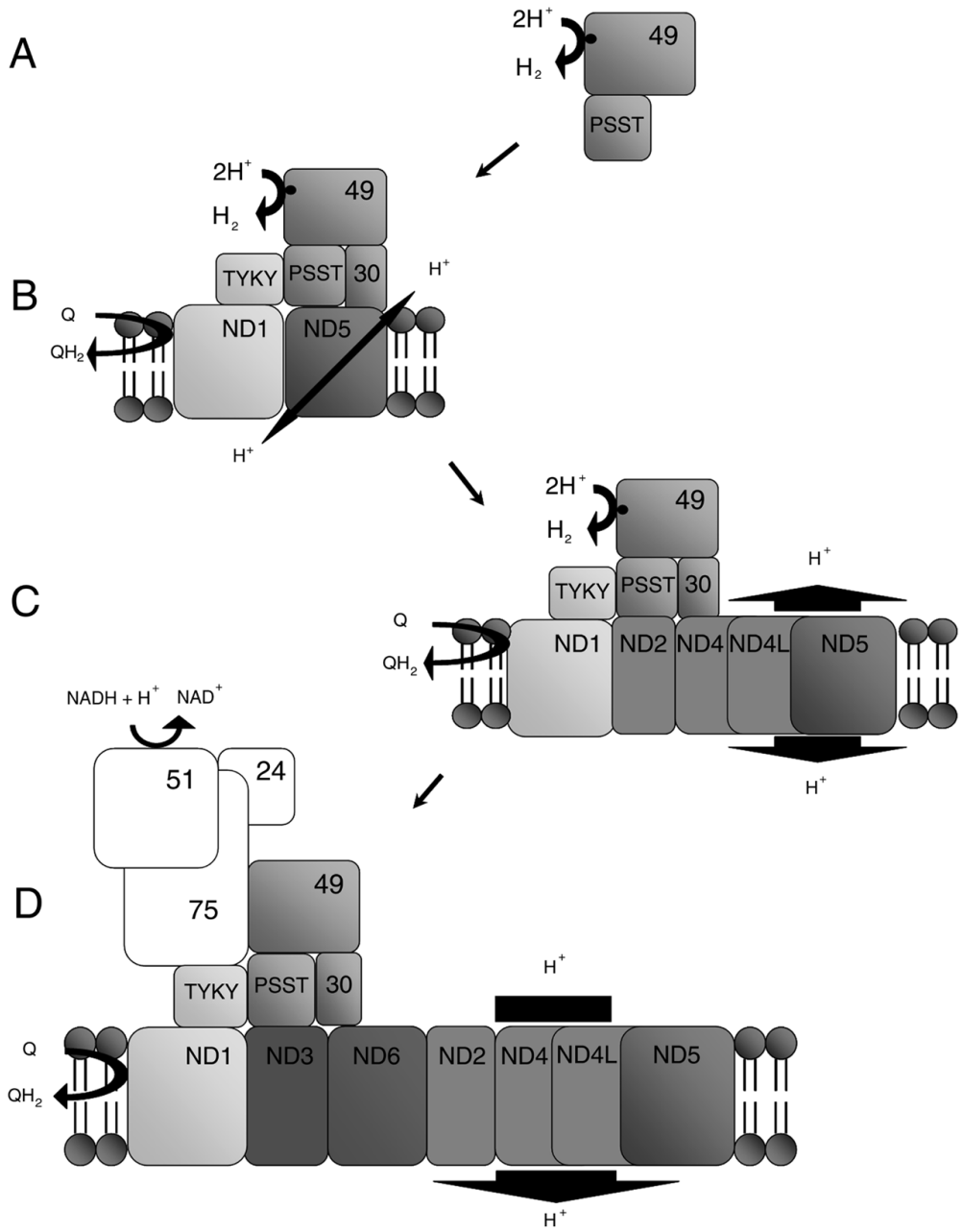


Figure 2: Modular evolution of complex I (adapted from (Mathiesen and Hagerhall, 2003))
The proposed modular evolution is depicted in figures A-D. Please mind that this does not necessarily represent the order of assembly. According to the theory, a soluble [NiFe] hydrogenase (A) combined with a ferrodoxin and a transport module to form the progenitor of membrane-bound hydrogenases (B). Expansion of the transport module resulted in the common ancestor of complex I. Addition of the NADH dehydrogenase module resulted into present-day bacterial complex I (D).

Complex I assembly in Neurospora crassa

The first organism in which the biogenesis of complex I has been studied in detail is the fungus *Neurospora crassa* (Schulte, 2001). Assembly intermediates were observed after inhibition of mitochondrially encoded subunits by chloramphenicol (the peripheral arm accumulates) (Friedrich et al., 1989) and under manganese limitation (the membrane arm accumulates) (Schmidt et al., 1992). By inducing mutations in, and deletion of, many genes encoding complex I subunits, assembly was blocked and more assembly intermediates were observed. Analysis and pulse-chase labelling of these assembly intermediates (Videira, 1998; Tuschen et al., 1990) resulted in the first assembly pathway for complex I. By knocking out the TYKY homologue in the fungus, two proteins were found to be associated with a large membrane arm assembly intermediate: CIA (Complex I Intermediate Associated) proteins CIA30 and CIA84. These proteins are not part of the final structure of complex I and have been shown to cycle between the bound and unbound state (Kuffner et al., 1998). These two proteins are the first examples of putative assembly factors of complex I. The human homologue for CIA30 was found (Janssen et al., 2002) but has to date not been shown to be associated with disease.

In total, four assembly intermediates were observed by combined investigations: a small and a large membrane arm intermediate, the complete membrane arm and a peripheral segment. Assembly is proposed to occur via several steps. The first step is the combining of the small and large membrane arm intermediates (possibly via the interaction with the chaperones CIA30 and CIA84) to a membrane arm intermediate. This is combined in the second step with the assembled peripheral arm to result in holo-complex I (Schulte, 2001).

Complex I assembly in *Homo sapiens*

The group of Attardi was among the first to investigate complex I assembly in humans (Chomyn, 2001). By the analyses of mitochondrial DNA mutations affecting subunits of complex I it was shown that ND4 and ND6 are essential for the assembly of the enzyme complex. Since then, the occurrence of assembly intermediates in patients with a mitochondrial disorder has resulted in several studies for the assembly of complex I.

Assembly profiles of human complex I were researched by Triepels and colleagues (Triepels et al., 2001). In this study a set of monoclonal antibodies was tested on western blots of complex I deficient patients. By comparing the intensities of different antibodies in different patients, patient samples were classified by their assembly profiles. Subunits appeared to behave as three classes. The levels of 39 and 30 kDa subunits varied in the same manner as did the 20 and 18 kDa as did the levels of the 15 and 18 kDa subunits.

Recently, two dimensional blue native electrophoresis (2D-BN/SDS-PAGE) was used to detect partially assembled complex I subcomplexes in a cohort of four complex I deficient patients (Antonicka et al., 2003). Analysis of the observed subcomplexes led to the first description of the assembly pathway for complex I in humans. By using a set of 11 antibodies against nuclear and mitochondrial encoded subunits, a set of five subcomplexes was identified. Observed groupings of subunits were the 49, 39 and 30 kDa subunits, the 24, 20 and 18 kDa subunits, the ND1 and 8 kDa subunits, the 15 and 14 kDa subunits and a large subcomplex containing ND1, the 49, 39, 30, 20, 18, 15 and 14 kDa subunits. The 17 kDa subunit could not be assigned to any of the groups.

These findings led to the proposal of the following assembly pathway. The first step is the partial assembly of the peripheral arm by combining two intermediates (including the 49, 39 and 30 kDa subunits). This complex is then coupled to several subunits of the membrane arm (including ND1) resulting in a membrane bound protein. Subsequently, the last part of the peripheral arm is added (including the 20, 24 and 18 kDa subunits) and the membrane arm is completed.

The finding of the 39 and 30 kDa subunits together as well as the 20 and 18 kDa subunits can be correlated to the findings of Triepels and colleagues (Triepels et al., 2001), described earlier. However, the assembly pathway differs significantly from the pathway in *N. crassa* as many more intermediates are observed in humans than in the fungus.

Do experimental models of assembly fit with the modular evolution theory?

The *N. crassa* model of assembly correlates only crudely with the modular evolution scheme. After comparison with modular evolution, one could envisage the four subcomplexes in *N. crassa* as follows. The peripheral arm should include the NADH dehydrogenase module. The small membrane arm intermediate includes the hydrogenase and/or transporter module. The large membrane arm intermediate includes the transporter module. By analogy, in *N. crassa*, the transporter and hydrogenase modules are coupled prior to attachment of the NADH dehydrogenase fragment. This order of assembly is consistent with the modular evolution model. Nevertheless, this is still speculative and the *N. crassa* model is yet too unrefined to speculate about a more detailed comparison.

The model of human complex I assembly is hard to reconcile with the modular evolution theory. There is an important conflict between the two models. The separate assembly of the 49 kDa and the PSST subunits is inconsistent with the conserved collocation of these two subunits in the nowadays soluble [NiFe] hydrogenases (Friedrich, 2001). In the light of modular evolution, one would not expect these two subunits to be present in different assembly intermediates. The appearance of the 39 kDa subunit together with the 30 kDa subunit in one subcomplex is inconsistent with recent findings in our laboratory (unpublished results). A possible explanation for this discrepancy is the presence of different subcomplexes of a similar size. Finally, the presence of degradation products which can exist simultaneously with assembly intermediates, can lead to ambiguity in the interpretation. The fact that relative amounts of subunits in subcomplexes found by Antonicka and colleagues differ between patients might be an indication for the occurrence of degradation products.

Using a greater variety of antibodies may (especially of the 51 and 75 kDa subunits) ease the comparison between ‘theory’ and ‘practice’. More patient studies will continue to contribute to the elucidation the complex I assembly pathway. We recently performed an assembly study (Ugalde et al., 2003) in which we found a novel mutation in the ND6 gene in a patient with Leigh syndrome which seems to have a profound effect on complex I assembly and/or stability. More studies along these lines will help to elucidate a general assembly pattern in humans.

Combined deficiencies: possible link to supercomplex stability

As discussed, complex I assembly should be regarded in combination with its stability. An interesting link between combined OXPHOS deficiencies and supercomplexes has recently intensified the research on this area.

It has been known for some years that (some of) the protein complexes forming the respiratory chain are associated in higher order assemblies called 'supercomplexes' or 'respirasomes' (Schagger, 2002). Supercomplexes are thought to ease diffusion and reaction chemistry, also called 'substrate channelling' and may increase individual complex stability. After their discovery in yeast, supercomplexes were shown in mammalian mitochondria as well (Schagger and Pfeiffer, 2000). Supercomplexes composed of $I_1III_2IV_4$ and III_2IV_4 were identified as the two major fragments of the respirasome in healthy mitochondria.

Recently, a complete respirasome was isolated in *Parococcus denitrificans* containing complex I, III and IV in a 1:4:4 stoichiometry (Stroh et al., 2004). Complex I activity was found in membranes of mutants lacking complexes III or IV. Interestingly however, no assembled complex I (only dissociated subunits) was found after electrophoretic separation or chromatographic isolation of the supercomplex in the wild-type strain. This has the important implication that complex I is stabilized by assembly into the supercomplex.

These findings fit with observations of combined deficiencies. In two patients with different cytochrome *b* mutations, complex III was shown to be decreased in combination with a decrease in complex I activity and stability (Schagger, 2002). It seems that lack of assembled complex III causes a specific lack of assembled complex I. Interestingly, complex III deficiency is observed often in combination with deficiencies of other complexes. Vice versa, (Ugalde *et al*., unpublished data) find that mutations in complex I genes can also affect the stability of other mitochondrial complexes, with a specific decrease in the levels of fully-assembled complex III in patients with mutations in the 49 and 18 kDa subunits. These findings indicate that combined deficiencies may be related to a decrease in supercomplex stability and that functional analysis of isolated complexes I and III should be regarded with caution.

Recently, supercomplexes of complex III and IV in yeast have shown to be stabilised by cardiolipin, an anionic phospholipid (Pfeiffer et al., 2003). Likewise, cardiolipin may be involved in the stabilisation of supercomplexes in humans. Defects in cardiolipin remodelling have been described and result in the rare disorder X-linked cardioskeletal myopathy and neutropenia (Barth syndrome) (Vreken et al., 2000).

Accessory subunits involved in complex I biogenesis or activity regulation

During the last two decades, a great variety of pathogenic mitochondrial and nuclear mutations has been described in literature (Smeitink et al., 2001; DiMauro and Schon, 2003; Zeviani and Carelli, 2003). Mutations are found in seven nuclear encoded subunits of complex I (the 75, 51, 49, 24, 18 kDa, PSST and TYKY subunits) and in all the ND subunits in the mitochondrial genome. Remarkably, all of these subunits (except for the 18 kDa subunit) are part of the minimal 14 subunits constituting bacterial NDH-1. The affected 'minimal' nuclear encoded subunits all contain FeS clusters (responsible for electron channelling in the protein complex). It seems that mutations in the 'framework' formed by the modules in the modular evolution scheme have a dramatic effect on complex I function.

A lack of mutational data for 32 subunits that are not part of the minimal bacterial version of complex I have kept the function of most of these ´accessory´ subunits unclear. It has been proposed that these subunits function as a protective scaffold to escaping electrons hence preventing damage by radicals. However, there is increasing experimental evidence indicating that at least some of the accessory subunits may have another enzymatic function, e.g. in biosynthesis or activity regulation. These findings may link accessory subunits to complex I dysfunction.

An accessory subunit that may be linked to assembly is the SDAP subunit, the homologue of the Acyl Carrier Protein (ACP) described in *N. crassa*. This subunit is closely related to ACPs involved in fatty acid synthesis (Zhang et al., 2003) and has been proposed to play a role in lipoic acid biosynthesis (Brody et al., 1997; Jordan and Cronan, Jr., 1997; Wada et al., 1997). Interestingly, an ACP mutant of *N. crassa* cannot assemble complex I membrane arm. Fatty acid synthesis however is not influenced (Schulte, 2001). Mutants disrupted in the peripheral arm do form ACP, suggesting an independent function. Cardiolipin synthesis requires

phospholipids and fatty acids and may hypothetically link this subunit, to (super)complex stability.

Another example is the MWFE subunit, which is required for stable assembly of complex I. It is thought to be involved in species-specific compatibility of complex I assembly and is related to Na^+ transporters in *N. crassa* (Yadava et al., 2002).

Alternatively, there is recent data suggesting a role in activity regulation for some accessory subunits. The 18 kDa subunit of complex I (AQDQ in man) is the target of a mitochondrial cAMP dependent protein kinase. An increase of complex I activity was observed upon phosphorylation of this subunit (Papa et al., 1996). A recent description of a patient with a deletion in the phosphorylation site has linked the dysfunction of the phosphorylation process with impaired complex I assembly and a fatal neurological syndrome (Scacco et al., 2003). Both findings suggest a role of the subunit in activity regulation of complex I (Papa, 2002). Schulenberg and collegues also observed phosphorylation of the 42 kDa subunit (Schulenberg et al., 2003).

Additional support for the idea that accessory subunits can regulate complex I activity comes from studies on the kinetics of activation and de-activation of complex I. Complex I exists in an active and a de-active form, in which the active form can perform the NADH:quinone-reductase function and the de-active form cannot (Kotlyar and Vinogradov, 1990; Vinogradov, 1998). De-activation occurs under substrate limiting conditions whereas activation occurs by reduction of NADH and a slow reoxidation by quinone (Maklashina et al., 2003).

Although bacterial and mitochondrial complex I differ in the number of accessory subunits, the only functional difference is in their ability to undergo active/de-active transition (Kotlyar et al., 1998). Only complex I from vertebrate animals and fungi demonstrate the active/de-active transition. On the basis of kinetic experiments it was shown that activation energy required for activation differs not only between eukaryotes and prokaryotes but also between warm- and cold-blooded animals. Maklashina and colleagues suggest that regulation of complex I activity via active/de-active transition has been achieved in evolution by introducing additional protein subunits (Maklashina et al., 2003).

Other previously described accessory subunits with ‘extra’ functions are the 39 kDa and the bovine B16.6 and B14.7 subunits. The 39 kDa subunit is proposed to bind

NADPH (Schulte et al., 1999). Although the bound NADPH is not involved in electron transfer, lack of the 39 kDa subunit leads to inactivation of complex I. It is therefore thought that the subunit stabilizes a yet unknown redox factor involved in electron transfer in complex I (Schulte, 2001). Other data suggest that the 39 kDa subunit interacts with ACPs as a biosynthetic module (Friedrich et al., 2000). The bovine B16.6 subunit is highly (83%) homologous to the human GRIM19 protein, which is a product of a cell death regulatory gene induced by interferon-β and retinoic acid (Fearnley et al., 2001). This could provide a link between this subunit and apoptosis. The B14.7 subunit is homologous to Tim17, 22 and 23, proteins part of the import machinery of proteins into mitochondria (Carroll et al., 2003) but are further uncharacterised.

Conclusion

The increasing amount of data linking disturbances in complex I assembly to a broad spectrum of clinical phenotypes has resulted in a rapid increase of research in the field. The many aspects of biogenesis, the association of supercomplex stability with mitochondrial disorders and the increasing insight into the function of the accessory subunits contribute to the current momentum.

Interesting future perspectives for research on complex I assembly and stability are provided by recent technical advances. Creating knockouts of genes encoding complex I subunits is difficult in mammalian cells, either due to technical difficulties or due to lethality of the knockouts. However, with the development of the RNA interference technique, creating partial knockouts in mammalian cells is now possible. In combination with the rapidly progressing field of proteomics, intermediates can be rapidly identified and functional properties of unknown intermediates and accessory subunits can be ascertained.

The use of model systems and patient data is a continuing requirement and will contribute to the increasing insight into the possible mechanism underlying many of the yet uncharacterised mitochondrial complex I disorders. Insight into the assembly pathway may provide pre-natal diagnoses and insight into disease progression. Hopefully, it will also contribute to the development of possible therapies for patients suffering from mitochondrial disorders.

Acknowledgements

The authors thank the Prinses Beatrix Fonds and the Netherlands Organization for Scientific research (NWO) for continuous support of our research.

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Chapter 3

Human mitochondrial complex I assembles through the combination of evolutionary conserved modules: a framework to interpret complex I deficiencies

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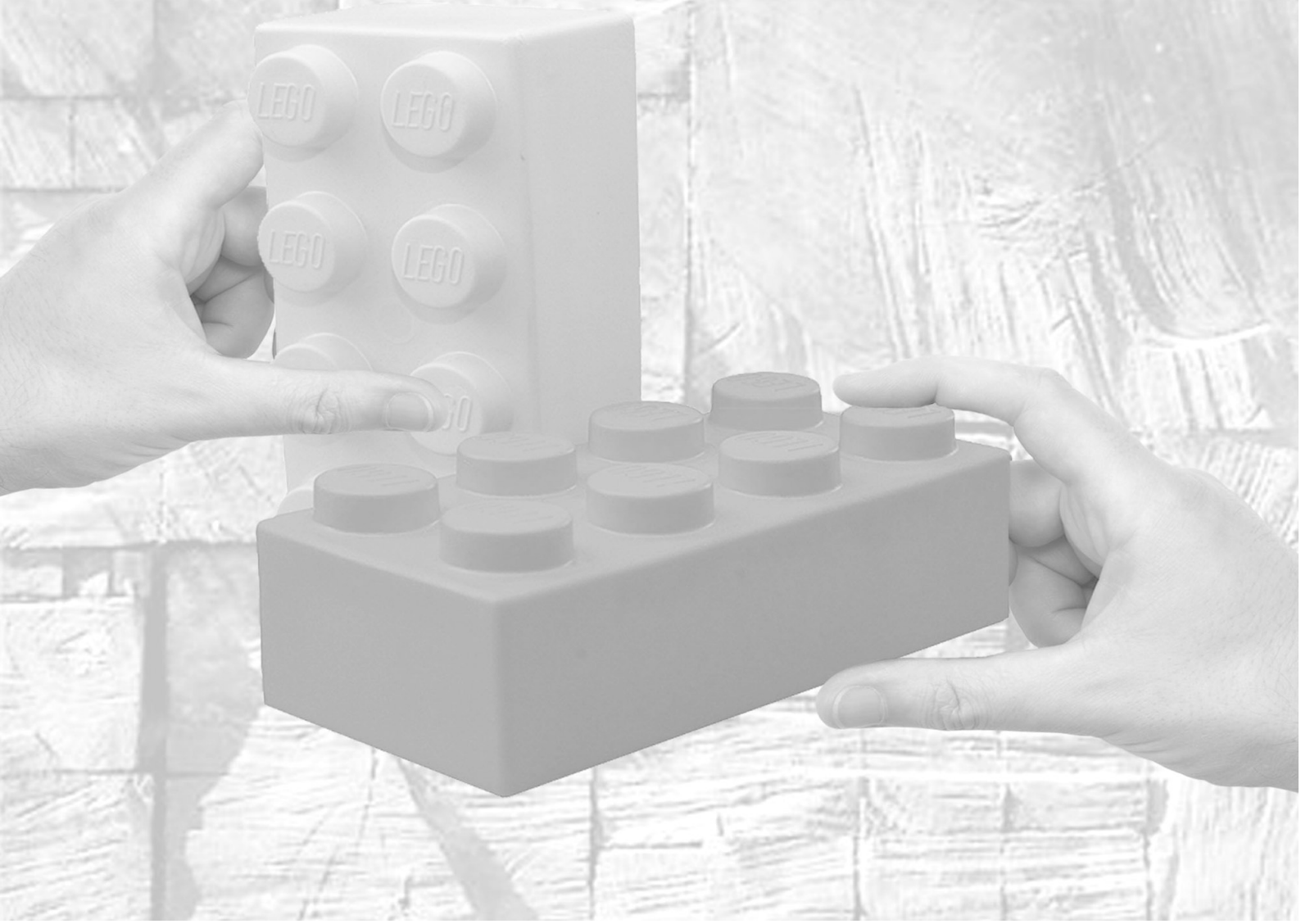
Human Molecular Genetics 2004; 13(2): 2461-2472



Abstract

With 46 subunits, human mitochondrial complex I is the largest enzyme of the oxidative phosphorylation (OXPHOS) system. We have studied the assembly of complex I in cultured human cells. This will provide essential information about the nature of complex I deficiencies and will enhance our understanding of mitochondrial disease mechanisms. We have found that 143B206 rho zero cells, not containing mitochondrial DNA, are still able to form complex I subcomplexes. To further address the nature of these subcomplexes, we depleted 143B osteosarcoma cells of complex I by inhibiting mitochondrial protein translation with doxycycline. After removing this drug, complex I formation resumes and assembly intermediates were observed by two-dimensional blue native electrophoresis. Analysis of the observed subcomplexes indicates that assembly of human complex I is a semi-sequential process in which different pre-assembled subcomplexes are joined to form a fully assembled complex. The membrane part of the complex is formed in distinct steps. The B17 subunit is part of a subcomplex to which ND1, ND6 and PSST are subsequently added. This is bound to a hydrophilic subcomplex containing the 30 and 49 kDa subunits, to which a subcomplex including the 39 kDa subunit is incorporated, and later on the 18 kDa and 24 kDa subunits. At a later stage more subunits, including the 15 kDa, are added and holo-complex I is formed. Our results suggest that human complex I assembly resembles that of *Neurospora crassa,* in which a membrane arm is formed and assembled to a preformed peripheral arm, and support ideas about modular evolution.

Introduction

Complex I (NADH-ubiquinone oxidoreductase, EC 1.6.5.3) is the most frequently affected complex of the oxidative phosphorylation (OXPHOS) system leading to mitochondrial disease (Smeitink et al., 2001; Nijtmans et al., 2004). The enzyme couples the transfer of two electrons from NADH to ubiquinone to the translocation of four protons across the mitochondrial inner membrane. The thus generated proton gradient is used by complex V to produce ATP. Mammalian complex I consists of 46 polypeptide subunits, seven encoded by the mitochondrial DNA and the remainder by the nuclear genome, a non-covalently bound flavomononucleotide (FMN) group and eight iron sulfur clusters (Hirst et al., 2003). Although several mutations have been found in both nuclear and mitochondrial subunits (Nijtmans et al., 2004; Triepels et al., 2001; DiMauro and Schon, 2003; Benit et al., 2004; Ugalde et al., 2003) (see Fig. 1), many complex I deficiencies remain to be explained (Smeitink et al., 2001). In striking contrast to complex IV, where the majority of deficiencies can be explained by mutations in genes encoding for specific assembly proteins, so far this has not been described for complex I. This discrepancy is probably caused by the absence of complex I in the yeast *Saccharomyces cerevisiae*, a model organism which enabled the identification of a dozen of complex IV assembly genes (Barrientos et al., 2002). Given the intricacy of complex I, it is very well possible that defective assembly proteins account for a number of complex I enzyme deficiencies. In a previous study, we have shown a decrease in the levels of intact complex I in six patients harboring mutations in nuclear-encoded complex I subunits, indicating that complex I assembly and/or stability is compromised (Ugalde et al., 2004a). Different patterns of low molecular weight subcomplexes are present in these patients; a finding that has also been demonstrated in other patient studies (Ugalde et al., 2003; Antonicka et al., 2003; Scacco et al., 2003). Insight in complex I assembly in human cells will aid interpretation of these patient data and lead to a better understanding of the molecular mechanisms underlying these disorders.

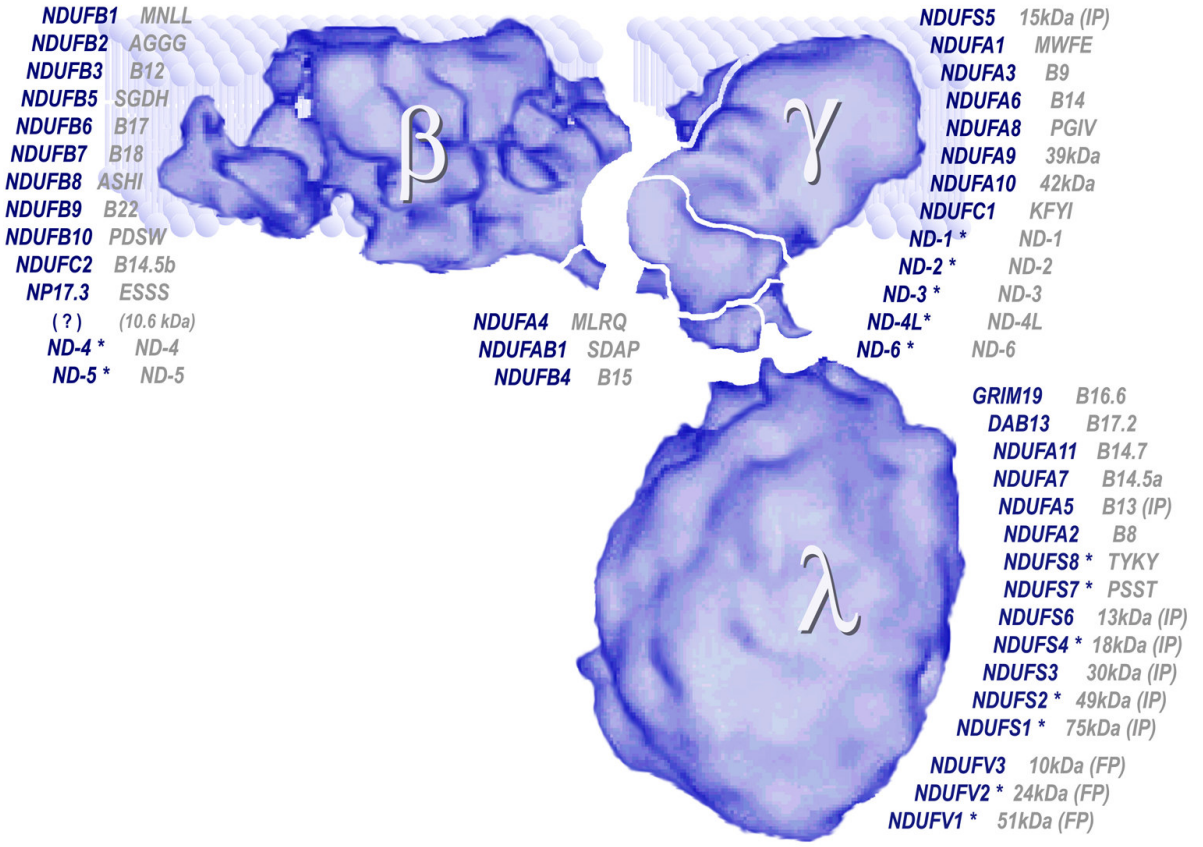


Figure 1: Schematic representation of human complex I subunits and their putative topology within the complex

*Adapted from (Carroll et al., 2002). Bovine homologues are written in gray and human subunits in which mutations are found are marked with *. The unknown (10.6 kDa) subunit has been identified in bovine, but its sequence has not been elucidated yet (from (Nijtmans et al., 2004) © Springer, reprinted with permission of the publisher).*

Complex I, which awaits a crystal structure, is an L-shaped molecule with one arm embedded in the mitochondrial inner membrane and one arm protruding into the matrix, the peripheral arm (Grigorieff, 1999). Although our knowledge of the biosynthesis of complex I is still limited, much information about the subunit topology has been obtained. Hatefi and coworkers were able to fractionate the bovine enzyme into three functional parts: the FMN containing part, the iron-sulfur cluster containing part and a membrane part (Galante and Hatefi, 1978). Over the years the group of Walker refined this fractionation into α, β, γ and λ parts (Sazanov et al., 2000; Carroll et al., 2002) and more importantly, they were able to identify all subunit components of these fractions by mass-spectrometry (Fig.1) (Carroll et al., 2003). By using an immunocapture method in combination with mass spectrometry, Murray and colleagues were able to identify the human homologues

of 42 polypeptides of the 46 beef heart complex I subunits, suggesting an identical composition of the human and bovine complex I (Murray et al., 2003). To avoid confusion we use the bovine subunit-nomenclature in this paper. The conversion to the human nomenclature is given in Fig. 1. Although the location of subunits within the complex only reveals physical associations of subunits, which does not necessarily reflect the physiological assembly, it provides vital information to construct an assembly pathway. Another aspect which can be taken into account when studying the assembly of complex I is the theory that the basic bacterial form of complex I has evolved from several functional modules: an NADH-dehydrogenase, a hydrogenase and a transporter module (Friedrich and Weiss, 1997). It is temping to assume that at least part of the evolutionary origin of complex I is conserved in the mammalian assembly pathway (Vogel et al., 2004).

Most of what is known about the assembly of complex I comes from studies carried out in the fungus *Neurospora crassa*, which contains 35 subunits (Videira and Duarte, 2001). It has been shown that the peripheral arm can still be formed in the absence of mitochondrially encoded subunits (Tuschen et al., 1990). This independent formation of the membrane and protruding arm of the complex was also demonstrated in disruption mutants of this organism (Duarte et al., 1995). In addition, the membrane arm appeared to be formed out of two subcomplexes, designated as the small and large intermediates (Schulte et al., 1994). Interestingly, two non-subunit proteins, named CIA30 and CIA84, were bound to the large membrane arm assembly intermediate. Disruption of either of the proteins led to a specific block of complex I assembly and CIA30 and CIA84 are therefore regarded as complex I assembly proteins (Kuffner et al., 1998). A human homologue has only been found for CIA30 and despite sequence analysis of this gene in complex I-deficient patients, no pathogenic mutations have been described so far (Janssen et al., 2002). To date, it is unclear whether complex I assembly in mammalian cells is comparable to the *N. crassa* model. Consistent with this model is the finding that in metabolic labelling studies in combination with immunoprecipitations, some nuclear encoded subunits can preassemble before mitochondrially encoded subunits are added to the complex (Hall and Hare, 1990). Nevertheless, in this study it is not clear which nuclear subunits form this scaffold for the mitochondrially encoded subunits. Immunopreciptations using a 49 kDa antibody demonstrated that no other mitochondrial encoded subunits were co-precipitated in human 143B and mouse A9 cybrid cells containing mutations in the ND4 and ND6 subunits (Hofhaus and Attardi, 1993; Bai and Attardi, 1998). However, in cybrids which had mutations in ND5 all mitochondrial encoded

subunits were co-precipitated, except ND5 (Hofhaus and Attardi, 1995). This suggests that ND4 and ND6 are essential for assembly whereas ND5 is not. Compatible with these findings are observations in mutants of the green alga *Chlamydomonas reinhardtii,* lack of ND1 and ND6 led to the absence of fully assembled complex I, but still a 160-210 kDa subcomplex could be formed, which contained homologues of the 49 kDa and 75 kDa subunits and showed NADH-dehydrogenase activity. Besides the formation of this 160 kDa-210 kDa subcomplex, deletion of the ND4 and ND4/ND5 subunits also resulted in the formation of a 650 kDa subcomplex with NADH dehydrogenase activity (Cardol et al., 2002). An assembly pathway for human complex I has been recently described by performing two-dimensional blue native/sodium dodecyl sulphate gel electrophoresis (2D BN/SDS PAGE) of mitochondria from muscle biopsies of complex I-deficient patients. Several complex I subcomplexes were found depending on the antibodies used. Moreover, similar patterns of subcomplexes were found in different patients (Antonicka et al., 2003). This led to the suggestion that these subcomplexes are intermediates of assembly. By combining the patterns of the panel of antibodies an assembly pathway was deduced. In this model, subcomplexes of the peripheral arm and subcomplexes containing parts of both arms are found, suggesting that the peripheral and membrane arms are not assembled in separate ways. This is in sharp contrast to the *N. crassa* model, in which the peripheral arm is formed independently from the membrane arm.

In our study we have investigated whether partially assembled complex I subcomplexes could be formed in cells lacking mitochondrial DNA (143B206 rho zero cells), as is the case in *N. crassa*. By using 2D BN/SDS PAGE in combination with immunodetection several partially assembled subcomplexes were detected, illustrating that parts of the complex that do not contain any of the mitochondrially encoded complex I subunits can be formed. To address the dynamics of assembly, we created a conditional complex I assembly system by partially depleting 143B osteosarcoma cells of complex I and other OXPHOS complexes by treating them with doxycycline, an inhibitor of mitochondrial translation. After removal of this drug complex I assembly resumed and the appearance of assembly intermediates was investigated. This approach has been successfully used to study the assembly of complex V and IV (Nijtmans et al., 1995; Nijtmans et al., 1998). Besides the reappearing of complex I, we observed the appearance of partially assembled complex I intermediates, indicating that these subcomplexes were newly formed. Based on the alignment and analysis of these subcomplexes we propose a modular complex I assembly model, which largely resembles the assembly as

described for *N. crassa*, in which the membrane and peripheral parts can be pre-assembled independently.

Materials and methods

Cell Cultures

143B206 rho zero (ρ^0) cells were cultured in DMEM (Life Technologies) supplemented with 5% fetal calf serum (FCS), antibiotics, 1mM uridine and 100 □g/ml bromodeoxyuridine. HEK 293 human embryonic kidney cells and 143B osteosarcoma cells were cultured in DMEM supplemented with 10% FCS, antibiotics and 1mM Uridine (King and Attardi, 1989). 143B control cybrids (Ugalde et al., 2003) were cultured in DMEM (Life Technologies) supplemented with 10% fetal calf serum (FCS), antibiotics, glutamine and 1mM sodium pyruvate. To block mitochondrial translation doxycycline was added at a concentration of 15 µg per ml (Nijtmans et al., 2002). The cells were growth in exponential conditions and harvested at the indicated time points.

Blue Native electrophoresis and in-gel activity assays

Blue Native 5-15% or 5-13% gradient gels were loaded with 20-40 □g of digitonin-isolated mitochondria as described before (Nijtmans et al., 2002). After electrophoresis, the gels were further processed for in-gel activity assays, western blotting or second dimension 10% SDS PAGE as described before (Nijtmans et al., 2002). Proteins were transferred to a PROTAN® nitrocellulose membrane (Schleicher & Schuell).

SDS-PAGE analysis

Whole cell homogenates were prepared by resuspending 5 x 10^6 cells in 125 µl PBS containing 2% (w/v) n-dodecyl ß-D-maltoside. Following a 15 min incubation on ice, homogenates were centrifuged (30 min, 12000 g, 4°C). Next, the supernatant was mixed with an equal volume of Tricine sample buffer (Biorad laboratories, Hercules, USA) containing 2% (v/v) 2-mercaptoethanol. The mixture was kept at room temperature for 60 min. Protein (40 µg protein/lane) was separated on 10 % polyacrylamide gel. Gels were blotted to PROTAN® nitrocellulose membrane (Schleicher & Schuell).

Antibodies

Western blotting was performed using primary antibodies raised against the following subunits of the human mitochondrial OXPHOS complexes: NDUFS7 (PSST), NDUFA3 (30 kDa), NDUFA9 (39 kDa), NDUFB6 (B17), NDUFS5 (15 kDa), UQCRC1, MTCO2, SDHA, (Molecular Probes), ND1 (a gift from Dr. A. Lombes, France), ND6 and NDUFS4 (18 kDa) (a gift from Prof. R. Capaldi, USA), NDUFV2 (24 kDa) (donated by Prof. J. Walker, United Kingdom), NDUFS2 (49 kDa) (provided by Prof. B. Robinson, Canada) and Hsp70 antibody (Affinity Bioreagents, Golden, USA). Peroxidase-conjugated anti-mouse IgGs or Peroxidase conjugated anti-rabit IgGs were used as secondary antibody (Molecular Probes). The signal was detected with ECL® plus (Amersham Biosciences) and the quantification of the blots was performed using ImagePro-Plus 4.1 image analysis software (Media Cybernetics, Silver Spring, MD, USA).

Protein assay

The protein concentration for BN PAGE and SDS PAGE was determined in the n-dodecyl ß-D-maltoside solubilised supernatants before adding Coomassie Blue containing sample buffer, using a MicroBCA protein assay kit (Pierce).

Results

Cells without mitochondrial DNA form partially assembled subcomplexes

To investigate whether subcomplexes of complex I can be formed in cells that do not express mitochondrial subunits, we analyzed 143B206 rho zero (ρ^0) cells by 2D BN/SDS PAGE electrophoresis in combination with western blotting (Fig. 2). Signals were obtained with antibodies against the 30 kDa, 39 kDa, 24 kDa and B17 subunits, but not with antibodies against the 15 kDa, 18 kDa and PSST subunits (results not shown), suggesting that the incorporation of these three proteins into the complex requires the presence of mitochondrially encoded subunits. Antibodies against the mitochondrially encoded ND1 and ND6 subunits were tested as negative controls with which no signals were obtained (data not shown). In control cells most of the signal appeared at the place where complex I runs on the first dimension (indicated in Fig. 2 as CI). As expected no fully assembled complex I could be detected in the ρ^0 cells. However, in ρ^0 cells the 30 kDa, 39 kDa, 24 kDa and the B17 subunits are present at higher molecular weight spots than the

expected molecular weight of the monomeric subunit. This result shows that partially assembled subcomplexes can be formed in the absence of the mitochondrial complex I subunits. The 30 kDa subunit is part of at least five distinct subcomplexes. The first runs at the front and is probably the monomeric subunit. Next there are two subcomplexes at a molecular weight of approximately 80 and 150 kDa and two subcomplexes at a molecular weight of approximately 250 kDa and 600 kDa. All these subcomplexes are also visible in the control cells. The 39 kDa subunit is detected as a long smear, which might reflect the hydrophobic nature of this subunit. A high molecular weight subcomplex can be distinguished also at 600 kDa. A subcomplex of a similar size is found in the control cells. The hydrophobic B17 subunit results in a smeary pattern as well; nevertheless six different subcomplexes can be distinguished. The first intense spot is likely to represent the unassembled subunit. The next subcomplexes run at 50 kDa, 200 kDa, 400 kDa, 650 kDa and 800 kDa respectively. These B17-containing subcomplexes observed in ρ^0cells are unexpected and there are several reasons to think that they are not assembly intermediates. Firstly, the B17 subcomplexes in the ρ^0cells are not observed in other cell systems we tested (see further sections). Secondly, a subcomplex of a molecular weight of 800 kDa (largest B17 spot) is difficult to explain given the fact that many subunits are lacking. For some reason the B17 subunit seems less prone to degradation than other subunits. It is therefore possible that these subcomplexes in ρ^0cells reflect aggregates of this hydrophobic subunit or aggregates of subcomplexes that contain this subunit. The 24 kDa subunit appears in two spots, the first one is connected by an arch to the second spot. These spots probably represent the monomeric molecule and the excess of blue dye, which runs at the front, causes the peculiar shape. An additional spot containing the 24 kDa subunit just before the front is also detected.

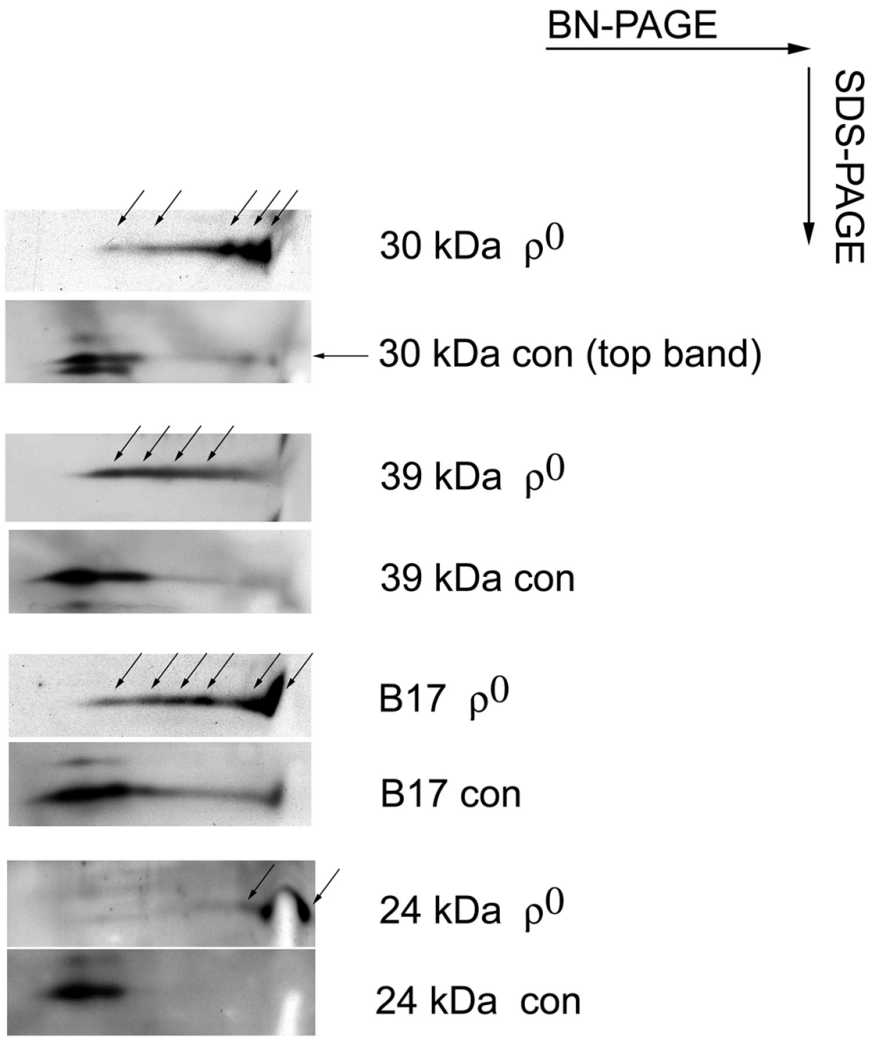


Figure 2: Complex I subcomplexes in ρ^0 cells
Crude mitochondrial fractions of control 143B osteosarcoma cells (con) and 143B206 ρ^0 cells (ρ^0) were separated by two dimensional Blue Native/ SDS electrophoresis (top-right arrows indicate the first and second dimension). The gels were blotted onto nitrocellulose and analysed with antibodies against the complex I subunits 30 kDa, 39 kDa, B17 and 24 kDa. Subcomplexes are indicated with arrows. Fully assembled complex I is indicated as CI. In the panel for the 30 kDa subunit, a residual band of a previous antibody incubation of ND1 is still visible just below the 30 kDa spots. One should therefore focus on the top band (indicated in the figure).

Reversibly blocking complex I assembly

Detection of assembly intermediates is difficult because these are transient products, which are likely to have short half-lives. Moreover, breakdown can occur simultaneously, complicating the identification of true assembly intermediates. To

circumvent these problems we decided to deplete 143B osteosarcoma cells of complex I and other OXPHOS complexes containing mitochondrially encoded subunits, by reversibly blocking mitochondrial protein translation with the drug doxycycline. After 6 days of doxycycline treatment, we observed an approximate 80% reduction of fully assembled complex I compared with untreated cells (Fig. 3A, 0 hours lane). In agreement with previous reports (Yadava et al., 2004), this indicates that the half-life of complex I is relatively long, especially taking into account that because of cell division the complex I pool is diluted. However, it needs to be mentioned that after two to three days of doxycycline treatment the growth of the cells slows down significantly and changes on metabolism likely influence the stability of complex I. Because further treatment with doxycycline affected cell viability in our culture conditions, we decided to treat the cells for 6 days. After the doxycycline treatment, the cells were washed, incubated with fresh medium and collected 3, 6, 12, 24, 48 and 125 hours after for BN PAGE analysis (Fig. 3A). In-gel complex I activity and western blotting using an antibody against the 39 kDa subunit, demonstrate the restoration of fully assembled complex I after 48-125 hours (Fig. 3A, 3B). This rate of complex I restoration does not necessarily reflect the time required for the biosynthesis of complex I, since the assembly can make use of pools of subunits and partially assembled subcomplexes. The observed time-course for complex I assembly is comparable with the findings of Yadava and colleagues in their conditional complex I assembly system in Chinese hamster fibroblasts (Yadava et al., 2004). The fact that no complex I could be detected by the in-gel activity assay after 3 and 6 hours can be explained by the lower sensitivity of this assay compared with immuno-detection.

At 125 hours after doxycycline removal, there is more fully assembled complex I compared to the control cells. Apparently, there is a compensatory mechanism in the cells as a response to the inhibition of mitochondrial protein synthesis. This might involve a general increase of mitochondrial mass because complex II (Fig. 3A), which only contains nuclear encoded subunits, also shows such an increase at 125 hours compared to the control cells. In this experiment we also tested complex III, since it has been recently demonstrated that complex I stability is severely hampered when there is no fully assembled complex III present (Acin-Perez et al., 2004). As shown in Fig. 3A and 3B, complex III is restored in a similar time course as complex I. It is therefore unlikely that in our system the absence of complex III negatively affects complex I stability.

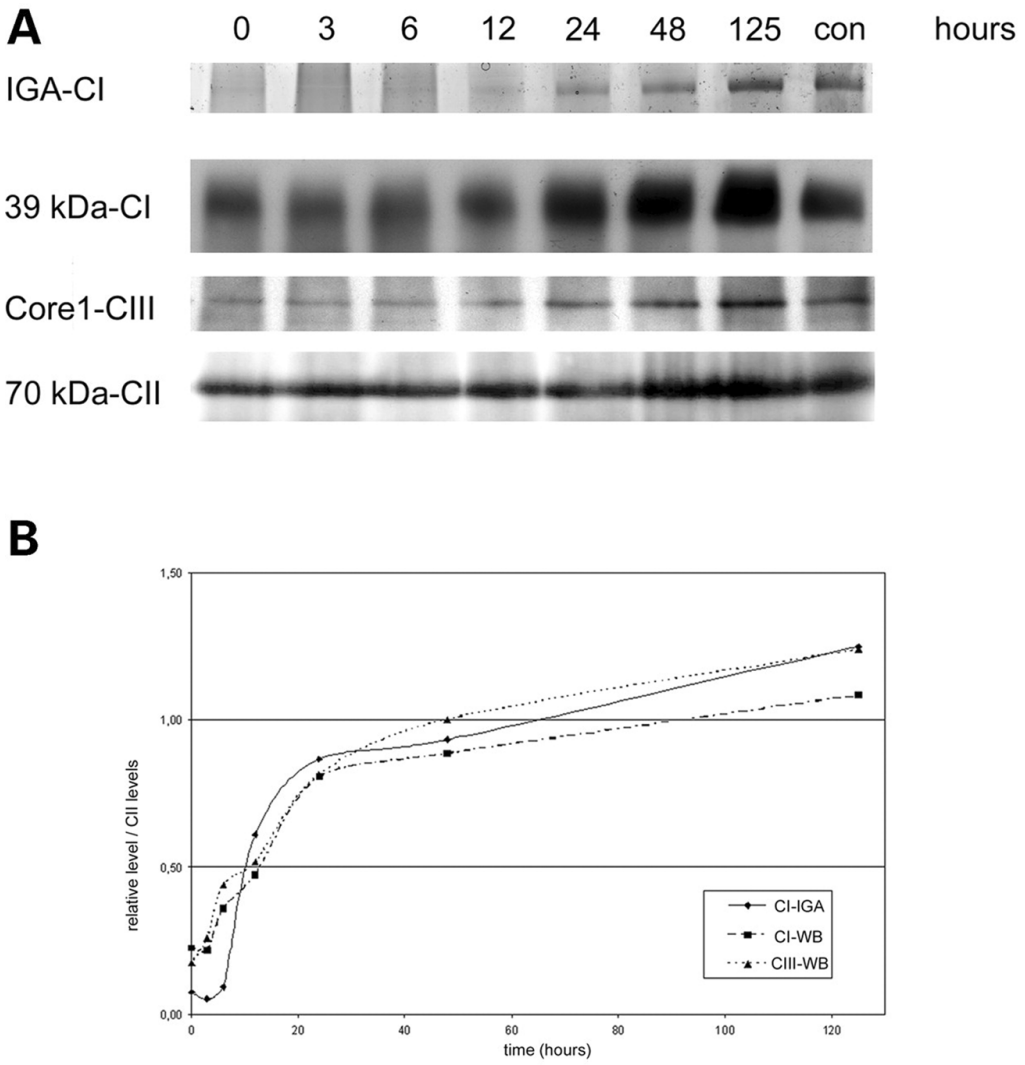


Figure 3: Appearance of complex I in 143B cells after doxycycline treatment

A. Osteosarcoma143B cells were treated for 6 days with doxycycline (an inhibitor of mitochondrial translation), the medium was replaced by doxycycline-free medium and cells were grown for the indicated time (in hours). 40 µg of crude mitochondrial pellets were analyzed by blue native electrophoresis in combination with complex I in-gel activity (IGA-CI, top panel). Duplicate gels were blotted and incubated with antibodies against the complex I subunit 39 kDa (39 kDa-CI, second panel), against complex III core1 protein (Core1-CIII, third panel) and against the complex II 70 kDa subunit (70 kDa-CII, bottom panel). Control untreated 143B cells are indicated as con. B. The signals for the in-gel activity assay and western blots were expressed as percentage of the untreated cells, normalized with the complex II-70 kDa subunit and plotted.

Reappearing of subunits

To follow the reappearance of subunits after the reversible block of assembly, samples taken at different time points after removal of doxycycline were run on a SDS-PAGE, blotted on nitrocellulose and incubated with a panel of nine complex I antibodies (Fig. 4). Cells deprived of mtDNA (ρ^0 cells) were run as a negative control and untreated cells were included in the panel as a positive control. We observe remarkable differences in the steady-state levels of the different subunits. As expected, ρ^0 cells lack the mitochondrially encoded ND6 and ND1 subunits. Consistent with previous findings, also the 18 kDa, 15 kDa and PSST subunits were not detectable in ρ^0 cells and after 6 days of doxycycline treatment (lane 0) their abundance was very low (18 kDa subunit) or not detectable (15 kDa and PSST). These subunits start to reappear at 12 to 24 hours and increase gradually to control levels at 125 hours after release of inhibition. This result indicates that these subunits might not be stabilized in a pre-assembled subcomplex and they would enter the assembly process relatively late. In contrast, the 39 kDa, 30 kDa, 24 kDa and B17 subunits were still visible in ρ^0 cells and after 6 days of doxycycline treatment, suggesting that these subunits are more stable possibly because of the formation of subcomplexes. The steady-state levels of these subunits are comparable to the control levels at 6 to 12 hours after removal of the doxycyline, which suggests that these subunits enter the complex I assembly pathway at a relatively early stage.

The mitochondrially encoded subunits ND1 and ND6 were still present at detectable levels after 6 days of doxycycline treatment, which is consistent with the small amounts (~20%) of assembled complex I observed (Fig. 3A, 0 hours lane). Results show that these subunits gradually increase from 6-12 hours until they reach the maximum at 125 hours after doxycycline treatment. Since these subunits were directly targeted by the drug, which inhibits mitochondrial translation, no pools of pre-assembled subcomplexes could have been formed after the 6 days of doxycycline treatment. All subunits are more abundant after 125 hours compared to the untreated cells, probably as a compensatory mechanism as discussed in the previous section.

Dynamics of complex I subcomplexes

Immediately after removing the drug doxycycline, the synthesis of mitochondrially encoded subunits resumes and the assembly of complex I can start again. In this

way complex I assembly is synchronized and intermediates are more likely to be detectable. To find out when subcomplexes start to appear and whether there is a change in the pattern during the assembly process, we monitored the subcomplexes by 2D BN/SDS PAGE at all time points. To allow a good overview about what is happening in time we grouped the subunits per time point (Fig. 5). The molecular weights of the subcomplexes and possible co-localization of subunits within the same subcomplex will be discussed in more detail in the next section.

For all subunits investigated we observe residual holo-complex I (indicated with arrows) after six days of doxycyline treatment (time 0 hours), which increases in amount in time. In addition we observe subcomplexes for all investigated subunits. These subcomplexes increase in amount in time and subsequently the lower molecular weight subcomplexes decrease again, suggesting that these subcomplexes are true assembly intermediates and not breakdown products. Consistent with the results from the ρ^0 cells (Fig. 2) and the SDS gel (Fig. 4) we do not find subcomplexes smaller than 600 kDa for the 15 kDa and 18 kDa subunits. This result suggests that these subunits enter the assembly pathway relatively late, possibly as monomeric subunit and not in a pre-assembled form. The subunits B17 and 30 kDa are present in low molecular weight subcomplexes, suggesting that these subunits enter the assembly route at an earlier stage. The B17 subunit is also detected as a smear with distinct thickenings suggesting subcomplexes. It is remarkable that shortly after the release of doxycycline there is much free unassembled subunit present at the front (right side) of the gel, suggesting that there is a pool waiting for partners to participate in the assembly process. This is nicely illustrated by the fact that by increasing time larger subcomplexes appear and smaller subcomplexes disappear, indicating that higher molecular weight subcomplexes are formed. The 30 kDa subunit is detected in three characteristic low molecular weight subcomplexes comparable with the ρ^0 cells. The 39 kDa subunit is detected as a similar smear at the low molecular weight range, as seen in the ρ^0 cells. Although the 24 kDa subunit is visible after 6 days of doxycycline treatment in the SDS blot (Fig. 4), in the 2D BN/SDS blots no low molecular weight subcomplexes can be observed besides the small amounts of the monomeric subunit.

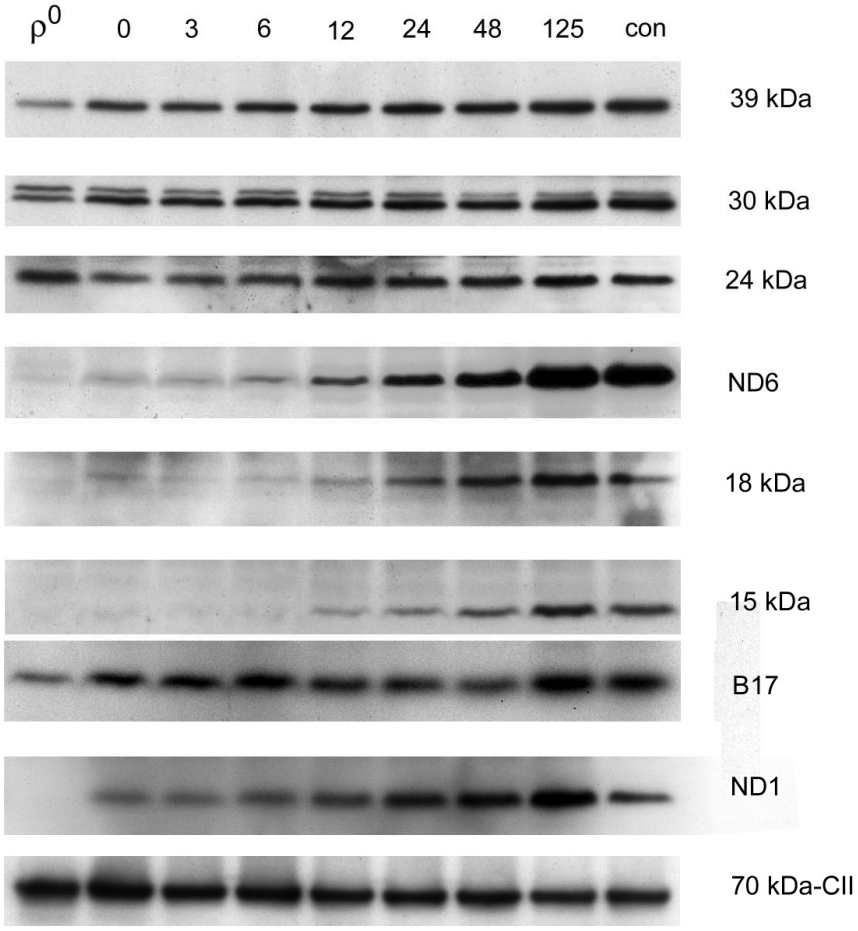


Figure 4: Kinetics of reappearance of complex I subunits

Forty µg of total cell lysate were loaded on a 10% SDS polyacrylamide gel, blotted on nitrocellulose filter and incubated with a panel of complex I antibodies (indicated on the right). An antibody against the complex II 70 kDa subunit was included as a loading control. The time (in hours) after removing the doxycyline is indicated on the top. Cells without mitochondrial DNA (ρ^0) and control cells (con) were included as negative and positive controls, respectively.

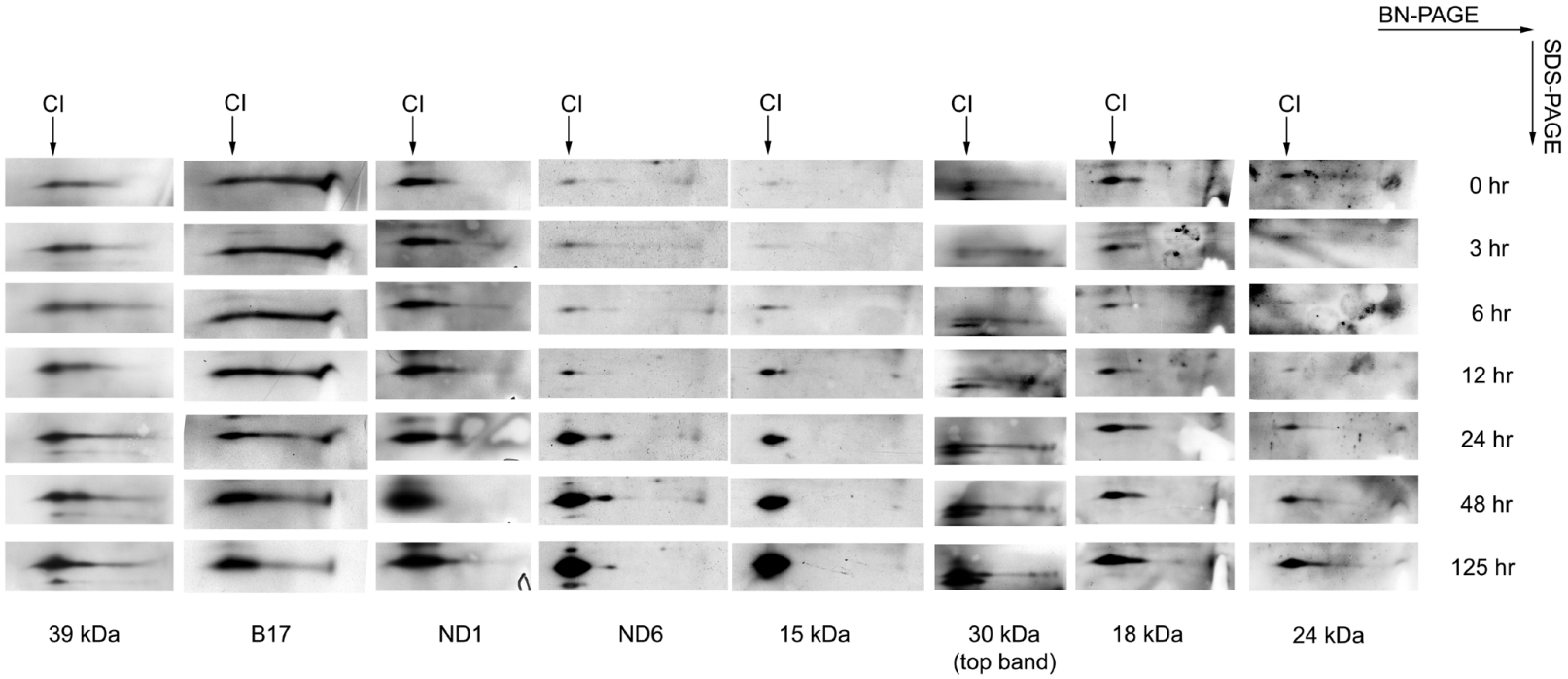


Figure 5: Dynamics of complex I subcomplexes

Cells were pretreated for 6 days with doxycycline and grown in the absence of the drug for the indicated times (specified on the right). 40 µg of crude mitochondrial pellets were analyzed by 2D BN/SDS PAGE (arrows indicate the first and second dimension). Relevant parts of the blots were grouped per subunit (indicated on the bottom). The mobility of complex I in the first dimension is indicated with arrows (top). Complex I is indicated as CI. In the 30 kDa subunit panel an additional ND1 band is seen (see also legends Fig. 2).

Composition of subcomplexes

In order to determine which subunits comigrate, we aligned the blots of several independent experiments and named A-G subcomplexes in which we observe more that one subunit (Fig. 6). The co-localization of subunits in a certain subcomplex is crucial for the interpretation of a possible assembly pathway (see discussion). For this reason we also show other representative examples of a similar analysis in a 2D BN/SDS blot of a crude mitochondrial preparation of human embryonic kidney cells, HEK 293 (Fig. 6B) and control 143B cybrid cells (Fig. 6D). We observe the subcomplexes A-G with the same subunit composition in the different cell lines, however the relative distribution of these subcomplexes differs, depending on the cell line and growth condition.

The 30 kDa subunit is part of three small subcomplexes, one running at the front, one running at approximately 80 kDa (subcomplex H) and one at approximately 150 kDa (subcomplex G) (Fig. 6A, B, D), as seen also in the ρ^0 cells (Fig. 2). The next co-localization is observed with subcomplexes F, D, B and fully assembled complex I (A). For the 49 kDa subunit we observe an identical pattern (Fig. 6D). As already discussed the 39 kDa subunit appears as a smear, possibly reflecting the hydrophobic nature of this subunit. The first distinguishable subcomplex appears at 250 kDa (Fig. 6B, subcomplex F). The next 39 kDa containing-subcomplex is subcomplex D, which has a molecular weigh of 600 kDa (Figs. 6A, 6B and 6C). Another broad spot spans mobility from 950 to 1000 kDa (subcomplexes A and B). Although it seems to be one spot, we believe it actually consists of two spots that run closely together, which can be seen for some subunits shortly after removing doxycycline (Fig. 5, see ND6 and 15 kDa subunit panels, 6 hours time-point). By adapting the gradient of the first dimension BN PAGE from 5-15% to 5-13%, a better separation of (sub)complexes A and B was obtained (Fig. 6C). Complex A represents fully assembled complex I. The 18 kDa and 24 kDa subunits seem to comigrate with subcomplexes D, B and A. (Fig. 6A, C). The 15 kDa subunit is only present in subcomplexes A and B (Fig. 6A). Besides a spot at the front of the gel, which likely represents the monomeric subunit, subunit B17 shows a spot at approximately 400 kDa (Fig. 6A, 6B, subcomplex E). Other subcomplexes in which this subunit is present are found at estimated molecular weights of 700 kDa (subcomplex C), 950 kDa (subcomplex B) and at 1000 kDa (holo-complex I, A). ND1 comigrates with subcomplexes C, B and A, the same as ND6 and the

PSST subunit (Fig. 6D). The subcomplexes and their subunit compositions are summarized in table 1.

Table 1: The presence of complex I subunits per observed subcomplex

Subunit	Subcomplex (Estimated Molecular Mass)							
	H (80 kDa)	G (150 kDa)	F (250 kDa)	E (400 kDa)	D (600 kDa)	C (700 kDa)	B (950 kDa)	A (1 MDa)
15 kDa							x	x
18 kDa					x		x	x
24 kDa					x		x	x
30 kDa	x	x	x		x		x	x
49 kDa	x	x	x		x		x	x
39 kDa			x		x		x	x
B17				x		x	x	x
ND1				x		x	x	x
ND6				x		x	x	x
PSST				x		x	x	x

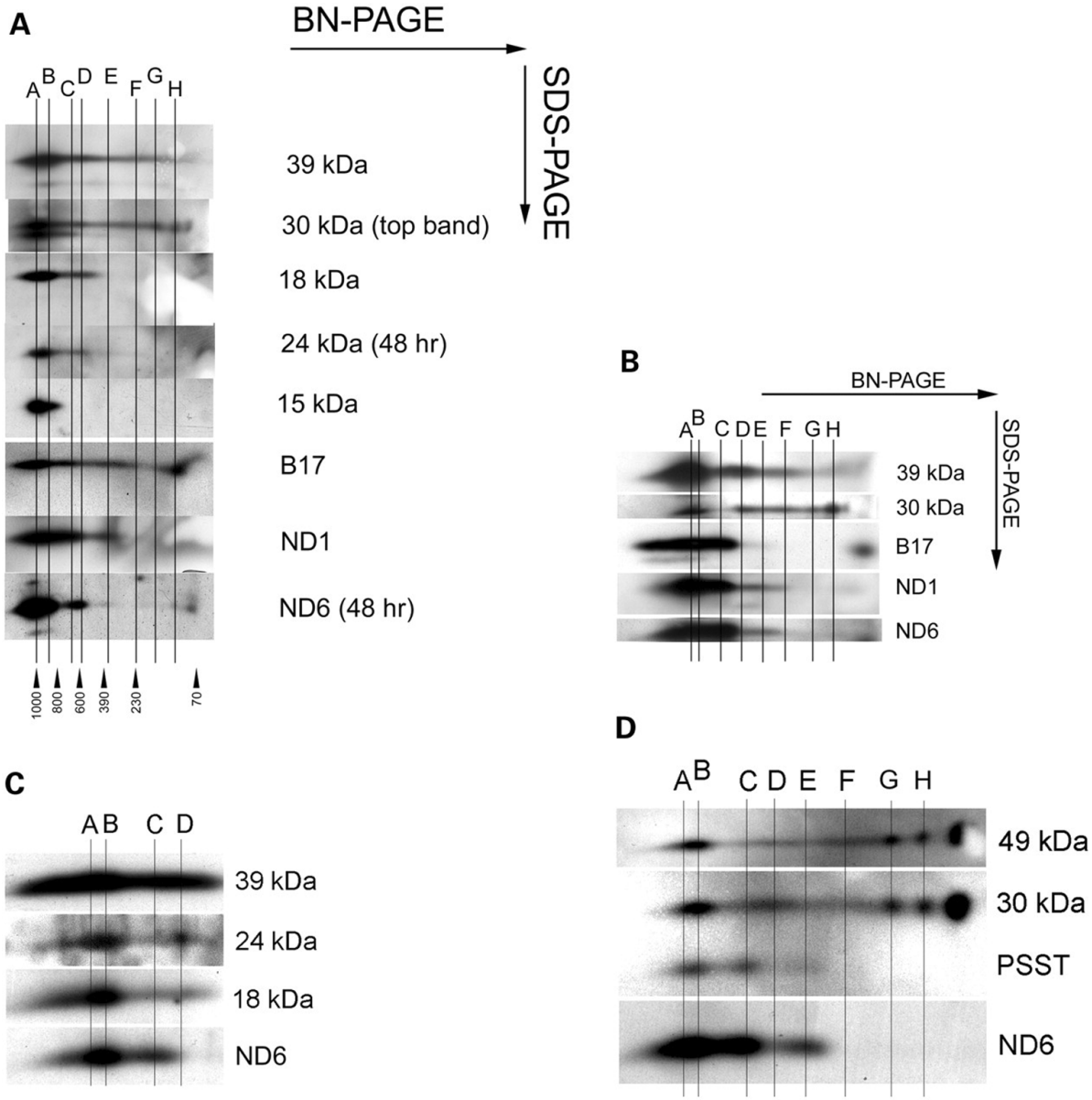


Figure 6: Co-localization of subunits in subcomplexes

A. Crude mitochondrial fractions of 143B cells were analyzed by 2D BN PAGE at 24 or 48 hours (indicated) after doxycycline removal. B. The same procedure was followed with untreated HEK 293 cells. C. Untreated 143B cells analysed on 2D BN/SDS PAGE. A 5-13% acrylamide gradient was used in the first dimension in order to separate the higher molecular weight (sub)complexes A to D. D. Control cybrids analyzed as described in A and B. Arrows indicate the first and second dimension. The antibodies used are indicated on the right. Complex I (A) and subcomplexes B-H are indicated. Bottom arrows in Fig. 6A indicate the molecular weights of selected markers (complex I, 1000 kDa, complex III dimer + complex IV= 800 kDa, complex III= 600 kDa, F1-ATPase= 390 kDa, complex IV = 230 kDa and HSP70 = 70 kDa).

Discussion

To better understand complex I-assembly defects as seen in patients with a complex I deficiency (Ugalde et al., 2004a; Antonicka et al., 2003), we aimed to identify important steps in the assembly pathway of human mitochondrial complex I. We have used 2D BN/SDS PAGE to investigate the appearance of complex I subcomplexes in cells devoid of mitochondrial DNA and in cells depleted of complex I and other OXPHOS complexes by the treatment of doxycycline, an inhibitor of mitochondrial translation. This approach has been successful in the identification of the assembly pathway of other respiratory chain complexes (Nijtmans et al., 1995; Nijtmans et al., 1998). These findings helped the interpretation of assembly intermediates in patient cells with defects in OXPHOS complexes, such as SURF1, ATP6, COX10, SCO1 and $tRNA^{leu}$ mutations (Nijtmans et al., 2001; El et al., 1998; Tiranti et al., 1999; Williams et al., 2004).

Subcomplexes containing at least the 30 kDa subunit are observed in ρ^0 cells, suggesting that the peripheral arm can partially be formed in the absence of a membrane arm. The subcomplexes observed in ρ^0 cells (Fig. 2) and doxycycline treated cells (Fig. 6) are not necessarily the same because ρ^0 cells are different from the doxycycline treated cells. The ρ^0 cells are adapted to the fact that they do not contain mitochondrial DNA and cannot assemble any mitochondrial gene product containing complex, whereas the doxycycline treated cells are only transiently partially depleted from mitochondrially encoded gene products. Also ρ^0 cells cannot make mitochondrial mRNA anymore whereas doxycyline treated cells can. For this reason we base our model solely on the experiments with doxycycline treated cells.

We have observed that the assembly of complex I starts with different low molecular weight subcomplexes that differ in their subunit composition. This confirms that complex I assembly is a semi-sequential process in which subunits preassemble in different subcomplexes that are joined later in the assembly pathway. We can distinguish at least two distinct parts of complex I which are pre-assembled independently (see table 1). The first one contains the peripheral arm 30 kDa subunit, which immediately associates with the 49 kDa subunit. Next the 39 kDa, and later 18 kDa and 24 kDa subunits, are assembled. The second one contains the membrane arm subunit B17, to which subsequently ND1, ND6 and the PSST subunits associate. This peripheral and membrane parts are joined and

additional subunits are inserted, including the 15 kDa subunit, to form a 950 kDa subcomplex which migrates closely to complex I. This 950 kDa subcomplex is completed to fully assembled complex I, possibly by the addition of subunits or conformational changes. In a recent paper Acín-Pérez and colleagues (Acin-Perez et al., 2004) observed also two closely migrating complex I bands on a BN-PAGE after pulse-labeling mitochondrial translation products, which resemble (sub)complexes A and B. After longer chase times the upper complex I band increases in intensity compared to the lower complex I band, suggesting that the lower complex I band is converted into the higher complex I band.

Our findings are consistent with the *N. crassa* model (Tuschen et al., 1990), for which it was proposed that complex I is assembled by combining different evolutionary modules (Videira, 1998). Based on our experiments and other cross-linking (Yamaguchi and Hatefi, 1993), fractionation (Carroll et al., 2002), 2D BN/SDS (Ugalde et al., 2004b; Antonicka et al., 2003) and evolutionary data (Friedrich and Weiss, 1997), we propose an assembly pathway of complex I in human cells which is consistent with a modular assembly (Fig. 7). This model entails the formation of a NADH-dehydrogenase module, a hydrogenase module and a transporter module.

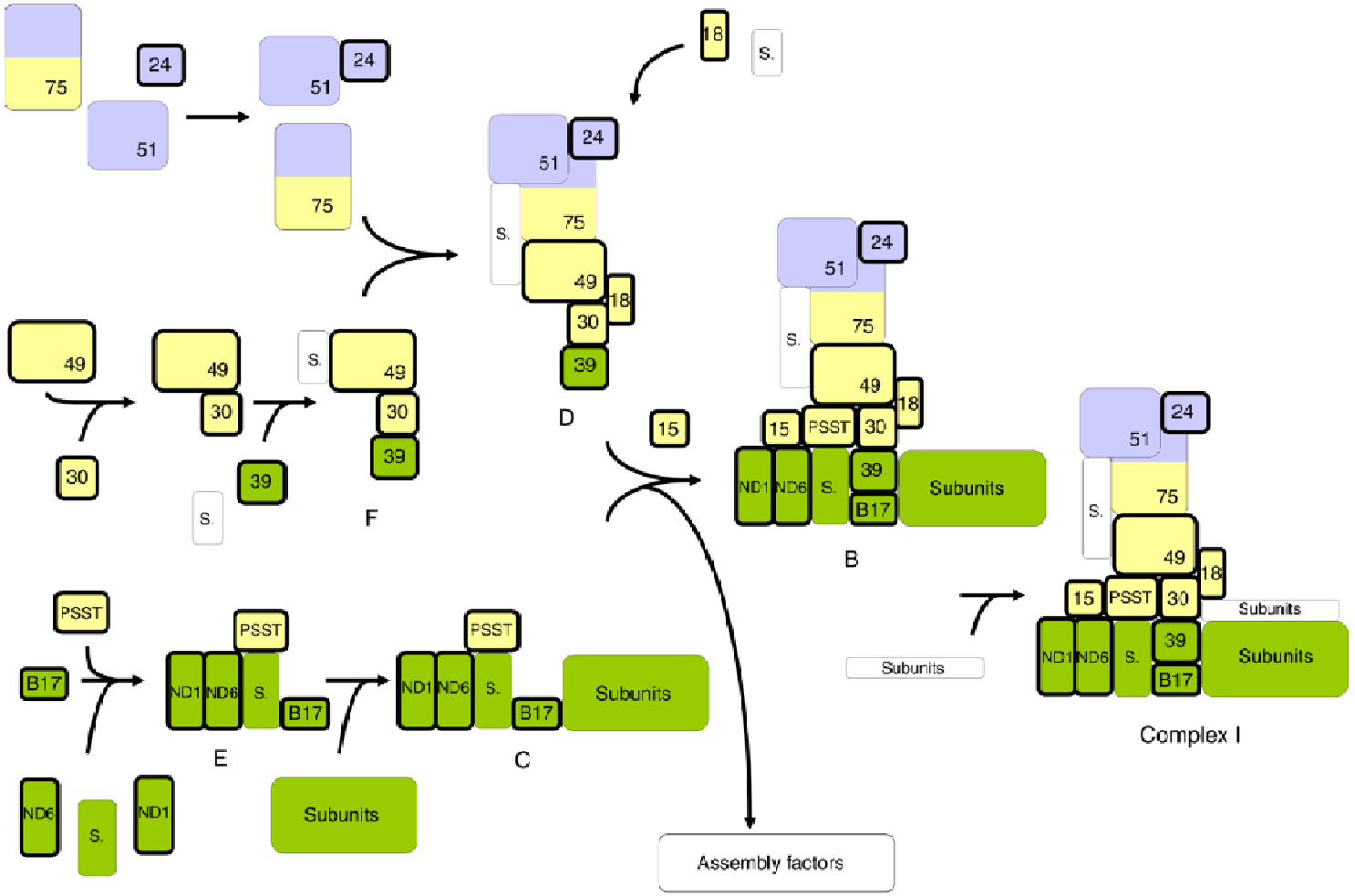


Fig. 7. Proposed modular model of human complex I assembly

Subunits investigated in our study are boxed with a thick line. Subunits proposed on the basis of literature data (see discussion) are boxed with a thin line. "S." and "Subunits" indicate unidentified subunits. Observed complex I (A) and subcomplexes B-H are specified. Their molecular mass based on their electrophoretic mobility on a BN PAGE is indicated between brackets (for more details see discussion).

Assembly of the evolutionary conserved hydrogenase module of complex I starts with the 30 and 49 kDa subunits, which are located in the peripheral arm of complex I, and are two of the 14 mammalian core subunit homologues present in the basic complex from *Escherichia coli* (Yagi et al., 1998). Three distinct low molecular weight subcomplexes were observed. Because the 30 kDa subunit has been shown to form a fusion protein with the 49 kDa subunit in *E. coli* (Finel, 1998) it is expected that these subunits are bound to each other early in assembly. The first two 30 kDa and 49 kDa spots might therefore represent the monomeric subunit and the subsequent association with each other to form subcomplex H. This is supported by the direct interaction of the 30 kDa and 49 kDa subunit in chemical cross-link studies in bovine (Yamaguchi and Hatefi, 1993). Consistent with this idea is the finding that in a complex I-deficient patient with a mutation in the 49 kDa subunit, the first 30 kDa spot accumulates, which suggests that the formation of the second spot is blocked (Ugalde et al., 2004a). The third 30 kDa-49 kDa spot (subcomplex G) represents the subsequent association of another subunit to this 30-49 kDa subcomplex. Next, we observe association of the 39 kDa subunit and likely other subunits to this 30-49 containing subcomplex to form subcomplex F (Fig. 6, 7). The 39 kDa subunit fractionates with the membrane γ-part of complex I (Fig. 1 and (Sazanov et al., 2000)) and might therefore serve as a membrane anchor for the connection of the peripheral arm with the membrane arm.

Other subunits, including the 24 kDa, are bound to subcomplex F to form subcomplex D. Because the 24 kDa subunit constitutes the NADH dehydrogenase module together with the 51 kDa subunit and the N-terminal segment of the 75 kDa subunit (Pilkington et al., 1991), it would be conceivable that these subunits preassemble in another intermediate complex. Although we do not observe a 24 kDa containing NADH-dehydrogenase subcomplex in our 2D-BN/SDS PAGE (Fig. 6), a 24 kDa-containing low molecular weight product occurs in the ρ^0 cells (Fig. 2). This could imply that such a subcomplex exists, but it is rapidly assembled into subcomplex D. The 75 kDa subunit is likely to play a crucial role in connecting the NADH-dehydrogenase module to the hydrogenase module, since cross-linking studies show association with the 30 kDa subunit of the hydrogenase module, and with the 51 kDa subunit of the NADH-dehydrogenase module (Yamaguchi and Hatefi, 1993). The finding of a 160-210 kDa subcomplex in complex I-deficient mutants of *Chlamidomonas reinhardtii*, which contains the homologues of 75 kDa and 49 kDa subunits and displays NADH-dehydrogenase activity, again supports a

previous assembly of the NADH-dehydrogenase/hydrogenase module (Cardol et al., 2002).

To this NADH-dehydrogenase/hydrogenase-containing peripheral arm (subcomplex D) other subunits associate, including the 18 kDa subunit. This is in accordance with the observed cross-links between the 18 kDa and 49 kDa subunits, and between the 18 kDa and 30 kDa subunits (Yamaguchi and Hatefi, 1993). Patient cells lacking subunit 18 kDa are still able to assemble an 800 kDa complex (Ugalde et al., 2004a; Scacco et al., 2003). This indicates that the 18 kDa subunit is acquired relatively late in the assembly, and that assembly can proceed without the 18 kDa subunit until an 800 kDa subcomplex is formed.

The membrane arm transporter module includes the B17 subunit, which is present in the β-part of the hydrophobic membrane arm (Fig. 1). B17 is accumulated as a monomeric subunit and in some possible low molecular weight subcomplexes. This subunit becomes part of the 400 kDa subcomplex E, to which other membrane arm subunits including ND1 and ND6 bind. Consistent with this finding is that there is a remarkable shift of B17 subcomplexes upon release of the mitochondrial translation block, indicating that mitochondrially encoded subunits are essential for this subunit to progress in the assembly. To subcomplex E, also the N2 iron-sulfur cluster containing PSST subunit associates. There are several reports which are compatible with this membrane arm association of the PSST subunit. Firstly in mouse cells which lack ND6, the PSST subunit is absent (Yadava et al., 2004). Secondly, chloramphenicol treated *N. crassa* cells (Tuschen et al., 1990) and disruption mutants (Nehls et al., 1992), in which only the peripheral arm was formed, also lack the N2 iron-sulfur cluster and thus the PSST subunit. Thirdly, a functional coupling of PSST with ND1 also suggests a close association of these subunits (Schuler and Casida, 2001). However, a PSST disruption mutant in *N. crassa* proved to be unable to assemble the peripheral arm (Duarte et al., 2002). This suggests that the PSST, which is located in the boundary between the peripheral and membrane arms, assembles to the membrane arm but is required for the stabilization of the peripheral arm. Interestingly, in methanogenic bacterium *Methanosarcina barkeri* (Kunkel et al., 1998) and photosynthetic bacterium *Rhodospirillum rubrum* (Fox et al., 1996), hydrogenases are organized in operons in which the gene for the PSST homologue is located next to the gene for the ND1 homologue, again illustrating a structural evolutionary conservation. Other genes of these operons are the homologues of ND5, TYKY, the 49 kDa and the 30 kDa subunits (only in *R. rubrum*). Subsequently, other subunits associate to the

membrane arm intermediate E form subcomplex C (Fig. 6, 7). The peripheral arm (subcomplex D) and the membrane arm (subcomplex C) come together and form subcomplex B at a molecular weight of around 950 kDa. Paradoxically, two subcomplexes of 600 kDa and 700 kDa add up to form a 950 kDa subcomplex, however there are plausible explanations for this. Firstly, the electrophoretic mobility of a protein or protein complex in a PAGE gel depends on charge and globular size of the molecule. When two protein complexes assemble into one complex, the resulting charge and globular size does not necessarily result in an electrophoretic mobility that corresponds to the sum of the two complexes. Secondly, it is described in *N. crassa* that complex I assembly proteins transiently associate with assembly intermediates (Kuffner et al., 1998), thereby contributing to the molecular weight of intermediate complexes but not to other subcomplexes which occur later in assembly. We also find in human cells co-localization of an assembly protein with intermediate subcomplexes but not with holo-complex I (unpublished results). In a last step additional subunits, including the 15 kDa, are assembled to subcomplex B and finally, fully assembled complex I is formed (A).

Recently, it has been demonstrated that the cyanobacteria *Synechocystis sp*. PCC 6803 contains a NDH-1 complex which resembles mitochondrial complex I (Prommeenate et al., 2004). Although the electron import module or NADH-dehydrogenase part is not acquired in this enzyme, it contains a hydrogenase part and a transporter part. The assembly of the cyanobacterial NDH-1 was studied using 2D BN/SDS PAGE. A hydrophilic subcomplex was observed containing the cyanobacterial homologues of the 30 kDa, 49 kDa, B13 and PSST subunits. A hydrophobic subcomplex was found which contains the homologues of ND1, ND2 and ND6. The finding of the PSST subunit in the hydrophilic part contradicts our findings, however, this can be explained by the chemical fractionation used. The authors propose that the separate modules are assembled independently. Our data support this conclusion and suggests that the modular assembly of complex I is preserved throughout evolution.

Recently, Antonicka and colleagues proposed a model for complex I assembly based on subcomplexes observed by 2D-BN/SDS PAGE in muscle samples of complex I-deficient patients (Antonicka et al., 2003). This model is in conflict with our proposed model at several points. Firstly, the Antonicka model describes a 24 kDa containing subcomplex, which also contains the 18 kDa and 20 kDa (PSST) subunits. The 20 kDa (PSST) subunit together with the 30 kDa, 49 kDa and ND1 subunit constitute the core of the hydrogenase module. It is therefore very unlikely

that the 20 kDa subunit is assembled in a subcomplex which does not contain any of these subunits and which is topologically located in another part of the complex (as discussed above). The 18 kDa subunit cross-links with the 49 kDa and 30 kDa subunits (Yamaguchi and Hatefi, 1993). For this reason, it is more likely that these three subunits assemble together to form a subcomplex. Secondly, they propose the occurrence of a 310 kDa subcomplex containing the membrane arm subunit ND1 and the peripheral arm subunits 30 kDa-39kDa-49 kDa, suggesting no separate formation of the peripheral and membrane arms. In contrast, we observed a co-localization of the 30 kDa and 39 kDa subunits with the ND1 subunit only late in the assembly, in the 950 kDa subcomplex B, which is compatible with the well-described *N. crassa* model. A possible explanation for these differences is that in the patient muscle samples investigated in the previous work, some of the proposed assembly intermediates are breakdown products resulting from an instable complex I. Increased instability of assembled complex I is known to occur for instance in cybrid cells containing an ND5 mutation (Hofhaus and Attardi, 1995).

In this study we present an alternative model for the assembly of complex I in cultured human cells. The use of doxycycline-treated cultured cells allows following the dynamics of the assembly process, therefore avoiding the interference of breakdown products. This might give a more representative picture of the physiological assembly pathway of complex I. We confirm that complex I assembly is a semi-sequential process in which pre-assembled subcomplexes are joined to form holo-complex I. A pre-assembled peripheral arm is formed, which associates with a pre-assembled membrane arm to form an intermediate complex, to which other subunits are attached to form holo-complex I. Still much work needs to be done to elucidate more details of the assembly of all of the 46 subunits into complex I. New approaches such as the construction of a viable complex I deletion strain of the complex I-containing yeast *Yarrowia lipolytica* (Kerscher et al., 2001), the generation of a mammalian conditional assembly system (Yadava et al., 2004), the application of protein mass spectrometry and analysis of newly identified CI-deficient patients, will aid this difficult task. Nevertheless, our proposed model for complex I assembly presents a starting point to further elucidate this intricate process and provides a framework to understand assembly defects in patients with a complex I deficiency. Moreover, information of the assembly status of complex I could provide a good pre-screening method in diagnostics.

Acknowledgements

We thank Dr. A. Lombes (Iserm, Paris, France), Prof. J. Walker (MRC, Cambridge, United Kingdom) and Prof. B. Robinson (Montreal, Canada) for kindly providing respectively the ND1,the 24 kDa and the 49 kDa subunit antibodies and Prof. R. Capaldi (University of Oregon, Eugene, USA) for giving the 18 kDa and ND6 subunit antibodies. This work was supported by “Het Prinses Beatrix Fonds” to J.S. and L.vdH. (grant number 02-0104). The Netherlands Organisation for Scientific Research supported L.N. with a “Vernieuwingsimpuls” grant.

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Chapter 4

Identification of mitochondrial complex I assembly intermediates by tracing tagged NDUFS3 demonstrates the entry point of mitochondrial subunits

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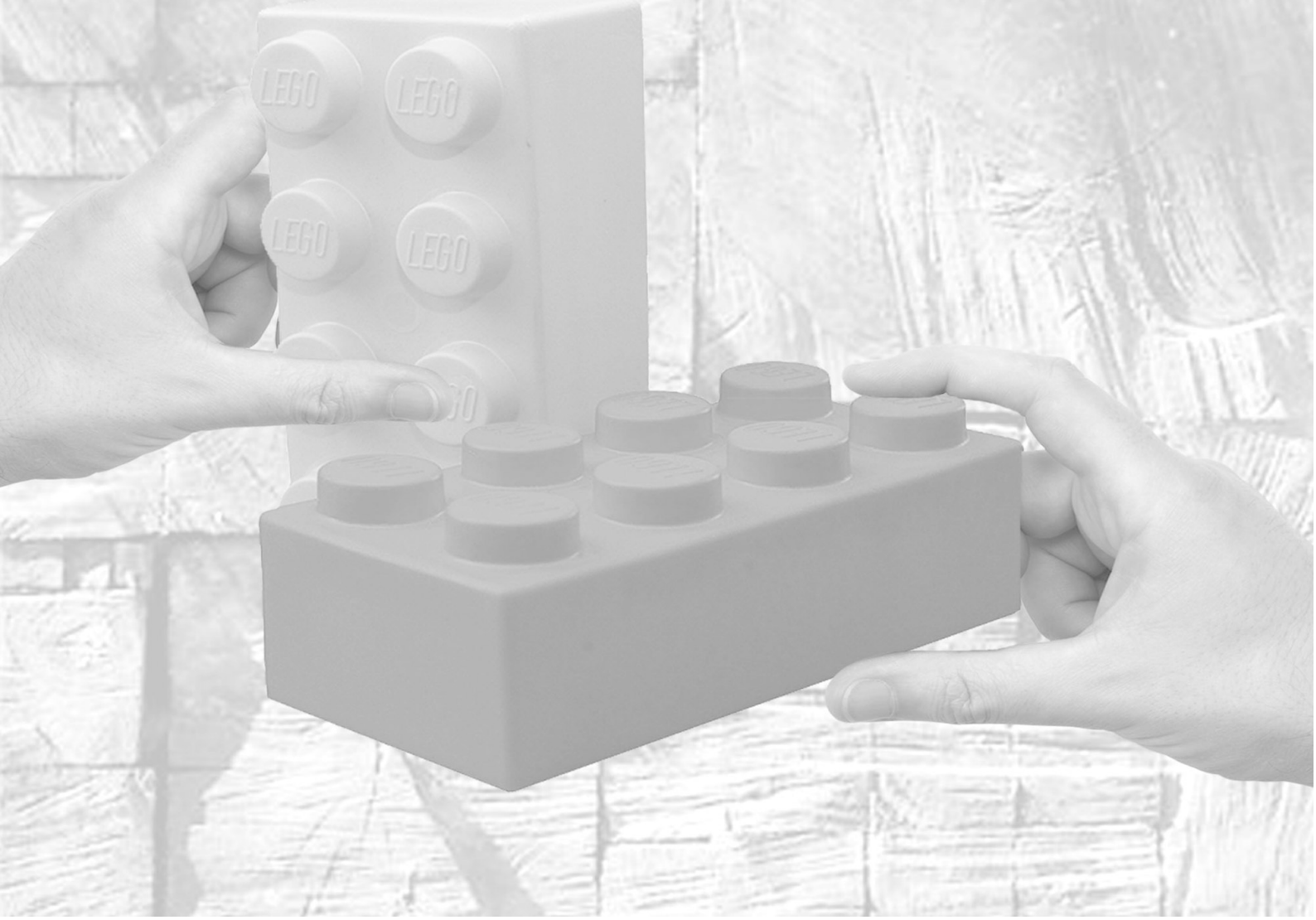
Journal of Biological Chemistry 2007; 282(10):7582-90



Abstract

Biogenesis of human mitochondrial complex I (CI) requires the coordinated assembly of 45 subunits derived from both the mitochondrial and nuclear genome. The presence of CI subcomplexes in CI deficient cells suggests that assembly occurs in distinct steps. However, discriminating between products of assembly or instability is problematic. Using an inducible NDUFS3-GFP expression system in HEK293 cells, we here provide direct evidence for the stepwise assembly of CI. Upon induction, six distinct NDUFS3-GFP-containing subcomplexes gradually appeared on a blue-native Western blot, also observed in wild-type HEK293 mitochondria. Their stability was demonstrated by differential solubilization and heat incubation, which additionally allowed their distinction from specific products of CI instability and breakdown. Inhibition of mitochondrial translation under conditions of steady state labeling resulted in accumulation of two of the NDUFS3-GFP-containing subcomplexes (100 and 150 kDa) and concomitant disappearance of the fully assembled complex. Lifting inhibition reversed this effect, demonstrating that these two subcomplexes are true assembly intermediates. Composition analysis showed that this event was accompanied by the incorporation of at least one mitochondrial DNA-encoded subunit, thereby revealing the first entry-point of these subunits.

Introduction

Mitochondrial ATP is produced by the oxidative phosphorylation (OXPHOS) system. This system consists of five complexes, composed of at least 75 nuclear DNA-encoded and 13 mitochondrial DNA (mtDNA)-encoded proteins, and is a prominent example of coordinated assembly. The first four OXPHOS complexes (CI-CIV) constitute the respiratory chain, which transfers electrons from substrates NADH (at CI) and $FADH_2$ (at CII) to the final electron acceptor molecular oxygen (CIV). Energy released by this electron transport is used to drive proton translocation across the mitochondrial inner membrane at CI, CIII and CIV. The resulting proton gradient is used to drive the conversion of ADP and inorganic phosphate into ATP by complex V (Smeitink et al., 2001).

CI (NADH:ubiquinone oxidoreductase; E.C.1.6.5.3) constitutes the largest and least understood of the OXPHOS complexes (Brandt, 2006; Janssen et al., 2006). Electron microscopy revealed that CI has an L-shaped structure that consists of a hydrophobic arm embedded in the lipid bilayer of the mitochondrial inner membrane and a hydrophylic peripheral arm exposed to the mitochondrial matrix (Grigorieff, 1999). Using chaotropic salts and the detergent *N,N*-dimethyldodecylamine *N*-oxide (LDAO), CI can be fractionated into several fragments (Galante and Hatefi, 1978; Sazanov et al., 2000), which together encompass 45 distinct subunits in bovine CI (Carroll et al., 2003; Carroll et al., 2006). The recent appearance of the first crystal structure of the hydrophilic domain of CI in *Thermus thermophilus* is an example of the increasing insight that is gained in this area of research (Sazanov and Hinchliffe, 2006).

In contrast, the many steps involved in the assembly of these 45 subunits still remain puzzling. Studies in the fungus *Neurospora crassa* demonstrated that the membrane and peripheral arms of CI are assembled independently and that the membrane arm, in its turn, is the product of the combination of a small and large assembly intermediate (Schulte et al., 1994; Tuschen et al., 1990; Videira and Duarte, 2001). Two models are described for the CI assembly pathway in human mitochondria: one is based on the subcomplex distribution in CI deficient patient cells, the other on the appearance of subcomplexes in a conditional CI assembly system (Antonicka et al., 2003; Ugalde et al., 2004). Although both models differ at several points, they agree in that assembly occurs rather via the combination of large pre-assembled fragments than via sequential addition of individual subunits.

Structural and phylogenetic data strongly suggest that certain CI subunits have co-evolved and are arranged in distinct structures, termed modules (Finel, 1998; Friedrich and Scheide, 2000; Friedrich and Weiss, 1997; Hedderich, 2004). Combination of these modules resulted in the 'minimal' CI structure consisting of the 14 most conserved subunits, of which a typical example is *Escherichia coli* CI: NDH-1. The proposed modules are the dehydrogenase module, consisting of the NDUFV2, NDUFV1 and NDUFS1 subunits (homologues of the nuoE, F and G subunits of bacterial NDH-1), the hydrogenase module, consisting of the NDUFS2, NDUFS3, NDUFS7, NDUFS8, ND1 and ND5 subunits (homologues of the nuoD, C, B, I, H and L subunits of NDH-1) and the proton translocation module, consisting of the ND2, ND3, ND4, ND4L and ND6 subunits (homologues of the nuoN, A, M, K and J subunits of NDH-1) (for further details concerning this subject, see (Friedrich and Weiss, 1997)). Our assembly model proposes that assembly in part reflects this evolutionary conservation of CI subunits (Ugalde et al., 2004). Assembly intermediates were identified by their appearance after the release of doxycycline inhibition of mitochondrial translation (allowing synthesis of the mtDNA-encoded ND subunits to resume). Membrane arm subunits seemed to be assembled in a different intermediate than peripheral arm subunits, and the presence of distinct early subassemblies suggested a link between the assembly process and co-evolution of different CI subunits (Ugalde et al., 2004).

Both assembly studies for human CI have used disturbed assembly systems, such as patient cell lines or cells treated with inhibtors of mitochondrial translation (Antonicka et al., 2003; Ugalde et al., 2004). Thus far, however, no subcomplexes have been identified in undisturbed systems. A useful strategy to trace assembly without disturbing its dynamics is by tagging a CI subunit, provided that the tag does not interfere with its biological function. This was previously done by Scheffler and colleagues, who used inducible tagged versions of the human homologues of the MWFE and ESSS subunits of CI to study their incorporation and function in assembly by complementation of CI deficient Chinese Hamster cell lines (Potluri et al., 2004; Yadava et al., 2004). However, assembly intermediates were not studied.

In this study, we have used leakage expression of an inducible HEK293 cell line expressing monomeric Green Fluorescent Protein (AcGFP1)-tagged NDUFS3 subunit. This strategy allowed detection of the NDUFS3 containing subcomplexes under steady-state labeling conditions and at relatively low expression levels on native one-dimensional gels. Moreover, it allowed the analysis of accumulation or

disappearance of individual subcomplexes upon interference with the assembly process. The NDUFS3 subunit was selected for labeling given the apparent ease with which its eYFP-HIS-tagged *Yarrowia* homologue can be used to isolate CI, demonstrating that the C-terminal tag does not disturb assembly (Kashani-Poor et al., 2001). Additionally, it has been argued that this core subunit is incorporated in an early stage of assembly (Antonicka et al., 2003; Ugalde et al., 2004), so that tagging will provide more insight into onset- and subsequent CI assembly steps. We demonstrate the existence of at least six distinct NDUFS3-containing intermediates, and distinguish these stable intermediates from products of CI instability and breakdown by differential solubilization and heat-incubation. Furthermore, inhibition of mitochondrial translation reveals an essential step, in which two NDUFS3-containing subcomplexes of 100 and 150 kDa require mtDNA-encoded proteins for progression in assembly. Determination of the constitution of all subcomplexes by two-dimensional (2D-SDS) immunodetection demonstrates that mtDNA-encoded subunit ND1 makes its first appearance in this key step, indicating that this is the first step in the assembly process that requires the availability of mtDNA-encoded proteins.

Materials and methods

Generation of an inducible NDUFS3—AcGFP1 stable cell line

The NDUFS3 open reading frame sequence (NM_004551; without stopcodon) flanked by Gateway® AttB sites (Invitrogen) was created by PCR following manufacturer instruction and cloned into pDONR201 by using Gateway® BP Clonase II Enzyme Mix (Invitrogen). A Gateway® Destination vector was produced by subcloning the BamHI/NotI restriction fragment of pAcGFP1-N1 (Clontech) in frame behind Gateway® Reading Frame Cassette B (Invitrogen) in pcDNA5/FRT/TO (Invitrogen). To obtain an inducible vector containing c-terminally GFP-tagged NDUFS3 (NDUFS3-GFP), the pDONR201-NDUFS3 vector was recombined with the AcGFP1-Destination vector using the Gateway® LR Clonase II Enzyme Mix (Invitrogen). Flp-In T-Rex293 cells (Invitrogen) were stably transfected using Superfect® Transfection Reagent (QIAGEN) following manufacturer protocols. Clones with low leakage levels (noninduced expression of the transgene) were selected to obtain steady state NDUFS3-GFP labeling. To induce NDUFS3—AcGFP1 overexpression in these cell lines, doxycycline (Sigma) was added to the medium at a final concentration of 0.1 µg/ml. To reversibly block mitochondrial translation chloramphenicol (Sigma) was used at a final

concentration of 40 µg/ml. For blue-native analysis, cells were harvested at the indicated time points.

Cell culture and mitochondria preparation

Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Biowhitaker) supplemented with 10% fetal calf serum (v/v) and 1% penicillin/streptomycin (v/v) (Gibco). HEK293 cells were harvested and washed twice in cold PBS (Gibco) prior to resuspension in 2% digitonin (w/v) (Calbiochem)/PBS and incubation for 10 minutes on ice. For the amount of cells used (5×10^6), this amount is comparable to 1 g of digitonin per g protein and results in disruption of the plasma membrane without disruption of mitochondrial inner membranes (Klement et al., 1995). After centrifugation (10 min, 10,000 g, 4°C), the mitochondria enriched pellet was washed twice in PBS and the protein concentration was determined using the MicroBCA protein assay kit (Pierce).

Sample preparation - DDM solubilizations – Mitochondrial protein was mildly solubilized (common solubilisations are performed using 1.6 g/g DDM) by 10 min incubation on ice using 0.3, 0.6 and 1 g of n-Dodecyl β-D-maltoside (DDM) (Sigma-Aldrich) per g of protein in solubilization buffer (1.75 M 6-aminocaproic acid; Fluka), 75 mM bis-tris HCl (pH 7.0; Fluka). After centrifugation (30 min, 10,000 g, 4°C) the supernatant containing solubilized mitochondrial proteins was used for blue-native analysis. *Digitonin solubilizations* – Mitochondrial protein was solubilized by 30 min incubation on ice with 4, 8 and 16 g of digitonin per gram of protein in solubilization buffer (30 mM HEPES pH 7.4, 150 mM potassium acetate (Merck), 10% (w/v) glycerol (Sigma-Aldrich) and 1 mM phenylmethylsulphonylfluoride (Sigma-Aldrich)). After gentle centrifugation (2 min, 600 g, 4°C) the supernatant containing solubilized mitochondrial proteins was used for blue-native analysis. *37 °C incubations* – For temperature incubation experiments, mitochondrial protein was mildly solubilized using 1 g of n-Dodecyl β-D-maltoside (DDM) (Sigma-Aldrich) per g of protein in solubilization buffer (1.75 M 6-aminocaproic acid; Fluka), 75 mM bis-tris HCl (pH 7.0; Fluka). After centrifugation (30 min, 10,000 g, 4°C) the supernatant containing solubilized mitochondrial proteins was divided into three samples. One sample was kept on ice for 60 min, one was kept at 37 °C for 10 min and on ice for 50 min and another was kept at 37 °C for 60 min. After this period, mitochondrial lysates were used for blue-native analysis.

Blue-native electrophoresis and in-gel activity assays

Blue-native gradient gels (5-15%) were cast as described previously (Nijtmans et al., 2002) and run with 40 or 80 µg of solubilized mitochondrial protein. After electrophoresis, gels were further processed for in-gel activity assays, Western blotting or second dimension 10% SDS-PAGE as described in (Nijtmans et al., 2002). In addition to CI, in-gel activity assay typically resulted in a previously described smaller band at the bottom half of the gel, which was not shown (Potluri et al., 2004). Proteins were transferred to a PROTAN® nitrocellulose membrane (Schleicher & Schuell).

SDS-PAGE analysis

Mitochondrial lysates were prepared as described under "Cell culture and mitochondria preparation" and "DDM solubilizations". Subsequently the supernatant was mixed with an equal volume of Tricine sample buffer (Biorad) containing 2% (v/v) 2-mercaptoethanol. The mixture was incubated at room temperature for 60 min. Protein (20 µg/lane) was separated on 10% polyacrylamide gel. Proteins were blotted to PROTAN® nitrocellulose membrane (Schleicher & Schuell).

Antibodies and ECL detection

Protein immunodetection was performed using the following primary antibodies directed against EGFP (a gift from Dr. Frank van Kuppeveld, Nijmegen), NDUFS2 (a gift from Professor Brian Robinson, Toronto), NDUFA9 (Invitrogen), NDUFS3 (Invitrogen), ND1 (a gift from Dr. Anne Lombes, Paris), NDUFB6 (Mitosciences), NDUFA13 (Mitosciences), NDUFA6 (Mitosciences), NDUFA1 (a gift from Professor Immo Scheffler, San Diego), the CII 70 kDa subunit (SDHA; Invitrogen), the CIII Core2 subunit (UQCRC2; Invitrogen), CIV coxII subunit (COXII; Invitrogen) and CV ATPase α (ATPA1; Invitrogen). For generation of the NDUFA2-specific antibody, rabbits were immunized with peptide CDQVTRALENVLSGKA that was KLH-coupled using the Imject® Maleimide Activated mcKLH Kit (Pierce). The obtained antiserum was found suitable for specific NDUFA2 detection on Western blots (data not shown). Secondary antibodies used peroxidase-conjugated anti-mouse or anti-rabbit IgGs (Invitrogen). The signal was generated using ECL® plus (Amersham Biosciences). Sizes of the observed subcomplexes were determined by their relative migration compared to the OXPHOS complexes (CII: 150 kDa; CIII:

600 kDa (dimer); CIV: 260 kDa; CV: 800 kDa (dimer)), previously described CI subcomplexes (Ugalde et al., 2004) and to each other on the same two-dimensional blot.

Quantitative data analysis

After Western blotting, luminescent signals were quantified using Image Pro Plus 5.1 (Media Cybernetics, San Diego, CA, USA). The integrated optical density (I.O.D.) of each band was determined and corrected for background. The resulting numerical values were expressed relative to the CII-SDHA signal to correct for loading differences

Results

Induction of NDUFS3—GFP expression results in GFP labeling of CI and six distinct subcomplexes

To trace the appearance of CI subcomplexes we have made stable HEK293 clones containing a tetracycline inducible vector for the NDUFS3 subunit with C-terminal monomeric GFP (AcGFP1). To exclude that the GFP-tag disturbs CI assembly, we first investigated the effects of induction on CI assembly and activity (figure 1). After 4 h of induction, the NDUFS3-GFP protein was clearly immunodetectable on an SDS Western blot of mitochondrial lysates stained with a anti-NDUFS3 antibody and increased thereafter (figure 1A, top panel). Coinciding with the increase of induced protein, endogenous NDUFS3 decreased in time, possibly due to competition, often observed for inducible expression systems (Vogel et al., 2005; Yadava et al., 2004). Blue-native gel electrophoresis (BN-PAGE) followed by Western blot analysis with anti-NDUFA9 antibody and measurement of in-gel NADH-NBT oxidoreductase activity, revealed that expression of NDUFS3-GFP did not alter the amount of fully assembled CI nor its in-gel activity (figure 1A, panels CI-NDUFA9 and CI-IGA).

Figure 1B, depicting a BN-PAGE Western blot of mitochondrial lysates at various times after induction probed with anti-EGFP antibody, revealed the appearance of monomeric induced NDUFS3-GFP (m), six distinct GFP-labeled subcomplexes (indicated with 1-6) and GFP-labeled fully assembled CI (indicated with 7, figure 1B). In combination with the finding that neither the amount nor the activity of CI is altered this indicates that the labeled subunit is gradually incorporated in the 6

subcomplexes and the fully assembled complex without disturbing this process or the activity of the holocomplex.

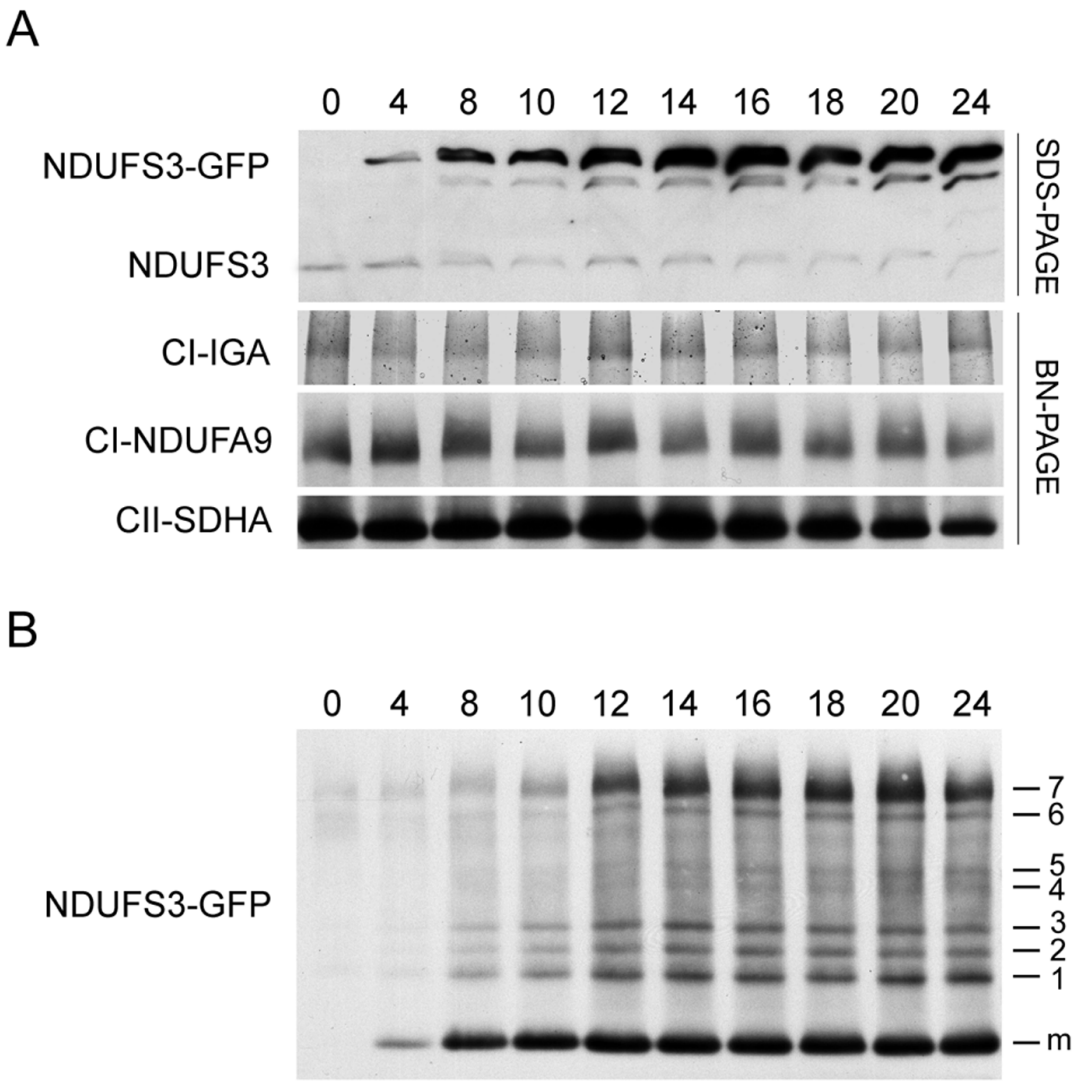


Figure 1: Induction of NDUFS3-GFP expression does not disturb CI assembly and results in the appearance of GFP-tagged subcomplexes

Mitochondrial lysates of inducible NDUFS3-GFP cells were isolated for SDS- and blue-native gel electrophoresis after continuous induction with 0.1 µg/ml doxycycline for several durations up to 24 h. The time points of harvesting the cells (in h) after starting induction are indicated at the top of each lane. (A) Shown are immunodetections of NDUFS3-GFP and endogenous NDUFS3 expression on 1D-SDS Western blot, CI in-gel activity (CI-IGA), CI immunodetection on 1D-BN Western blot (CI-NDUFA9) and CII immunodetection on 1D-BN Western blot (CII-SDHA). (B) GFP-tagged subcomplexes are made visible by immunodetection using a GFP antibody on 1D-BN Western blot. Monomeric NDUFS3-GFP is indicated with "m", (sub)complexes are numbered 1-7.

MtDNA-encoded proteins are required for the formation of subcomplexes larger than subcomplex 3

To highlight the role of mtDNA-encoded CI subunits (ND subunits) in the appearance of the subcomplexes production of these subunits was blocked by specifically inhibiting mitochondrial translation with chloramphenicol. Using translation inhibitors such as chloramphenicol or doxycycline has previously shown to result in strong reduction of mitochondrial translation and thus ND subunit synthesis (Ugalde et al., 2004). In the absence of ND subunits, CI assembly will seize at the point at which incorporation of these subunits is essential for formation of the next assembly intermediate. To obtain steady state labeling of NDUFS3-GFP we specifically selected a clone with low leakage level (non-induced expression) resulting in steady state labeling. Cell lines were incubated with chloramphenicol during 24, 36 or 60 h (figure 2A). Prolonged chloramphenicol incubations induced the specific accumulation of NDUFS3-GFP-labeled subcomplexes 2 and 3 and the disappearance of subcomplex 6 and the holocomplex. This accumulation was paralleled by a decrease in the total amount of CI (as revealed using the CI-NDUFA9 antibody) and a reduced in-gel NADH-NBT oxidoreductase activity (CI-IGA) on a 1D-BN PAGE gel. Expression of the nuclear DNA-encoded SDHA subunit of CII was not affected by chloramphenicol treatment (CII-SDHA). These results support the conclusion that CI assembly cannot proceed beyond the formation of subcomplexes 2 and 3 in the absence of ND subunits. Subsequent quantification of the ECL signals of figure 2A in figure 2C demonstrates that whereas subcomplexes 2 and 3 accumulate 10- to 20-fold, fully assembled CI decreases only 5-fold. This strongly suggests that subcomplexes 2 and 3 predominantly accumulate due to new synthesis. However, we cannot exclude that subcomplexes 2 and 3 to some extent also originate from partial CI breakdown or recycling of its constituents.

Next, we analyzed the effects of releasing chloramphenicol inhibition on the distribution of NDUFS3-GFP-containing subcomplexes. Contrary to the induction pattern shown in figure 1, these conditions do not disturb endogenous NDUFS3 expression (figure 2B, bottom panel) and prevent possible saturation of the assembly process as no monomeric NDUFS3-GFP accumulates (figure 2B, BN-PAGE NDUFS3-GFP signal). Following 60 h of chloramphenicol treatment, cells were washed and chased for 0, 4, 8 and 18 h (figure 2B, time points 0, 4, 8, 18 h). Re-initiation of mitochondrial translation resulted in a gradual return of CI amount and activity (figure 2B, panel CI-NDUFA9 and CI-IGA). Remarkably, subcomplex 1

appeared 4 h after chloramphenicol removal, whereas it was absent from figure 2A. In addition, a shift in the NDUFS3—GFP signal occurred from subcomplexes 2 and 3 towards subcomplexes 4-6 and CI. As subcomplexes 2 and 3 decrease while larger intermediates appear, subcomplexes 2 and 3 must represent products of assembly and not instability. Furthermore, these findings imply that ND subunits are required for the formation of subcomplexes 4-6 and fully assembled CI. Quantification of the ECL signals of figure 2B in figure 2D demonstrates that, directly after translation progresses, the levels of subcomplexes 2 and 3 rapidly decrease and then reach a rather constant level. This suggests that the accumulated subcomplexes 2 and 3 are rapidly incorporated into higher molecular weight complexes upon active translation of mitochondrial DNA-encoded subunits. The comparable kinetics and amounts of the two subcomplexes in figures 2C and 2D suggest that subcomplex 2 reaches a rapid equilibrium with subcomplex 3, which underlines the dynamic nature of the assembly process and allows the possibility that subcomplex 2 originates from subcomplex 3 and *vice versa*.

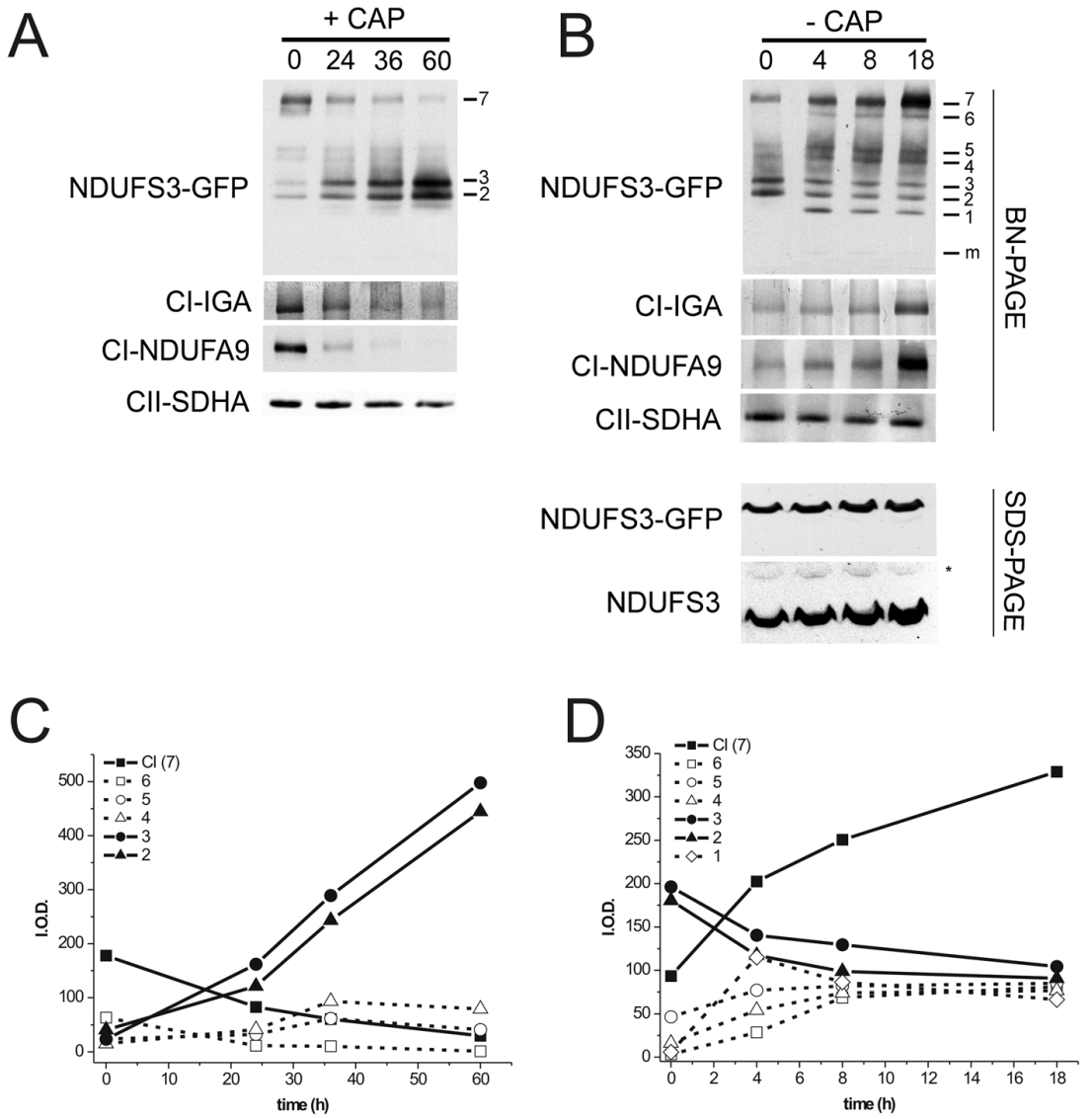


Figure 2: Subcomplexes 2 and 3 accumulate upon inhibition of mitochondrial translation and require mtDNA-encoded subunits for progression in assembly

(A) Inhibition of mitochondrial translation results in the accumulation of subcomplex 2 and 3. Mitochondrial lysates of inducible NDUFS3-GFP cells were analyzed by blue-native gel electrophoresis after inhibition of mitochondrial translation using 40 µg/ml chloramphenicol for time periods of 0, 24, 36 and 60 h under leakage expression of NDUFS3-GFP. After Western blotting, immunodetection was performed for CII (CII-SDHA), CI (CI-NDUFA9) and GFP. Furthermore, CI in-gel activity was assayed (CI-IGA). Complexes 2,3 and 7 are indicated on the right. (B) Redistribution of subcomplexes after the release of inhibition of mitochondrial translation. Mitochondrial lysates of inducible cells were analyzed by blue-native gel electrophoresis. Cells were treated with 40 µg/ml chloramphenicol for 60 h (0 h time point), followed by washing and growth without chloramphenicol for 4, 8 and 18.

(Legend to figure 2, continued)
Shown from top to bottom are GFP-labeled complexes on 1D-BN Western blot (incubated anti-GFP), CI in-gel activity (CI-IGA), CI on 1D-BN Western blot (incubated anti-NDUFA9), CII on 1D-BN Western blot (incubated anti-CII-SDHA). GFP-tagged complexes (1-7) and the position of monomeric NDUFS3-GFP ("m") are indicated on the right. Furthermore, the bottom two panels show minute NDUFS3-GFP expression on SDS Western blot (anti-GFP) and endogenous NDUFS3 expression on SDS Western blot (anti-NDUFS3). Minute NDUFS3-GFP expression is also detected using the NDUFS3 antibody and is indicated with an asterisk. (C-D) Quantitative analysis of the GFP-tagged subcomplexes in (A) and (B) respectively. The integrated optical density (I.O.D.) of the anti-EGFP signals was corrected for background and normalized to the I.O.D. of the CII-SDHA signal. For presentation purposes, the obtained I.O.D. values were divided by a factor of 1000.

Composition of CI subcomplexes

To confirm that the subcomplexes identified using NDUFS3-GFP are also present in wild type HEK293 cells and to determine the composition of these subcomplexes we used two-dimensional blue-native SDS PAGE (2D-SDS PAGE) followed by Western blotting and specific immunodetection of CI subunits. Use of 2D-SDS PAGE was essential since this method, in contrast to 1D-BN PAGE, allowed detection of wild type NDUFS3 protein using a commercially available antibody, likely because the NDUFS3 antibody binds more efficiently to the unfolded protein. Figure 3A shows that the subcomplexes identified by GFP tagging of the NDUFS3 subunit (figure 2B) are also present in two independent isolations of wild type HEK293 mitochondria. To analyze the composition of these complexes, we performed immunodetection of nine CI subunits (figure 3B). Subcomplex 1 is poorly visible, but subcomplexes 2-6 and CI (indicated with 7) can be clearly discriminated. Alignment between the different subunits shows that subcomplexes 2-6 and CI contain the NDUFS2 and NDUFS3 subunits with the addition of ND1 in subcomplexes 4-7 and the addition of NDUFA13 in subcomplex 5-7. Close inspection furthermore reveals the "appearance" of an additional subcomplex termed 'a1' ("appearing subcomplex 1") that only contains membrane subunits ND1, NDUFB6, NDUFA13, NDUFA6 and NDUFA1. Composition analysis of subcomplex 6 is difficult due to the close vicinity of CI and a1. OXPHOS complexes II (~150 kDa), III (600 kDa in dimeric form), IV (~240 kDa) and V (~750 kDa) are shown to allow estimation of the sizes of the CI subcomplexes.

Table 1: Immunodetected proteins in this study

This table lists the proteins investigated by immunodetection for each OXPHOS complex. For the CI subunits, bovine homologue names are indicated as well as their distribution after fractionation as described in (Hirst et al., 2003).

OXPHOS Complex	Subunit (human)	Subunit (bovine)	CI fraction (bovine)
CI	NDUFS2	49 kDa	Iα (Iλ)
CI	NDUFA9	39 kDa	Iα
CI	NDUFS3	30 kDa	Iα (λ)
CI	ND1	ND1	Iγ
CI	NDUFB6	B17	Iβ
CI	NDUFA13/GRIM19	B16.6	Iα (Iλ)
CI	NDUFA6	B14	Iα
CI	NDUFA2	B8	Iα (Iλ)
CI	NDUFA1	MWFE	Iα
CII	SDHA		
CIII	UQCRC2		
CIV	COXII		
CV	ATPA1		

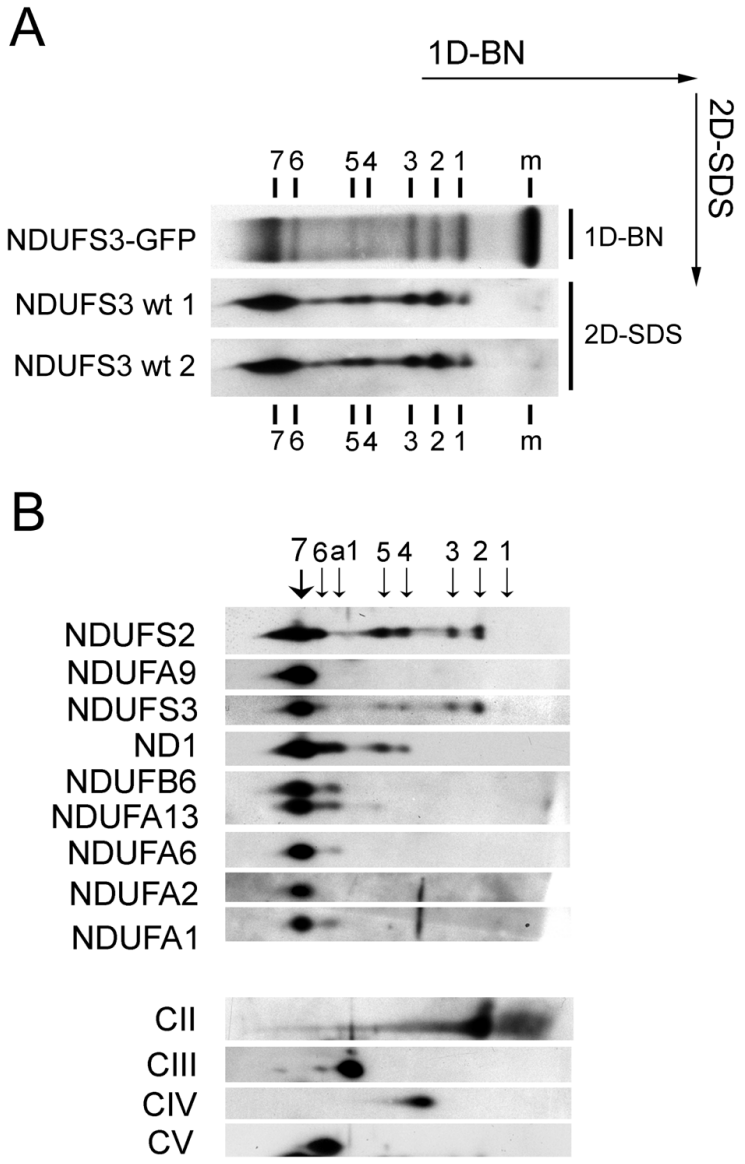


Figure 3: Investigation of the occurrence and composition of the NDUFS3 subcomplexes in wild type HEK293 cells

(A) Alignment between immunodetections of NDUFS3-GFP on a 1D-BN Western blot lane with NDUFS3 on two independent 2D-SDS Western blots of wild type HEK293 mitochondrial lysates. Monomeric NDUFS3, NDUFS3-GFP, subcomplexes 1-6 and CI (7) are indicated at the top of the 1D-BN lane and bottom of the 2D-SDS panels. (B) Analysis of the composition of the NDUFS3 subcomplexes by Western blot immunodetection after 2D-SDS PAGE analysis of wild type HEK293 mitochondrial lysates. Individual immunodetections are shown for the OXPHOS proteins indicated at the left of the figure (see also table 1). Complexes range from "1" (low molecular weight) to "7" (high molecular weight) and are indicated at the top of the panels. CI is indicated with the thick arrow (also with "7"), an additionally appearing subcomplex is indicated with "a1".

Stability of CI subcomplexes

In principle, BN analysis reveals native CI subcomplexes after detergent-mediated isolation. In order to determine whether the observed subcomplexes originate from partial CI solubilization, we investigated their presence in wild type HEK293 mitochondria under different, increasingly stringent, concentrations of n-Dodecyl β-D-maltoside (DDM) and digitonin, which are frequently used detergents in the isolation of membrane protein complexes. We performed immunodetection with the same antibodies as depicted in figure 3B. For simplicity, figure 4A only shows the NDUFS3 and ND1 subunits. The results obtained for the full set of antibodies are shown in supplemental figures 1 and 2 and are summarized in table 2. Core2 was used as a positive control for the effect of solubilization, as it allows investigation of the presence of complex III in various supercomplexes (described in (Schagger and Pfeiffer, 2000) and marked by S1 and S2). In these experiments the yield of subcomplexes 1-6 did not alter, whereas, in contrast, CI was increasingly solubilized from supercomplexes with ever more stringent conditions. Therefore, it is highly unlikely that the subcomplexes in our NDUFS3-GFP-expressing and wild type HEK 293 cells are artifacts of solubilization. On the contrary, subcomplex a1 became more predominant under more stringent solubilization conditions (figure 4A, ND1 signal), strongly suggesting that this subcomplex originates from the holocomplex due to solubilization.

Finally, we investigated the presence of these subcomplexes in conditions promoting CI breakdown (incubation at 37° C). This resulted, as shown in figure 4B, in the appearance of subcomplex “a2” (“appearing subcomplex 2”), seen with anti-NDUFS2, anti-NDUFA9, anti-NDUFS3 and anti-NDUFA2, and an increase in subcomplex “a1”, seen with anti-ND1, anti-NDUFB6, anti-NDUFA13 anti-NDUFA6 and anti-NDUFA1, and subcomplex 1, all suggestive of breakdown products (figure 4C). The occurrence and composition of subcomplexes in each of the three conditions (4A and B) is summarized in table 2.

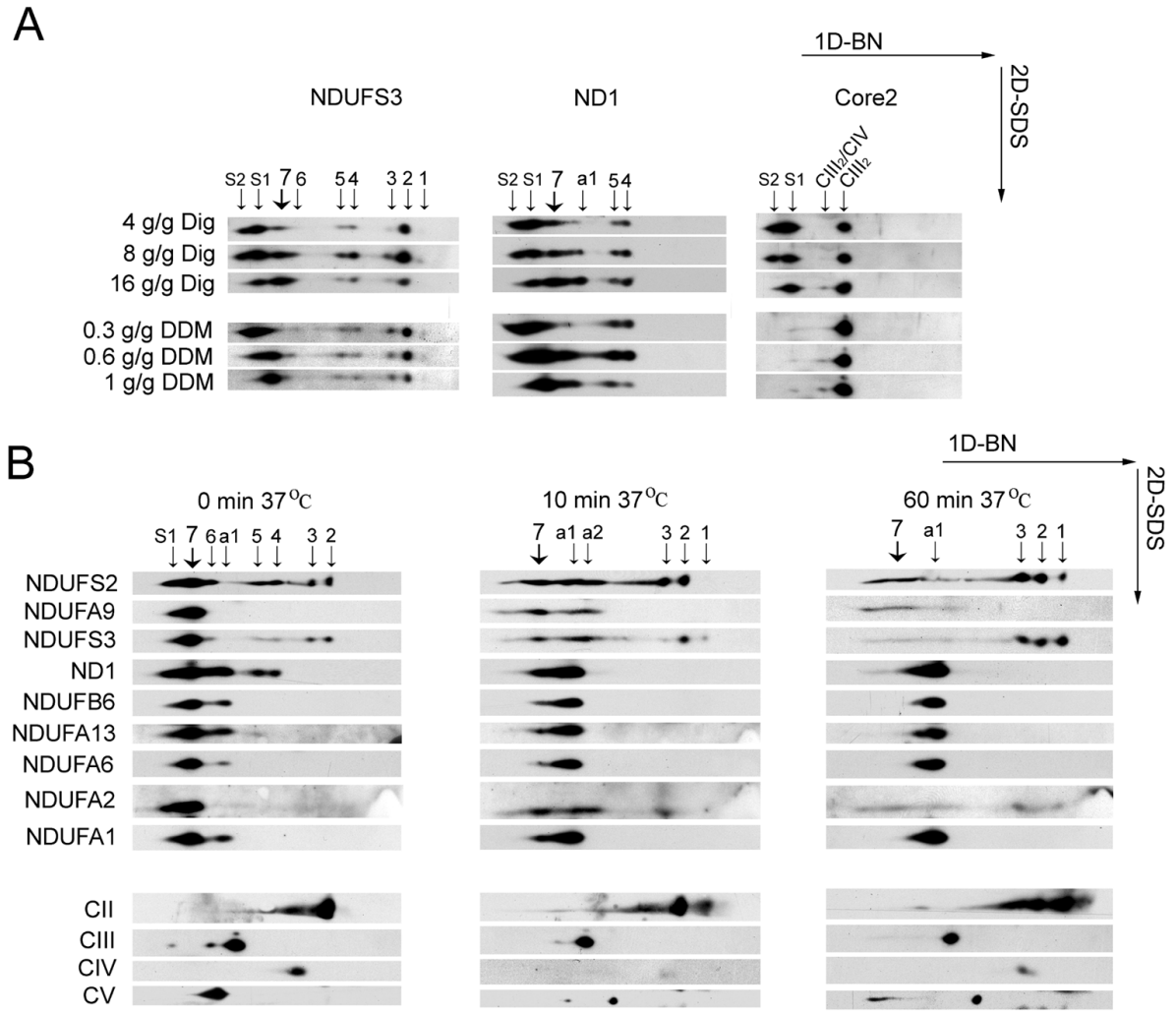


Figure 4: Subcomplex distribution after solubilization with increasing concentrations of DDM, digitonin and at different 37 °C incubations

(A) Analysis of the two-dimensional distribution of NDUFS3, ND1 and Core2 for DDM solubilizations of HEK293 mitochondria using 0.3, 0.6 and 1 g DDM/g protein and for digitonin solubilizations using 4, 8 and 16 g digitonin/g protein (indicated at the left of the lanes). More subunits were tested but their distribution or abundance remained unaltered throughout all of the solubilizations, summarized in table 2 (see also supplementary figures 1 and 2). Complexes range from "1" (low molecular weight) to "7" (high molecular weight). CI is indicated with the thick arrow (also with "7"). Supercomplexes are indicated with S1 (CI/$CIII_2$/CIV) and S2 (CI/$CIII_2$/CIV_x) (Schagger and Pfeiffer, 2000). An appearing subcomplex is indicated with "a1". (B) Analysis of the two-dimensional distribution of the NDUFS3 subcomplexes after different incubations at 37 °C. DDM solubilized mitochondrial lysates were treated as follows: 60 min on ice (indicated "0 min 37 °C"); 10 min at 37 °C followed by 50 min on ice (indicated "10 min 37 °C"); or 60 min at 37 °C (indicated "60 min 37 °C"). For each incubation period (indicated at the top of the 2D panels), individual immunodetections are shown for the OXPHOS proteins indicated at the left of the figure (see also table 1). Complexes range from "1" (low molecular weight) to "7" (high molecular weight) and are indicated at the top of the panels. CI is indicated with the thick arrow (also with "7"), additionally appearing subcomplexes are indicated with "a1" and "a2".

Table 2: Overview of the distribution of the NDUFS3 subcomplexes per solubilization condition
S1/S2: supercomplex variants. a1 and a2: appearing subcomplex numbers 1 and 2, respectively. A: appearing subcomplex. D: disappearing subcomplex. X: unchanged subcomplex.

	Subunit	1	2	3	4	5	a2	a1	6	7 (CI)	S1/S2
DDM solubilization											
	NDUFS2	A/D	X	X	X	X			X	X	D
	NDUFA9									X	D
	NDUFS3	A/D	X	X	X	X			X	X	D
	ND1				X	X		A		X	D
	NDUFB6							A		X	D
	NDUFA13					X		A		X	D
	NDUFA6							A		X	D
	NDUFA2									X	D
	NDUFA1							A		X	D
Digitonin solubilization											
	Subunit	1	2	3	4	5	a2	a1	6	7 (CI)	S1/S2
	NDUFS2	A/D	X	X	X	X			X	X	X
	NDUFA9									X	X
	NDUFS3	A/D	X	X	X	X			X	X	X
	ND1				X	X		A		X	X
	NDUFB6							A		X	X
	NDUFA13					X		A		X	X
	NDUFA6							A		X	X
	NDUFA2									X	X
	NDUFA1							A		X	X
37 ˚C solubilization											
	Subunit	1	2	3	4	5	a2	a1	6	7 (CI)	S1/S2
	NDUFS2	A	X	X	D	D	A/D		D	D	D
	NDUFA9						A/D			D	D
	NDUFS3	A	X	X	D	D	A/D		D	D	D
	ND1				D	D		A		D	D
	NDUFB6							A		D	D
	NDUFA13					D		A		D	D
	NDUFA6							A		D	D
	NDUFA2						A/D			D	D
	NDUFA1							A		D	D

Discussion

A better understanding of how nuclear- and mtDNA-encoded subunits of human mitochondrial CI are combined not only aids elucidation of how macromolecular assemblies are formed in the mitochondrial inner membrane, but also helps to clarify the molecular background of many unexplained CI deficiencies. Although the finding of subassemblies in CI deficient patients hints towards the existence of distinct steps in the assembly pathway, proper interpretation of such data requires detailed insight into assembly under normal conditions. Especially the dynamics, rate-limiting steps and specific assembly proteins involved in this process might deliver important clues.

In this study, we have used an inducible NDUFS3-GFP HEK293 cell line to investigate the CI assembly process. We show that upon induction six GFP-labeled subcomplexes appear in addition to GFP-labeled CI itself. Importantly, this labeling procedure did not alter expression and/or activity of the fully assembled complex. The same subcomplexes were detected in mitochondria of wild type HEK293 cells, excluding that they were labeling artifacts. We furthermore show that inhibition of mitochondrial translation under conditions of steady-state “leakage” labeling resulted in accumulation of the smaller subcomplexes 2 and 3, but not 1, and the disappearance of subcomplex 6 and CI. This finding unambiguously demonstrates the requirement of mitochondrial translation products for the assembly process to proceed beyond subcomplexes 2 and 3. Because rigorous testing demonstrated that it is highly unlikely that subcomplexes 2 and 3 are isolation artifacts, their accumulation during inhibition of mitochondrial translation shows that these two subcomplexes are true assembly intermediates.

The existence of CI subassemblies in the absence of mtDNA-encoded CI subunits has been described previously (Hall and Hare, 1990; Hofhaus and Attardi, 1993; Hofhaus and Attardi, 1995; Bai and Attardi, 1998; Potluri et al., 2004). In our previous assembly study, we analyzed 143B cells that were depleted of mitochondria DNA (rho-0 cells) and found that NDUFS3 was present in three subcomplexes very similar to subcomplexes 1, 2 and 3 described in the present study (Ugalde et al., 2004). We termed these subcomplexes H, G and F. We now learn that subcomplex H is not monomeric NDUFS3 but already includes the NDUFS2 subunit. Intriguingly, this subcomplex appears not only during induction (figure 1B), but also after degradation (figure 4B) of CI, which makes it difficult to

determine its exact origin. As it does not accumulate after chloramphenicol inhibition (figure 2A), it most likely does not originate directly from subcomplexes 2 and 3. It may originate from larger assemblies, but as yet its exact origin remains enigmatic.

Based on their composition and size and their accumulation in the absence of mitochondrial translation, we propose that subcomplexes 2 and 3 represent early stages in the assembly process. It is conceivable that assembly reflects the conserved evolutionary structural relationship of CI subunits via the formation of distinct modules (Ugalde et al., 2004; Vogel et al., 2004). The evolutionary conserved relation between the NDUFS2 and NDUFS3 as fused proteins in several bacterial species and as part of the hydrogenase module of CI (Braun et al., 1998; Clark et al., 1998) corresponds well with their early association during the assembly process. The hydrogenase module is important early in assembly, as illustrated by the finding that the overexpressed dehydrogenase module of *Escherichia coli* NDH-1 is only incorporated when the homologues of the NDUFS3, NDUFS2 and NDUFS7 subunits are also overexpressed (Braun et al., 1998). Based on their size of about 100-150 kDa, subcomplexes 2 and 3 likely include additional subunits, such as NDUFS7 and NDUFS8. Prommeenate and colleagues describe the existence of distinct subcomplexes containing the homologues of NDUFS2, NDUFS3 and NDUFS7 subunits in cyanobacteria (Prommeenate et al., 2004). In addition, Bourges and colleagues have co-immunoprecipitated subcomplexes containing IP-fraction subunits NDUFS2, NDUFS3 and NDUFS7 using anti-NDUFS3 antibody in wild-type and rho-0 143B osteosarcoma cells and in cell lines devoid of ND4 and ND5 (Bourges et al., 2004).

The accumulation of subcomplexes 2 and 3 after inhibition of mitochondrial translation, and the specific appearance of larger subcomplexes after releasing this inhibition, suggest that mtDNA-encoded subunits are incorporated after subcomplex 3 is formed. The additional presence of an ND subunit in subcomplexes 4 and 5 (figure 3B), which reappear after assembly has resumed (figure 2B), may represent expansion of the hydrogenase module and anchoring to the mitochondrial inner membrane. Unfortunately, due to the lack of proper antibodies, we were not able to investigate the presence of other ND subunits in subcomplexes 4 and 5 except ND1. Membrane subunit ND1 is one of the first subunits clearly expressed after release of inhibition of mitochondrial translation in 143B osteosarcoma cells (Ugalde et al., 2004) and absence of ND1 and ND6 results in severe assembly disturbances (Bai and Attardi, 1998; Chomyn, 2001;

Cardol et al., 2002). In contrast, lack of ND4 or ND5 does not have such a 'null' effect on assembly. For example, in *Chlamydomonas* CI, loss of ND4 or ND4/ND5 still allows formation of substantial portion of CI (Cardol et al., 2002). Also in humans, absence of ND4 or ND5 still allows formation of nuclear DNA-encoded subcomplexes (Bourges et al., 2004), possibly even displaying activity (Hofhaus and Attardi, 1993; Hofhaus and Attardi, 1995; Bai and Attardi, 1998). ND3 mutation and absence of ND5 seem to have an effect on activity rather than assembly (Hofhaus and Attardi, 1995; McFarland et al., 2004). Therefore, as opposed to ND3, ND4 and ND5, it appears that ND1 and ND6 are incorporated early in assembly and that the appearance of ND1 in subcomplex 4 may represent the first incorporation of mtDNA-encoded CI subunits, in line with what is observed after the chloramphenicol inhibition experiments (figures 2A and B).

As opposed to the detergent-stable intermediates 1-6, differential detergent solubilization results in subcomplex a1 as a product of CI instability (figure 3A). That this intermediate most likely does not represent an assembly intermediate is supported by its composition in relation to subcomplexes 4 and 5. Subcomplexes 4 and 5 contain at least the NDUFS2, NDUFS3 and ND1 subunits (table 2). Subcomplex a1, which migrates at a higher molecular weight, consists primarily of membrane arm CI subunits, including ND1 but not NDUFS2 and NDUFS3 (table 2). This means that subcomplex a1 has either specifically 'lost' two of its subunits or is of different origin altogether. This discrepancy illustrates that subcomplex a1 is not simply a successive intermediate of CI assembly and that different subcomplexes seem to have different origins, possibly in assembly but possibly also in instability and breakdown, as also demonstrated for cyanobacterial CI (Prommeenate et al., 2004).

Additional heat-incubation at 37 °C shows that membrane arm subcomplex a1 also results from breakdown and is stable, possibly protected from proteases due to its lipid environment. It appears in conjunction with subcomplex a2, which consists of peripheral arm subunits and is rapidly broken down in time. Judging from their size and composition, observation of subcomplexes a1 and a2 points towards fractionation of CI into its membrane and peripheral arms. The disappearance of subcomplexes 4 and 5 and intensification of subcomplexes 1-3 after incubation at 37° C remains puzzling (figure 4C). This finding suggests that subcomplexes 1-3, under certain conditions, can originate from subcomplexes 4 and 5 or a2.

Somewhat to our surprise, the NDUFA9 subunit is not detected in any of the observed subcomplexes 1-6, which may illustrate its late assembly into CI. This is supported by the absence of this subunit in accumulated intermediates of nearly the size of CI in NDUFS6 and B17.2L patients (Kirby et al., 2004; Ogilvie et al., 2005). The presence of the NDUFA1 subunit (homologue of the bovine MWFE subunit) in CI subcomplexes was not investigated previously. In Chinese hamster cells, this subunit is speculated to serve as a membrane anchor to which membrane arm subunits attach during CI assembly (Yadava et al., 2004). Although NDUFA1 is detected in a membrane arm fraction of CI (subcomplex a1), it is consistently not detected in subcomplexes 4 and 5. It therefore seems that the hydrogenase core subomplex has already acquired at least one membrane arm subunit (ND1) prior to the addition of NDUFA1. Two other previously unstudied subunits in relation to their presence in subassemblies are NDUFA2 and NDUFA13. The co-migration of the NDUFA2 subunit with peripheral arm subunits NDUFS2, NDUFS3 and NDUFA9 in subcomplex a2 (that appears during breakdown) is in line with its fractionation with the Iλ fragment (Hirst et al., 2003). The NDUFA13, or GRIM19, subunit of CI comigrates with membrane arm subunits and, in addition to its presence in subcomplex a1, shows a particular presence in subcomplex 5 (consisting of the NDUFS2/NDUFS3/ND1/NDUFA13 subunits). It seems that a subunit that is considered ‘accessory’ and involved in regulation of cell death, is already present in a smaller structure containing the conserved ‘basic’ core subunits of CI (Huang et al., 2004).

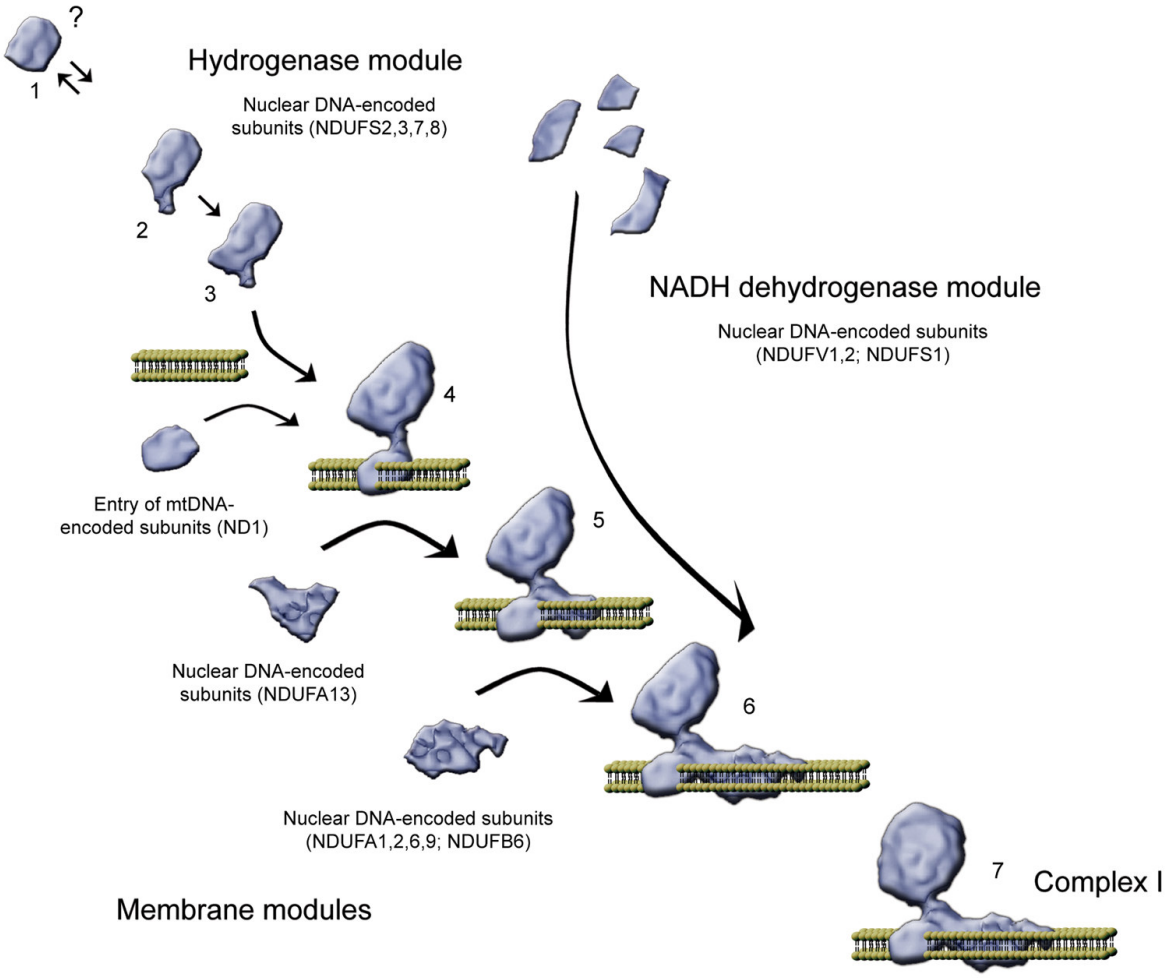


Figure 5: An updated model for human mitochondrial CI assembly

CI assembly initiates with the formation of the hydrogenase core module, which is, in several steps, anchored to the mitochondrial inner membrane and expanded with the NADH dehydrogenase module and the further addition of membrane fragments. Comparison with our previous assembly model (Ugalde et al., 2004) leads to the following 'translation': subcomplexes 1, 2, 3 represent subcomplexes H, G, F; subcomplexes 4, 5 represent subcomplex E; subcomplexes 6, 7 represent subcomplex A (possibly also B).

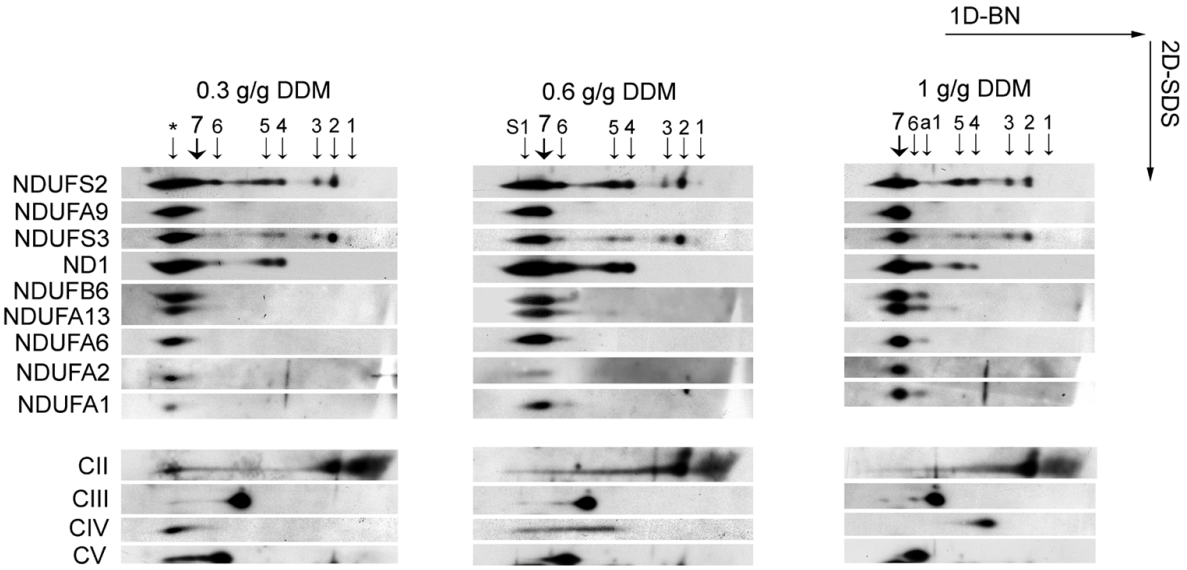
In conclusion, subcomplexes 1-6 represent equilibriums in the assembly pathway of CI and highlight important steps in the process. It is likely that more assembly intermediates exist but were not detected using our set of antibodies or have escaped detection because incorporation during assembly occurs too rapidly. The existence of these particular intermediates could be demonstrated in CI deficient patient cell lines, when assembly disturbance leads to accumulation of an assembly step. We have updated our previous assembly model to incorporate our current findings, which supports modular CI assembly on the basis of evolutionary conservation (figure 5). Future analysis of the exact composition of each subcomplex will allow further verification and refinement of existing CI assembly models which, in turn, will aid the understanding of many yet unexplained CI assembly disturbances.

Acknowledgements

This work was supported by 'Het Prinses Beatrix Fonds' to J.S. and L. v.d. H. (grant number 02-0104) and the European Community's sixth Framework Programme for Research, Priority 1 "Life sciences, genomics and biotechnology for health" (contract number LSHMCT-2004-503116). The Netherlands Organization for Scientific Research supported L.N. with a 'Vernieuwingsimpuls' grant.

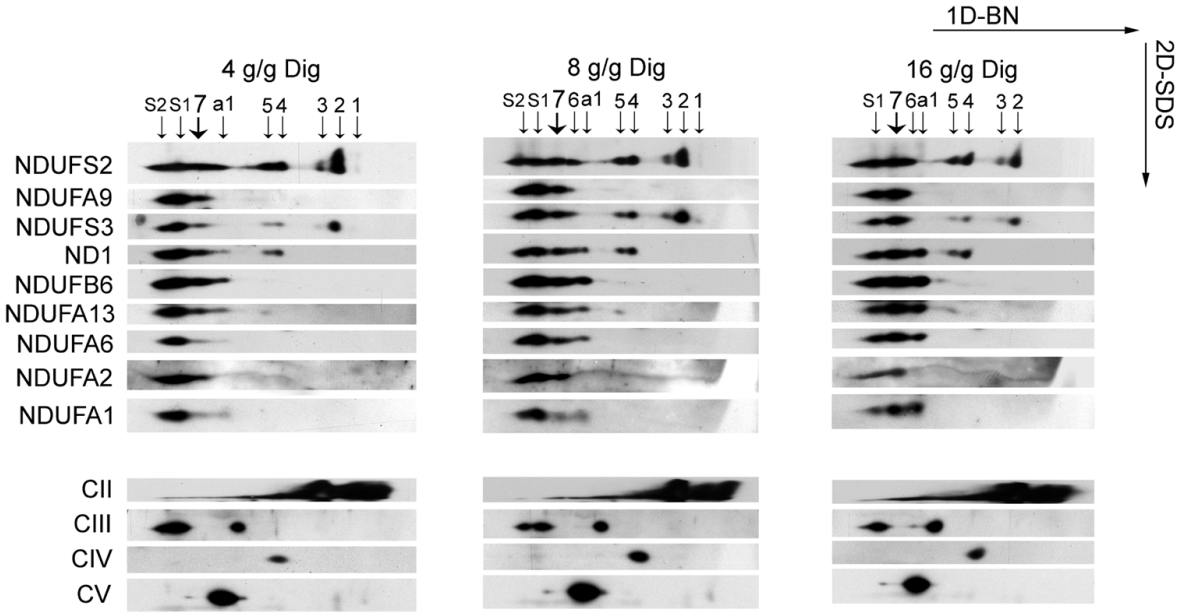
The authors wish to thank Dr. Ronald Roepman for excellent technical support on Gateway® Cloning. We are grateful to Dr. Frank van Kuppeveld (University of Nijmegen, Nijmegen, The Netherlands), Dr. A. Lombes (Inserm, Paris, France), Professor I. Scheffler (University of California, San Diego, USA) and Professor B. Robinson (University of Toronto, Toronto, Canada) for providing, respectively, the anti-EGFP, anti-ND1, anti-NDUFA1 and anti-NDUFS2 antibodies.

Supplemental data



Supplemental figure 1: Analysis of the two-dimensional distribution of OXPHOS proteins for DDM solubilizations of HEK293 mitochondria using 0.3, 0.6 and 1 g DDM/g protein (indicated at the top of the 2D panels)

Per solubilization condition, individual immunodetections are shown for the OXPHOS proteins indicated at the left of the figure (see also table 1). Complexes range from "1" (low molecular weight) to "7" (high molecular weight). CI is indicated with the thick arrow (also with "7"). Supercomplexes are indicated with S1 (CI/$CIII_2$/CIV) and S2 (CI/$CIII_2$/CIV_x) (Schagger and Pfeiffer, 2000), and a possible aggregate (also containing CII) is indicated with an asterisk. An additionally appearing subcomplex is indicated with "a1".



Supplemental figure 2: Analysis of the two-dimensional distribution of OXPHOS proteins for digitonin solubilizations of HEK293 mitochondria using 4, 8 and 16 g digitonin/g protein (indicated at the top of the 2D panels)

Per solubilization condition, individual immunodetections are shown for the OXPHOS proteins indicated at the left of the figure (see also table 1). Complexes range from "1" (low molecular weight) to "7" (high molecular weight). CI is indicated with the thick arrow (also with "7"). Supercomplexes are indicated with S1 (CI/CIII2/CIV) and S2 (CI/CIII2/CIVx) (Schagger and Pfeiffer, 2000). An additionally appearing subcomplex is indicated with "a1".

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PART II: Complex I assembly chaperones

Chapter 5

Human mitochondrial complex I assembly is mediated by NDUFAF1

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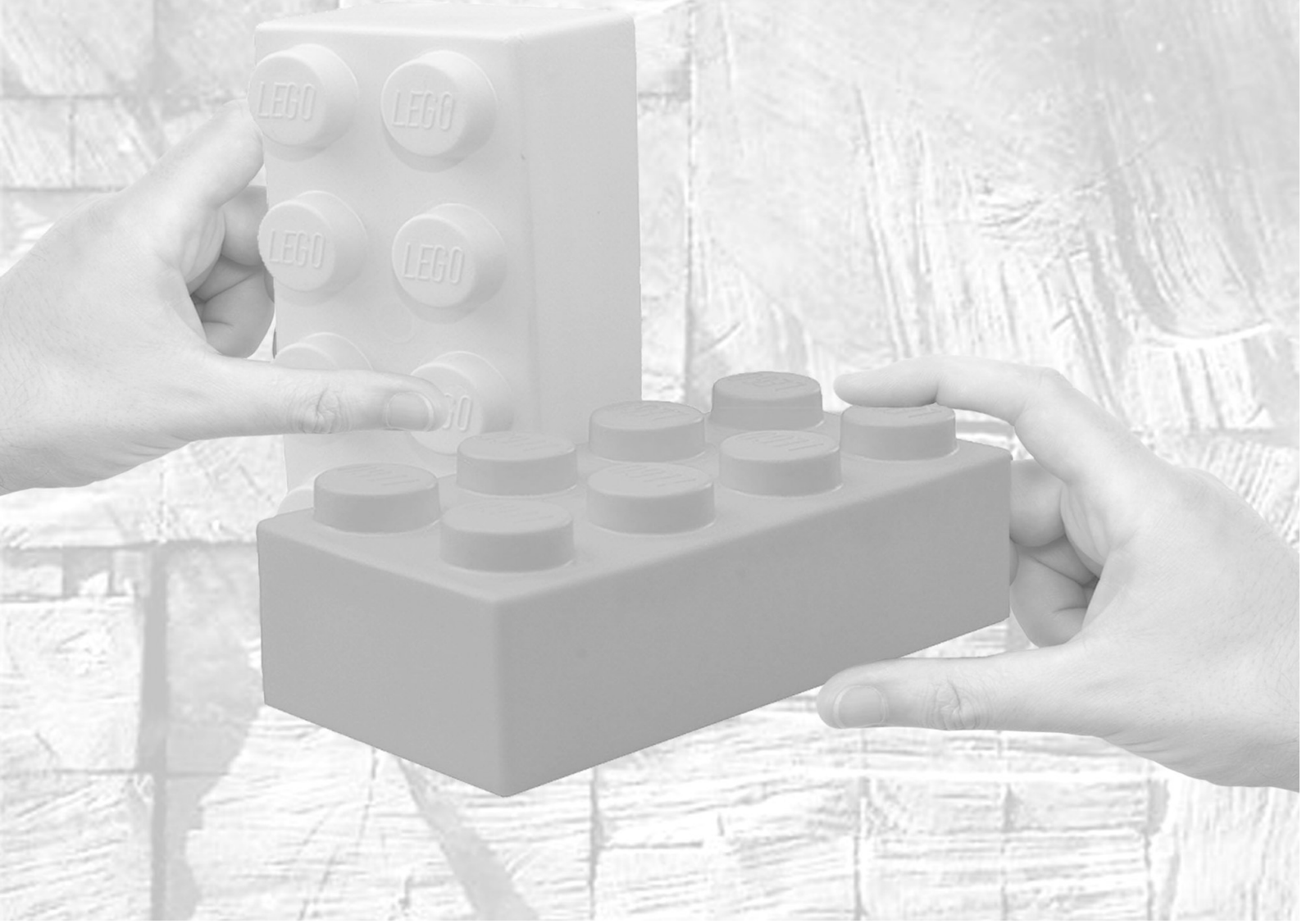
FEBS Journal 2005; 272(20): 5317-5326



Abstract

Complex I (NADH:ubiquinone oxidoreductase) is the largest multi-protein enzyme of the oxidative phosphorylation system. Its assembly in human cells is poorly understood and no proteins assisting this process have yet been described. A good candidate is NDUFAF1, the human homologue of *Neurospora crassa* complex I chaperone CIA30. Here, we demonstrate that NDUFAF1 is a mitochondrial protein that is involved in the complex I assembly process. Modulating the intra-mitochondrial amount of NDUFAF1 by knocking down its expression using RNA interference leads to a reduced amount and activity of complex I. NDUFAF1 is associated to two complexes of 600 and 700 kDa in size of which the relative distribution is altered in two complex I deficient patients. Analysis of NDUFAF1 expression in a conditional complex I assembly system shows that the 700 kDa complex may represent a key step in the complex I assembly process. Based on these data, we propose that NDUFAF1 is an important protein for the assembly/stability of complex I.

Introduction

The failure to assemble a properly functioning complex I (NADH:ubiquinone oxidoreductase, EC 1.6.5.3) results in complex I deficiency. This is a major contributor to mitochondrial disease and frequently results in early childhood death (Smeitink et al., 2001). Since complex IV (cytochrome *c* oxidase) deficiency is frequently caused by mutations in an assembly factor (Barrientos et al., 2002), it is likely that a similar situation holds for complex I. However, in contrast to the 15 assembly chaperones already found for complex IV, so far no assembly proteins have been described for mammalian complex I. Given the complexity of the enzyme, many assembly factors are likely to be needed and still await detection. We have described a candidate gene, NDUFAF1, which has 28% homology with *Neurospora crassa* complex I assembly chaperone CIA30 (Janssen et al., 2002). The present study is the first to investigate the possible role of NDUFAF1 in the assembly of complex I in human cells.

Complex I is the first of five multi-protein complexes which together constitute the oxidative phosphorylation system. In this system NADH is oxidised by complex I, after which electrons are transferred via electron carriers (ubiquinone and cytochrome *c*) and via complexes III and IV to the final electron acceptor molecular oxygen. The energy of this transfer is used to translocate protons across the mitochondrial inner membrane. The thus generated proton gradient is used by complex V (ATP synthase) to generate ATP.

Mammalian complex I is an L-shaped structure consisting of at least 45 subunits (Grigorieff, 1999; Carroll et al., 2003; Hirst et al., 2003) of which seven are encoded by the mitochondrial genome. The complex can be subdivided into three functionally distinct fragments. The NADH dehydrogenase segment includes the redox cofactor flavomononucleotide, which is involved in the oxidation of the NADH substrate. The hydrogenase part contains several iron sulfur clusters which are involved in electron transfer to the electron transporter ubiquinone. The membrane bound transporter part of complex I is involved in proton translocation. Whether and how electron transport and proton translocation are coupled is yet uncertain (Vinogradov, 2001).

Assembly of complex I is an intricate process which has been studied in several organisms (Cardol et al., 2002; Stroh et al., 2004; Yadava et al., 2004). The most

extended investigation of complex I assembly was performed for the fungus *Neurospora crassa* (Videira, 1998; Schulte, 2001). In the proposed model, the hydrophilic peripheral arm and the hydrophobic membrane arm of complex I are assembled independently before being joined together. The search for a more detailed description of the assembly pathway has recently resulted in the publication of the first models for human complex I assembly (Antonicka et al., 2003; Ugalde et al., 2004). Both models differ considerably, illustrating the fact that the complex I assembly pathway is far from solved. The model we describe shows considerable homology to *N. crassa* complex I assembly. We propose that, analogous to the situation in *N. crassa*, complex I is assembled semi-sequentially: discrete functional modules are assembled independently and are joined in several steps to form a peripheral arm and a membrane arm assembly intermediate. In more detail, this entails the formation of perhipheral arm assembly intermediate D (600 kDa) from intermediates H (80 kDa), G (150 kDa) and F (250 kDa) and the formation of membrane arm assembly intermediate C (700 kDa) from the combination of membrane proteins with intermediate E (400 kDa). Peripheral arm intermediate D consists of a core or highly conserved hydrophilic subunits (such as the 49 kDa, 39 kDa and 30 kDa subunits), while membrane arm intermediate C consists of highly conserved hydrophobic subunits (such as ND1 and ND6). These two key intermediates are combined to form assembly intermediate B (950 kDa) and finally holo-complex I (A, 1 MDa) (Ugalde et al., 2004).

Two candidate assembly proteins for complex I were found in *N. crassa*: Complex I Intermediate Associated proteins CIA30 and CIA84 (Kuffner et al., 1998). Knockouts of the *cia* genes in *N. crassa* resulted in membrane arm subunit knockout phenotypes. The CIA proteins are thought to chaperone the combination of the small and large membrane arm intermediates of complex I via binding to the large membrane arm intermediate. The binding of CIA84 is transient as the protein cycles between a bound and unbound state. Immunoprecipitations using a CIA84 antibody have resulted in the identification of associated subunits in the membrane arm (Kuffner et al., 1998).

So far nothing is known about the possible involvement of CIA30 homologue NDUFAF1 in human complex I assembly. In this paper, we demonstrate that NDUFAF1 acts as an assembly protein for complex I in human cells, in line with the proposed function of its homologue CIA30 in the fungus *N. crassa.*

Materials and methods

Generating NDUFAF1 antibody

To produce antibodies against NDUFAF1, two oligopeptides were selected. The first peptide corresponds to the mid-portion of the NDUFAF1 sequence, amino acids 43V-57G; the second peptide corresponds to the C-terminus of the protein, amino acids 314F-327K. A mixture of both peptides was coupled to keyhole limpet hemocyanin, serving as an immunogenic carrier. Rabbits were immunised with an injection of the antigen-carrier conjugate, followed by three subsequent boosters, one every three weeks. The two antisera were collected from a final bleeding and tested for specific detection of the NDUFAF1 protein by Western blotting. Both antisera displayed comparable specificity.

Cell cultures

JM109 *E.coli* cells (Promega) were cultured in Luria Burtani (LB) medium and the appropriate antibiotic was added to the medium. 143B206 ρ^0 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Bio Whitaker) supplemented with 5% fetal calf serum (FCS), antibiotics, 1 mM uridine and 100 µg/ml bromodeoxyuridine. HeLa cells and 143B osteosarcoma cells were cultured in DMEM supplemented with 10% FCS and antibiotics. HEK293 T-REx^{tm} cells without the TO/NDUFAF1 construct were cultured in the same medium to which 5 µg/ml blasticidin (Invitrogen) was added to maintain the repressor construct. To HEK293 T-REx^{tm} cells containing the TO/NDUFAF1 construct additionally 300 µg/ml Zeocin^{tm} (Invitrogen) was added to maintain the inducible construct.

siRNA transfection

For transfection, HeLa cells were plated in DMEM supplemented with 10% FCS (without antibiotics) in 24-well plates with a cell density of 1.5 x 10^4 cells per well. The next day, cells were transfected with siRNA duplex (control: Cyclophilin B (Dharmacon), NDUFAF1: #1 antisense strand: 5'- ACUAACAUCAGGCUUCUCC dTdT -3', #2 antisense strand: 5'- UAACUAUACAUCUGAUUCG dTdT -3') in 3 µl oligofectamine (Invitrogen) to achieve a final concentration of 10 nM siRNA per well. Cells were incubated at 37°C in a CO_2 incubator for 48-72 hours until they were ready to assay for gene knockdown or to perform a second transfection.

Cellular fractionation

Approximately 5 x 10^6 HEK293 cells were harvested, washed in PBS, resuspended in an appropriate isotonic buffer (0.25 M sucrose, 5 mM Tris-HCl, pH 7.5, and 0.1 mM PMSF) and homogenised using a glass teflon homogeniser. Unbroken cells and nuclei were pelleted by centrifugation at 600 *g* for 15 minutes. Supernatants were centrifuged at 10,000 *g* for 25 minutes. The resulting supernatant was isolated as the cytoplasmic fraction and the mitochondrial pellet was washed once with the isotonic buffer containing 1 mM EDTA, pH 8.

Creating the inducible NDUFAF1 construct and transfection into HEK293 T-REx™ cells

A PCR of total cell cDNA was performed to specifically amplify NDUFAF1 cDNA using Native Pfu DNA polymerase (Stratagene). Primer sequences are (5' – 3'):
NDUFAF1forward: CGCGGAATTCATGGCTTTGGTTCACAAATTGC
NDUFAF1reverse: CGCGTCTAGATTTAAAAAGCCTTGGGTTAAGCTC
The amplified fragment and pcDNA4/TO/myc-His A (Invitrogen) (TO) were digested with *Eco*RI and *Xba*I restriction enzymes (Gibco) and subsequently ligated using T4 DNA Ligase (Invitrogen) and transformed into JM109 competent *E. coli* cells (Promega). Clones containing the plasmid were obtained by kanamycin (Sigma Aldrich) selection and were cultured to obtain sufficient construct for transfection. After sequence verification, transfection of the construct was performed on HEK293 T-REx™ cells (Invitrogen), which stably repress the tetracycline repressor operon, using the Superfect® Transfection Reagent (Qiagen). Cells were grown to a confluency of 60-70% in a 6-well plate and cells in each well were transfected by adding 2 µg of the construct according to the protocols described in the Superfect Transfection Reagent Handbook (Qiagen). Stable clones were obtained by culturing under selective pressure of 300 µg/ml Zeocin™ (Invitrogen). HEK293 T-REx™ clones containing the TO/NDUFAF1 construct were induced for expression of the transgene by adding 0,1-1 µg/ml doxycycline to the growth medium for the times indicated in the results section.

Immunofluorescence assay

A culture of a TO/NDUFAF1 containing HEK293 T-REx™ clone was seeded onto a coverslip in a 24-wells plate and grown overnight to achieve a confluency of 50% on the day of the assay. Cells were prestained with Mitotracker Red (Molecular Probes) prior to paraformaldehyde fixation. For fluorescence imaging, cell

preparates were attached to the stage of an inverted microscope (Axiovert 200 M, Carl Zeiss, Jena, Germany) equipped with a x63 Plan NeoFluar (NA 1.25) objective (Carl Zeiss). Alexa Fluor® 488 and Mitotracker Red were excited at 488 nm and 570 nm respectively, using a monochromator (Polychrome IV, TILL Photonics, Gräfelfing, Germany). Fluorescence emission light was either directed by a 505DRLPXR dichroic mirror (Omega Optical Inc., Brattleboro, USA) through a 535AF26 emission filter (Alexa Fluor® 488) or by a 595DRLP dichroic mirror through a 645AF75 emission filter (Mitotracker Red) onto a CoolSNAP HQ monochrome CCD-camera (Roper Scientific, Vianen, The Netherlands). All hardware was controlled with Metafluor 6.0 software (Universal Imaging Corporation, Downingtown, USA) running on a PC equipped with 1 Gb RAM running Windows XP Professional.

Digitonin isolation and solubilisation of mitochondria

Approximately 1 x 10^6 trypsin-harvested cells were pelleted and resuspended in 100 µl of cold PBS to which 100 µl of 4% digitonin (w/v) was added to achieve a final concentration of 2% digitonin. This sample was shortly vortexed and incubated on ice for 10 minutes to solubilize cell membranes. After this, 1 ml of cold PBS was added and the sample was centrifuged at 10000 g for 10 minutes at 4°C to obtain a mitochondria-enriched organelle pellet. To remove traces of digitonin, this pellet was washed twice with 1 ml of cold PBS. Mitochondrial proteins were solubilised by the addition of 100 µl of AC/BT (1.5 M aminocaproic acid, 75 mM Bis-Tris pH 7.0) and 20 µl of 10% *n*-dodecyl β-D-maltoside (w/v) and incubation on ice for 10 minutes. The solubilised proteins are retained in the supernatant after centrifugation at 10000 g for 25 minutes at 4°C, which was used for further analysis.

Gel electrophoresis and in-gel activity assays

The protein concentration for BN-PAGE and SDS-PAGE was determined in the *n*-dodecyl β-D-maltoside solubilised supernatants before adding coomassie blue containing sample buffer, using a MicroBCA protein assay kit (Pierce). Blue-native 5-15% gradient gels were loaded with 40 µg of digitonin-isolated mitochondria and, after electrophoresis, were further processed for Western blotting, second dimension 10% SDS-PAGE or in-gel activity assays as described earlier (Nijtmans et al., 2002). SDS-PAGE analysis was performed by loading 40 µg of protein/lane on 10% SDS-PAGE gels as described before (Ugalde et al., 2004).

Blotting, detection

Blue-native and SDS gels were blotted onto PROTRAN Nitrocellulose Transfer Membrane (Schleicher and Schuell BioScience) according to standard procedures. Primary antibodies used are against the following complex I subunits: NDUFA3 (30 kDa), NDUFA9 (39 kDa), NDUFB6 (B17) (Molecular Probes), ND1 (a gift from Dr. A. Lombes, France), ND6 (a gift from Professor R. Capaldi, USA), Complex II 70 kDa subunit (Molecular Probes), CoreII (Molecular Probes), NDUFS5 (15 kDa) (Molecular Probes), HSP70 (Abcam), c-myc (Invitrogen) and NDUFAF1. Secondary antibodies used are Swine Anti-Rabbit PerOxidase (SWARPO), Goat Anti-Rabbit PerOxidase (GARPO) and Goat Anti-Mouse PerOxidase (GAMPO) (Molecular Probes). Signal detection was performed using the ECL® plus Western Blotting Detection System (Amersham Biosciences). After ECL, the blots were exposed to X-OMAT UV film (KODAK). ECL signals were quantified using the ImagePro-Plus 4.1 image analysis software (Media Cybernetics, Silver Spring, MD, USA).

Results

NDUFAF1 localizes in the mitochondrion

To analyse the subcellular localization of NDUFAF1 we harvested HEK293 cells at 90% confluency and prepared protein samples of total cell lysate, isolated mitochondria and the cytoplasmic fraction. A Western blot of this gel was incubated with antibodies against NDUFAF1, a cytoplasmic marker (GAPDH), a marker for the mitochondrial inner membrane (COXII), a marker for the mitochondrial outer membrane (porin) and a marker for both cytoplasm and the mitochondrial matrix (HSP70) (Fig. 1). NDUFAF1 is clearly more abundant in isolated mitochondria than in the total cell protein extract and is absent in the cytoplasmic protein extract. These data show a specific localization of NDUFAF1 in the mitochondrion.

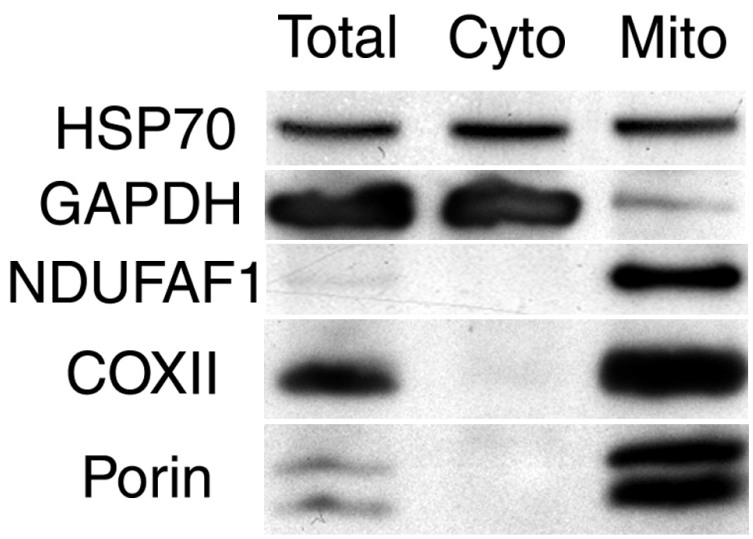


Figure 1: Western blot analysis of cellular fractionation of HEK293 cells
A 10% SDS-PAGE gel was loaded with 40 µg protein/lane as follows. Lane 1, total cell protein extract. Lane 2, cytoplasmic protein extract. Lane 3, mitochondrial protein extract. The Western blot of this gel was treated with antibodies against NDUFAF1, GAPDH (cytoplasmic marker), COXII and porin (mitochondrial markers) and HSP70 (loading control).

To confirm this result and to use another antibody for localization studies, we decided to create a construct in which the NDUFAF1 gene was tagged with an immunogenic epitope. We cloned NDUFAF1 into an inducible vector in-frame with a c-myc and 6xHis tag and transfected this construct into a human embryonic kidney cell line (HEK293), which stably expresses the tetracycline repressor gene. Stable clones were selected and induction of protein expression was tested using Western blot analysis. Digitonin-isolated mitochondria were obtained from cultures of an inducible clone after 0, 1, 2, 4, 24, 48 and 72 hours of induction with 0.1 µg/ml of doxycycline and analysed by Western blotting (Fig. 2). This concentration of doxycyline does not interfere with mitochondrial protein translation (Ugalde et al., 2004). Using both the NDUFAF1 and the c-myc antibodies both the endogenous and the induced (slightly larger than the endogenous protein due to the C-terminal tags) NDUFAF1 could be observed after induction.

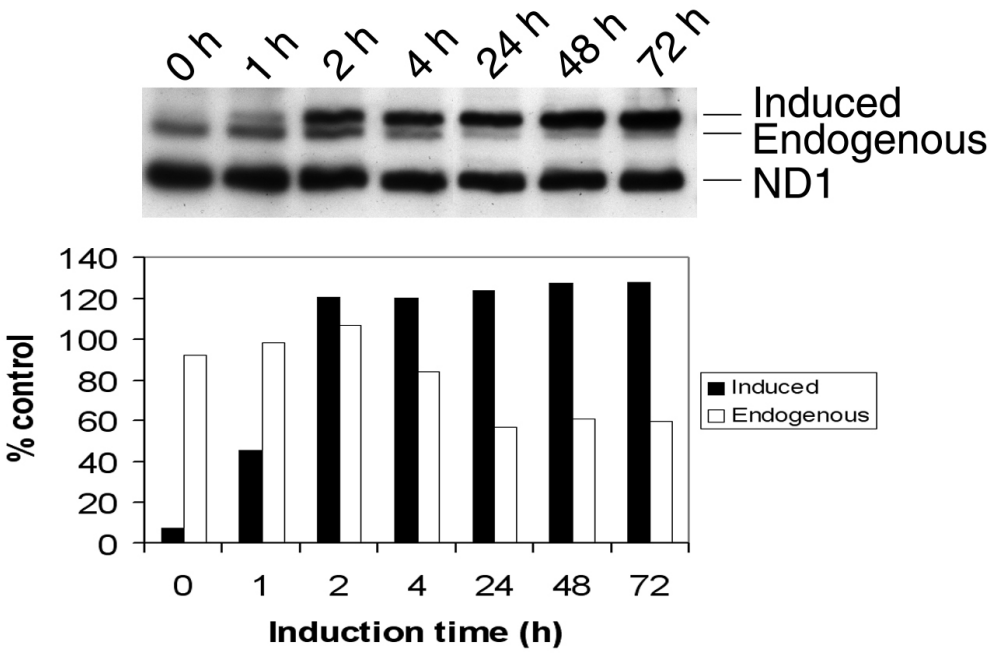


Figure 2: Western blot analysis of induction of NDUFAF1 in HEK293 cells
A 10% SDS-PAGE gel was loaded with 40 µg protein/lane of a mitochondrial protein extract of an inducible HEK293 clone and blotted. Cells were induced with 0.1 µg/ml of doxycycline for 0 (control), 1, 2, 4, 24, 48 and 72 hours. Antibodies used are against NDUFAF1 and complex I membrane arm subunit ND1. Induced and endogenous NDUFAF1 and ND1 signals are indicated on the right. Quantification of the ECL signals is shown in the histogram below, each bar corresponding to the lane above. The signal is expressed as percentage of the 0 hour time point signal for both endogenous NDUFAF1 (white bars) and induced NDUFAF1 (black bars).

To investigate the cellular localization of the NDUFAF1-c-myc fusion protein we used a monoclonal c-myc antibody in a immunohistochemical experiment, further incubated with a secondary antibody to which a fluorescent Alexa probe was coupled. The overlay of the Alexa dye staining with mitochondrial control Mitotracker Red shows that induced NDUFAF1 indeed migrates to the mitochondrion (Fig. 3). Biochemical fractionation of the NDUFAF1-c-myc expressing cells indicates that this fusion protein also exclusively localizes in mitochondria (results not shown).

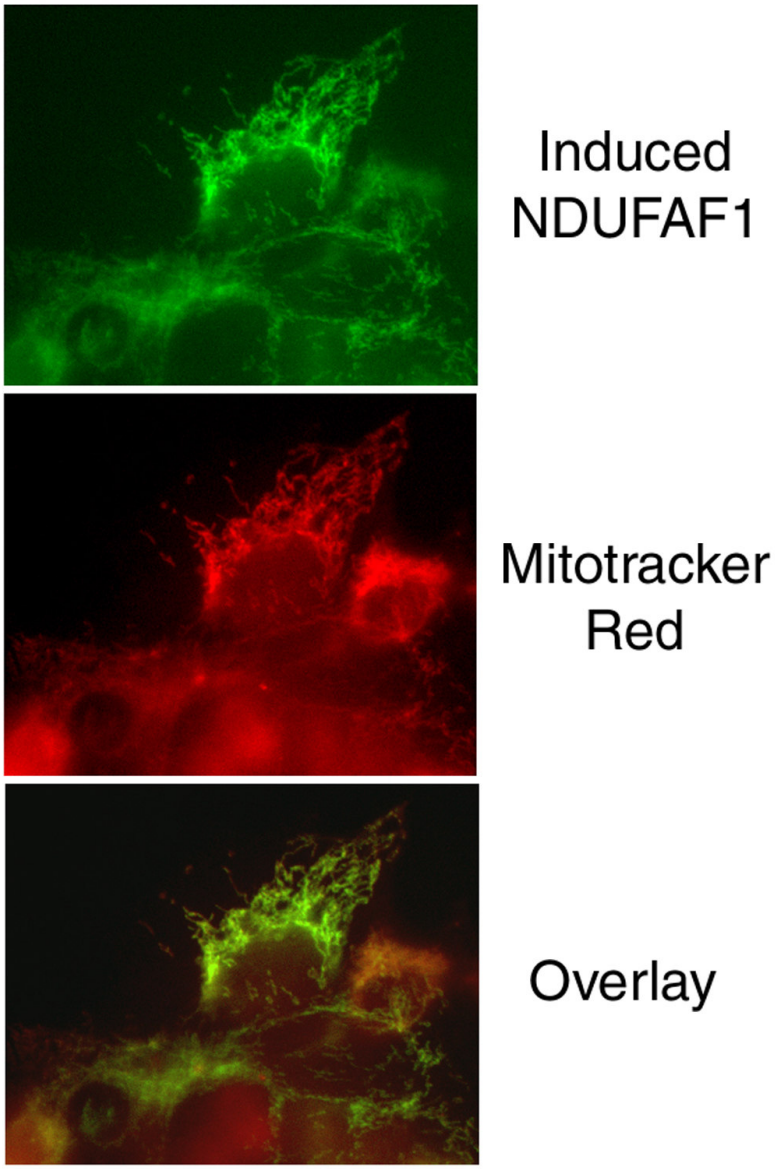


Figure 3: Immunofluorescence mitochondrial localization of induced NDUFAF1
Cells that are induced for NDUFAF1 expression by 1 µg/ml of doxycycline for 24 hours were treated with either anti-myc antibody coupled to Alexa Fluor 488 to verify presence of induced NDUFAF1 (top panel) or mitochondrial control Mitotracker Red (middle panel). The bottom panel shows an overlay of the two signals.

NDUFAF1 knock down reduces the complex I level and activity

Based on its homology with alleged complex I chaperone CIA30 in *N. crassa*, NDUFAF1 is an interesting candidate assembly protein for human complex I. The possible involvement of NDUFAF1 in complex I assembly/stability was investigated by performing RNA interference experiments. To knock down NDUFAF1 protein expression, two different small interfering RNA (siRNA) oligonucleotides were designed for targeting NDUFAF1 mRNA. Both siRNA's displayed similar knock down effects. Fig. 4 shows a transfection of 48 hours, followed by consecutive transfections of 48, 72 or 96 hours with 10 nM of siRNA #2 (data for siRNA #1 not shown). RNA interference effects are analysed for NDUFAF1 protein (Fig. 4A), fully assembled complex I (Fig. 4B) and complex I in-gel activity (Fig. 4C). NDUFAF1 expression can be knocked down to less than 30% of the control signal (Fig. 4A, lanes 4 and 5). This leads to a 40% decrease of fully assembled complex I (Fig. 4B, lane 5). Complex I activity is compromised as well, as can be seen by the 50% decrease of signal in Fig. 4C (lane 5). Interestingly, longer second incubations with siRNA lead to a greater inhibitory effect. Control transfections with a siRNA targeting cyclophilin B performed under the same circumstances did not lead to a reduction of the NDUFAF1 signal, nor to a reduction in the amount of fully assembled complex I (data not shown). This analysis shows that knockdown of NDUFAF1 protein leads to a decrease in the amount and activity of fully assembled complex I. Conversely, preliminary evidence suggests that overexpression of NDUFAF1 leads to an increase in the expression of complex I (data not shown).

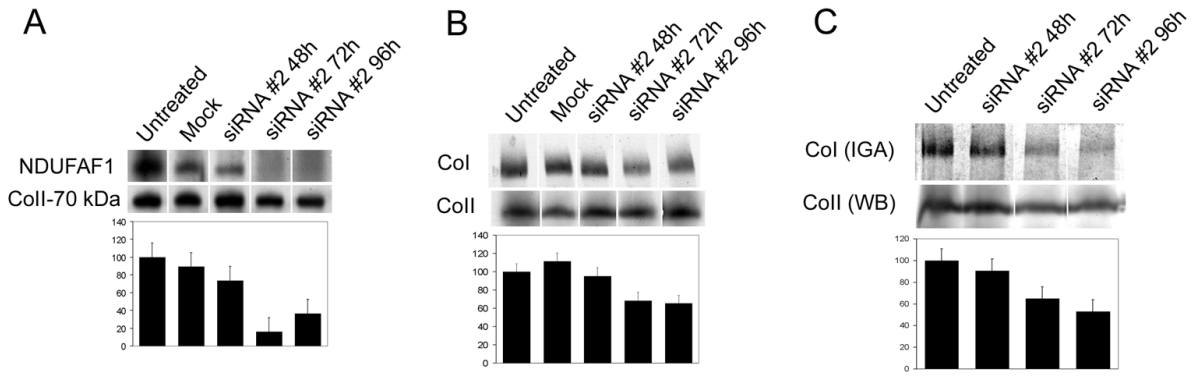


Figure 4: RNA interference of NDUFAF1 in HeLa cells

A, B and E, HeLa cells were transiently transfected twice for 48 and 48 hours, 48 and 72 hours, and 48 and 96 hours, respectively. Quantification of the signals is represented in the histograms below each panel. Each bar corresponds to the lane above it, representing the percentage of signal compared to the "untreated" signal, corrected for the loading control signal (CoII). A, Western blot of SDS-PAGE gel. B, Western blot of blue-native gel. C, In-gel activity assay. For these panels: Lane 1, untreated cells. Lane 2, mock transfection (no siRNA). Lane 3, transfection with NDUFAF1 siRNA #2 for 48 and 48 hours, consecutively. Lane 4, transfection with NDUFAF1 siRNA #2 for 48 and 72 hours, consecutively. Lane 5, transfection with NDUFAF1 siRNA #2 for 48 and 96 hours, consecutively. Antibodies used are against NDUFAF1, NDUFA9 (complex I) and CoII-70 kDa (complex II, loading control). In-gel activity results are indicated with IGA, Western blot results are indicated with WB.

NDUFAF1 is present in two high molecular weight protein complexes

To investigate whether NDUFAF1 is present in high molecular weight protein complexes, its expression pattern was analysed on Western blots of two-dimensional blue-native/SDS gels (2D BN/SDS-PAGE). Control HEK293 cells were harvested at 90% confluency and lysates of digitonin-isolated mitochondria were run on 2D BN/SDS-PAGE gels before blotting and antibody detection (Fig. 5). Two NDUFAF1-containing high molecular weight complexes of about 600 and 700 kDa can be observed (Fig. 5, complexes 2 and 1, respectively). As a size reference, complex I (1 MDa) is shown by using an antibody against the NDUFA9 (39 kDa) subunit of complex I. The expression pattern of NDUFAF1 when it is overexpressed was analyzed by using the doxycycline inducible expression system in combination with 2D BN/SDS-PAGE gels. After 4 hours of induction (Fig. 6), induced NDUFAF1 can be seen to migrate from its monomeric form towards the complexes of 600 and 700 kDa, confirming the data observed for endogenous NDUFAF1 in the control situation.

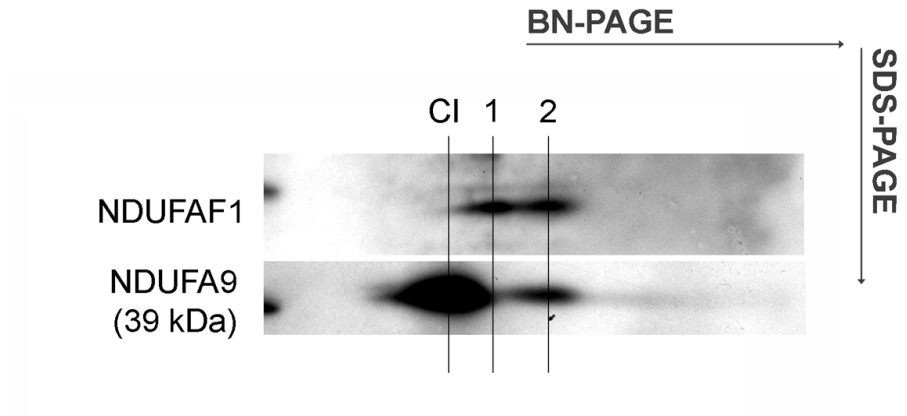


Figure 5: 2D BN/SDS-PAGE expression analysis of a mitochondrial protein extract from HEK293 cells

Forty µg protein per lane was loaded on a 5-15% blue-native gel (1st dimension) followed by 2nd dimension separation using 10% SDS-PAGE gels and Western blotting. Antibodies used are against NDUFAF1 and NDUFA9 (39 kDa), indicated at the left of each panel. CI refers to fully assembled complex I (complex A in (Ugalde et al., 2004)). Numbers 1 and 2 refer to NDUFAF1 complexes of 700 and 600 kDa, respectively.

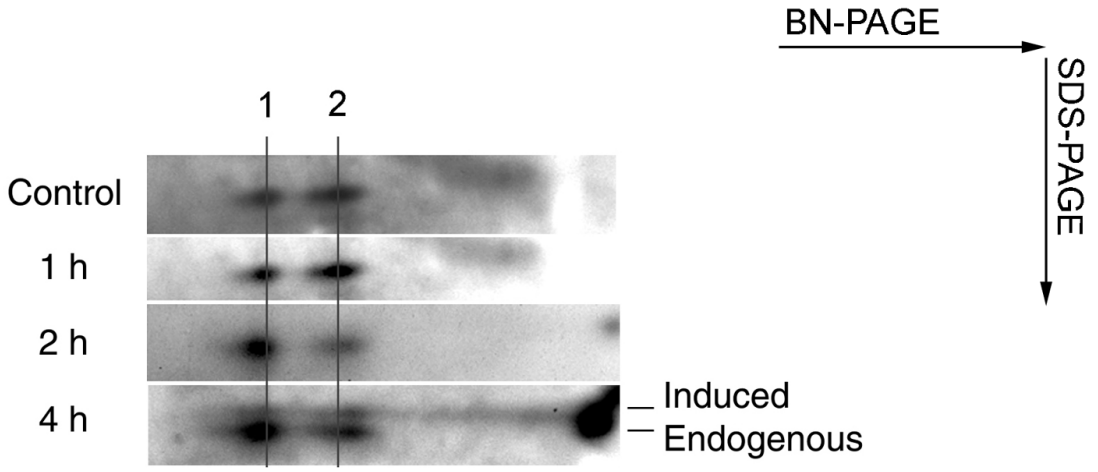


Figure 6: 2D BN/SDS-PAGE expression analysis of mitochondrial protein extracts from HEK293 control cells and a NDUFAF1 inducible HEK293 clone

The inducible clone was induced for NDUFAF1 expression for 1, 2 and 4 hours with 1 µg /ml of doxycycline. 40 µg protein per lane was loaded on a 5-15% blue-native gel (1st dimension) followed by 2nd dimension separation using 10% SDS-PAGE gels and Western blotting. Anti NDUFAF1 antibody was used for immunoblot detection. NDUFAF1 containing intermediates are indicated with 1 (700 kDa) and 2 (600 kDa). Induced and endogenously expressed NDUFAF1 are indicated on the right with "induced" and "endogenous", respectively to differentiate between the endogenous protein and the slightly larger tagged induced protein.

NDUFAF1 expression pattern changes in patients with mutations leading to complex I assembly defects

Our complex I assembly model (Ugalde et al., 2004) confirms the separate assembly of membrane and peripheral arms described in previous assembly models in *N. crassa.* A clue about the stage at which NDUFAF1 may operate in this human model can be obtained by screening for the NDUFAF1 expression pattern in complex I deficient patients. For this study, we have used fibroblasts from a NDUFS8 (TYKY) patient (manuscript in preparation) and a ND5 patient cybrid cell line with 90% of heteroplasmy (D393G, see (Corona et al., 2001)) as representatives of both a peripheral arm and a membrane arm subunit mutation. Cells were harvested to obtain mitochondrial lysates which were run on 2D BN/SDS-PAGE gels for Western blotting and antibody incubation (Fig. 7). The NDUFS8 (TYKY) patient cell line is represented in Fig. 7A and the ND5 cybrid cell line is represented in Fig. 7B.

For both patients, complex I expression analysis using NDUFA9 (39 kDa) antibody reveals a decrease in the amount of holo-complex I (represented by A, see reference 14 for detailed description of the composition of complex I assembly intermediates). Less NDUFAF1 is observed in the control fibroblast cell line used for the NDUFS8 (TYKY) patient compared to the cybrid control cell line, and the 700 kDa complex seems absent (Fig. 7A, NDUFAF1 panels). This difference in expression intensity is observed more often and appears to be cell type dependent (Ugalde et al., 2004). Irrespective of this, what can clearly be seen is the strong increase of NDUFAF1 in the 600 kDa complex in the patient compared to the control. So, interestingly, complex I and its assembly intermediates are less abundant whereas more NDUFAF1 is present in the 600 kDa complex.

Additionally, the ND5 patient displays a different relative distribution of NDUFAF1 between the 600 and 700 kDa complexes compared to the control (Fig. 7A, complexes 2 and 1). NDUFAF1 is more prominently present at 700 kDa in the 90% cybrid compared to the control. Since the observed effects are the consequence of complex I membrane arm subunit mutation, this allows the possibility that NDUFAF1 is involved in membrane arm assembly.

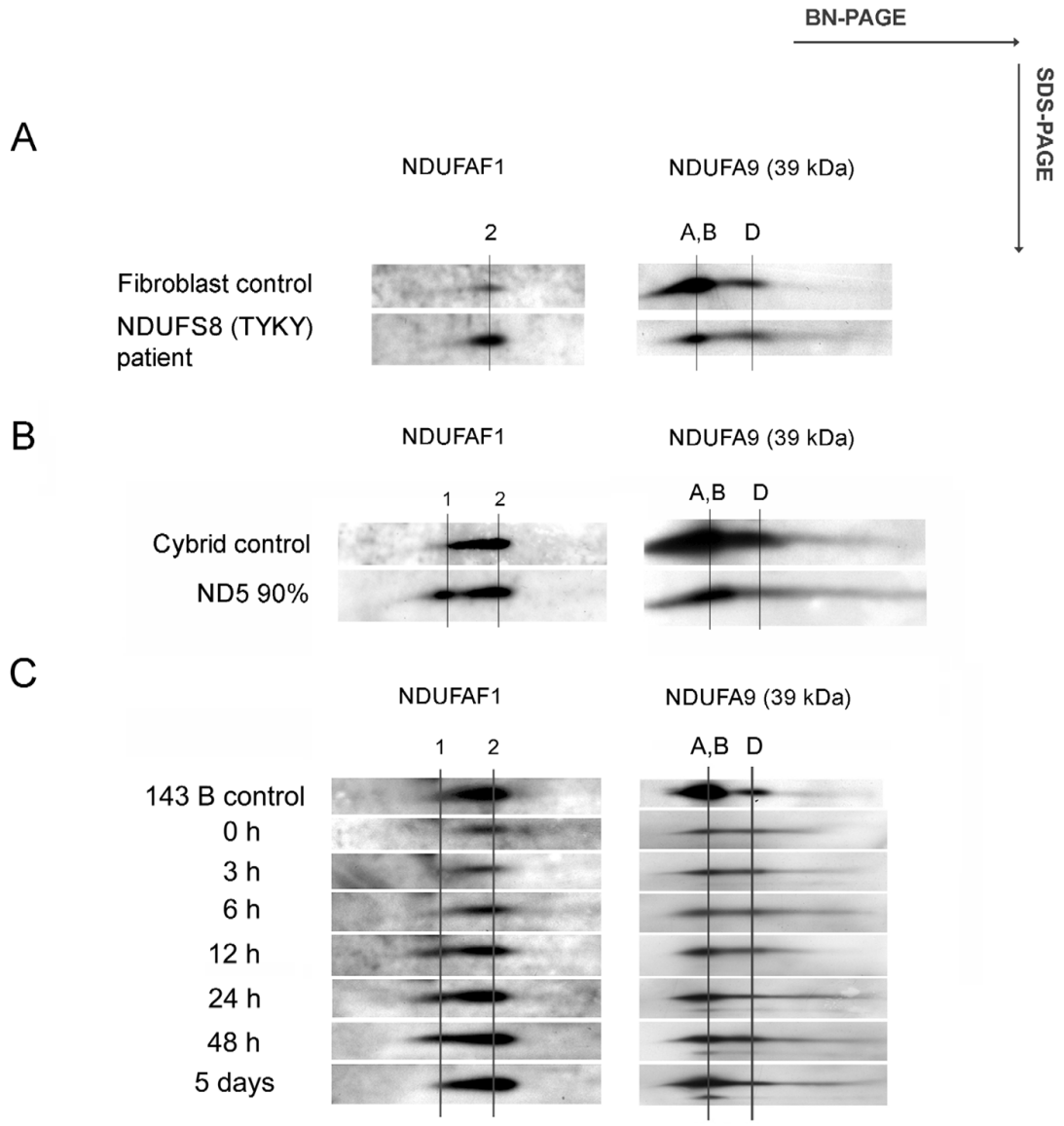


Figure 7: NDUFAF1 expression in two complex I deficient patients (panels A and B) and in a conditional complex I assembly system (panel C)

Mitochondrial pellets were solubilised and 40 µg of protein was loaded onto a 5-15% BN gel. Expression profiles were analysed by 2D BN/SDS-PAGE, Western blotting and antibody incubation. Antibodies used are against NDUFAF1 and NDUFA9 (39 kDa). Assembly stages described in ref. 14 are indicated at the top by A, B and D. NDUFAF1 containing complexes are indicated by 1 (700 kDa) and 2 (600 kDa). Panel A: expression analysis of control 143B osteosarcoma cells and a complex I peripheral arm subunit patient cell line (NDUFS8 mutation, manuscript in preparation)). Panel B: expression analysis of control cybrids (cybrid control) and a complex I membrane arm subunit patient cell line (ND5 mutation D393G (see (Corona et al., 2001)) with heteroplasmy level of 90%. Panel C: expression analysis of mitochondrial protein extracts from 143B osteosarcoma cells inhibited for mitochondrial protein synthesis by treatment with 15 µg/ml of doxycycline for 5 days. Samples were taken at 0, 3, 6, 12, 24, 48 hours and 5 days after release of doxycycline inhibition. Control (143B osteosarcoma cells) panel is at the top.

NDUFAF1 expression pattern changes in a conditional complex I assembly system

To further investigate this, we used a conditional complex I assembly system. A high concentration (10 µg/ml or more) of doxycycline results in the inhibition of mitochondrial protein synthesis. Releasing this inhibition allows investigation of the complex I assembly process and has recently been used in our group to establish a human complex I assembly model (Ugalde et al., 2004). Using this system in combination with 2D BN/SDS-PAGE analysis of NDUFAF1 expression allows investigation of the possible involvement of NDUFAF1 with complex I membrane arm assembly intermediates (Fig. 7C). The ratio of the 600 and 700 kDa complexes (Fig. 7C, complexes 2 and 1 respectively), shows a remarkable change after the resumption of mitochondrial protein synthesis. In control 143B osteosarcoma cells, NDUFAF1 is predominantly present in the 600 kDa complex (Fig. 7C, complex 2). After five days of inhibition of mitochondrial protein synthesis (Fig. 7C, t = 0 hours), a minor amount of NDUFAF1 is still present in this complex. At 12 hours after release of inhibition, NDUFAF1 becomes more predominant in the 700 kDa complex (Fig. 7C, complex 1) up to 48 hours after release of inhibition. After this time, the amount of NDUFAF1 in the 700 kDa complex decreases and finally returns to the wild-type state in the 600 kDa complex after five days. It seems that NDUFAF1 appears in the 700 kDa complex while complex I assembly proceeds and releases when the control complex I amount is assembled (Fig. 7C, complex A). These kinetics are not displayed for the 600 kDa NDUFAF1 complex, which is still present after 5 days of doxycycline inhibition and increases gradually while complex I assembly proceeds (Fig. 7C, complex 2). The expression kinetics described above support the notion that when complex I membrane arm assembly is either disturbed or induced, NDUFAF1 becomes more abundant in the 700 kDa complex.

Discussion

Studying complex I assembly factors will not only aid the development of an accurate assembly pathway but will also contribute to the elucidation of the molecular mechanism responsible for many of the genetically unexplained complex I deficiencies. Analogous to the numerous complex III and IV assembly proteins which are not part of the structural framework of these complexes, it is to be expected that such proteins also exist for complex I. However, so far only two candidate complex I assembly factors were found in the fungus *Neurospora crassa*: CIA30 and CIA84 (Kuffner et al., 1998). The human homologue of CIA30, named NDUFAF1, could be identified (Janssen et al., 2002), but has remained unstudied since its discovery.

The mitochondrial localization of NDUFAF1 shown by cellular fractionation (Fig. 1) and immunofluorescence microscopy (Fig. 3) is consistent with the *in silico* prediction of an N-terminal mitochondrial targeting sequence and the distribution pattern of expression in different tissues (Janssen et al., 2002).

Our findings support the role of NDUFAF1 in the regulation of assembly/stability of human complex I. Firstly, the knockdown of NDUFAF1 expression by RNA interference shows that the protein is required for correct complex I assembly/stabilisation (Fig. 4). After knockdown of NDUFAF1, the amount of fully assembled complex I is reduced and enzymatic activity is impaired. Surprisingly, no accumulation of assembly intermediates is observed in the NDUFAF1 RNAi experiments on 2D BN/SDS-PAGE gels, suggesting that NDUFAF1 may be involved in the stabilisation of these intermediates rather than in active combination of assembly intermediates (unpublished results). Secondly, prolonged overexpression of NDUFAF1 in HEK293 cells leads to an increased amount of fully assembled complex I.

NDUFAF1 occurs in two high molecular weight protein complexes of 600 and 700 kDa. Its expression in two complex I deficient patient cell lines differs greatly when either membrane arm or peripheral arm assembly is compromised by mutation (Fig. 7A and B). Mutation in membrane subunit ND5 results in a relative increase of the 700 kDa NDUFAF1 complex very similar to the shift in the conditional assembly system. Peripheral arm subunit NDUFS8 mutation results in a completely different expression profile. While in this patient complex I and its assembly intermediates are diminished, more NDUFAF1 is present at 600 kDa. This indicates that despite

the co-migration of this complex with peripheral arm assembly intermediate D (see (Ugalde et al., 2004)) they are different complexes.

Additional support for the involvement of NDUFAF1 in complex I assembly/stability comes from analysis of its 2D BN/SDS assembly profiles in a conditional complex I assembly system (Fig. 7C). Absence of NDUFAF1 from the 700 kDa complex after inhibition of assembly shows that association of NDUFAF1 to the complex is hampered. Since after doxycycline inhibition of mitochondrial protein synthesis no mitochondrial protein can be produced, this suggests that NDUFAF1 is bound to a mitochondrially encoded protein. The transient shift of NDUFAF1 to the 700 kDa complex after assembly resumes, suggests that NDUFAF1 is required in this complex while assembly proceeds. This is supported by the fact that when after five days the complex I amount is restored, the amount of associated NDUFAF1 in this complex is also reduced to control levels. The 700 kDa NDUFAF1 complex may therefore represent an important step in the process of complex I assembly.

The model for complex I assembly in humans proposed by our group supports the idea that complex I assembly occurs in a modular fashion, and is largely compatible with the *N. crassa* model for complex I assembly (Ugalde et al., 2004). In this system, complex I is assembled via the combination of pre-assembled evolutionary conserved modules like the bricks of a lego system. In more detail, this entails that the membrane arm appears to be assembled in several steps by combining a small and large intermediate. The peripheral arm is assembled independently and is joined to the complete membrane arm in a later stage. Based on the results presented in this paper, we propose that NDUFAF1 modulates this process. An active role in assembly may serve to ease the combination of assembly intermediates while, alternatively, a stabilising/scaffolding role may serve to prevent misfolding or degradation of assembly intermediates.

This function for NDUFAF1 is in line with the proposed mechanism described for the *N. crassa* homologue CIA30 (Kuffner et al., 1998). In this study CIA30 was suggested to aid the combination of the small and large membrane arm intermediates in complex I assembly by exclusive binding to the large membrane arm intermediate (Schulte, 2001; Duarte and Videira, 2000). Knockout of the *cia* genes in *N. crassa* resulted in a block in complex I assembly, characterised by the absence of the large membrane arm intermediate and the accumulation of the small membrane arm intermediate and the peripheral arm (Kuffner et al., 1998). The acquired data do not conflict with this idea. Both disturbance of membrane arm

assembly (ND5 cybrid) and pressurising the assembly system by releasing doxycycline inhibition of mitochondrial translation result in accumulation of the 700 kDa NDUFAF1 complex. However, to ascertain direct involvement in complex I membrane arm assembly, the exact composition of the NDUFAF1 intermediates is a prerequisite. In addition, the observed changes in NDUFAF1 assembly status in complex I deficient patients can be indicative for the possible gene defect and we are currently investigating more patients to test this.

It is quite conceivable that, analogous to NDUFAF1, many more proteins are involved in the stabilization of complex I assembly intermediates. A recent example is the possible function of apoptosis-inducing factor (AIF) in intra-mitochondrial assembly/maintenance of respiratory chain complexes in a mouse model system (Vahsen et al., 2004). Future investigation of the exact composition of complex I assembly intermediates will be a great step forward in studying the function of NDUFAF1 and finding new complex I assembly chaperones.

Acknowledgements

We thank Dr. A. Lombes (Inserm, Paris, France) and Professor R. Capaldi (University of Oregon, Eugene, USA) for providing, respectively, the anti-ND1 and anti-ND6 antibodies. This work was supported by 'Het Prinses Beatrix Fonds' to J.S. and B. vdH. (grant number 02-0104) and the European Community's sixth Framework Programme for Research, Priority 1 "Life sciences, genomics and biotechnology for health" (contract number LSHMCT-2004-503116). The Netherlands Organisation for Scientific Research supported L.N. with a 'Vernieuwingsimpuls' grant. CU is recipient of a research contract from Instituto de Salud Carlos III (ISC III CP04/00011).

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Chapter 6

Investigation of the complex I assembly chaperones B17.2L and NDUFAF1 in a cohort of CI deficient patients

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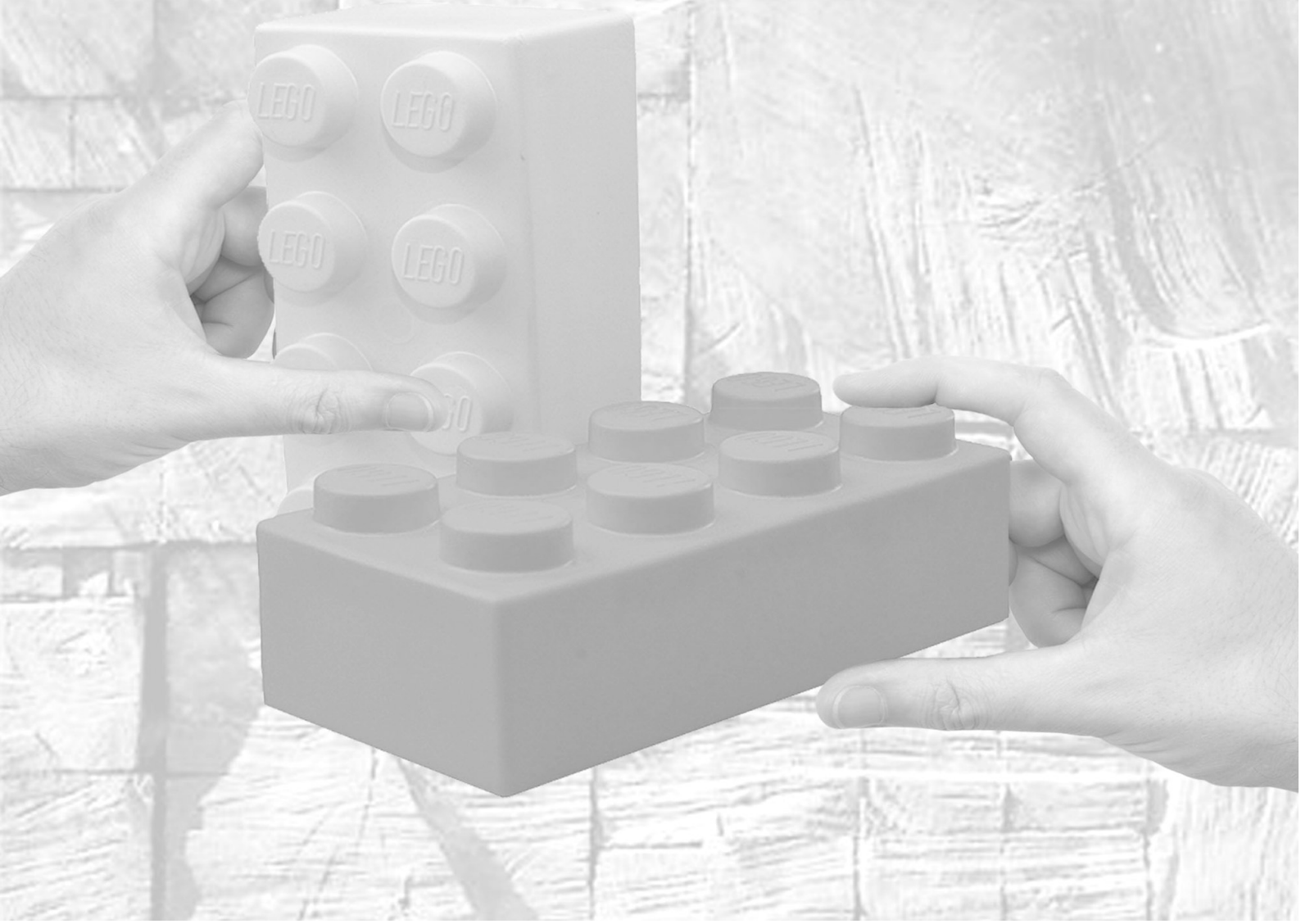
Molecular Genetics and Metabolism 2007; 91(2):176-182



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Abstract

Dysfunction of complex I (NADH:ubiquinone oxidoreductase; CI), the largest enzyme of the oxidative phosphorylation (OXPHOS) system, often results in severe neuromuscular disorders and early childhood death. Mutations in its 7 mitochondrial and 38 nuclear DNA-encoded structural components can only partly explain these deficiencies. Recently, CI assembly chaperones NDUFAF1 and B17.2L were linked to CI deficiency, but it is still unclear by which mechanism. To better understand their requirement during assembly we have studied their presence in CI subcomplexes in a cohort of CI deficient patients using one- and two-dimensional blue-native PAGE. This analysis revealed distinct differences between their associations to subcomplexes in different patients. B17.2L occurred in a 830 kDa subcomplex specifically in patients with mutations in subunits NDUFV1 and NDUFS4. Contrasting with this seemingly specific requirement, the previously described NDUFAF1 association to 500-850 kDa intermediates did not appear to be related to the nature and severity of the CI assembly defect. Surprisingly, even in the absence of assembly intermediates in a patient harboring a mutation in translation elongation factor G1 (EFG1), NDUFAF1 remained associated to the 500-850 kDa subcomplexes. These findings illustrate the difference in mechanism between B17.2L and NDUFAF1 and suggest that the involvement of NDUFAF1 in the assembly process could be indirect rather than direct via the binding to assembly intermediates.

Introduction

The importance of energy carrier ATP in each of our cells is inversely demonstrated in cases of mitochondrial disorders, which occur with an incidence of 1:5.000 newborns and display a broad clinical variety including Leigh syndrome, cardiomyopathy and encephalomyopathy (Smeitink et al., 2006). The most frequent cause of such disorders is deficiency of complex I (CI, NADH:ubiquinone oxidoreductase, E.C.1.6.5.3) (Janssen et al., 2006). This complex is the first of five complexes (CI-CV) that constitute the oxidative phosphorylation (OXPHOS) system, responsible for the generation of mitochondrial ATP.

In all cases studied thus far, deficient CI activity was associated with a decrease in the amount of intact CI signifying that assembly or stability of this enzyme was affected. CI assembly is an intricate process, which involves 45 subunit proteins, one FMN and eight iron-sulfur clusters (Carroll et al., 2003; Carroll et al., 2006). To date, two models have been published, both established on the basis of blue-native analyses (Antonicka et al., 2003; Coenen et al., 2004). The starting point in human CI assembly seems to be a core of highly conserved subunits that is anchored to the mitochondrial inner membrane and expanded with additional subunits (Vogel et al., 2007). This assembly mechanism at some points reflects the theoretical proposals that state that certain groups of subunits have co-evolved as distinct substructures (Friedrich and Weiss, 1997; Finel, 1998; Friedrich and Scheide, 2000; Mathiesen and Hagerhall, 2003). The proposed modules are a dehydrogenase module, which oxidizes NADH, a hydrogenase module, which transports electrons to the proton translocation module, which transports protons across the mitochondrial inner membrane. Combination of (parts of) these substructures is proposed to result in the assembly of holo-CI (Ugalde et al., 2004b; Vogel et al., 2004).

By analogy to the other OXPHOS complexes, CI assembly must involve the action of assembly chaperones. The recent addition of B17.2L (previously termed mimitin (Tsuneoka et al., 2005)), a paralogue of a CI subunit which is not incorporated into CI but is vital for its assembly, to the previously described assembly factor NDUFAF1 brings the number of CI specific assembly factors to two (Janssen et al., 2002; Vogel et al., 2005; Ogilvie et al., 2005). Recently, both chaperones have been linked to CI deficiency. A *B17.2L* gene null mutation resulted in less than 20% CI activity and concomitant early-onset progressing encephalopathy with vanishing

white matter (Ogilvie et al., 2005). Two novel heterozygous mutations in the *NDUFAF1* gene were described by Sugiana and colleagues, which resulted in a marked reduction in NDUFAF1 protein level in fibroblasts and EBV-lymphoblasts and clinically in cardiomyopathy, developmental delay and lactic acidosis (Sugiana et al., 2006).

In contrast to the assembly chaperones described for CIV, which play a role in the incorporation of prosthetic groups (e.g. COX10, COX11, Sco1, Sco2) or the maturation and membrane insertion of subunits encoded by mitochondrial DNA (e.g. Surf1 and Oxa1), the molecular role of the CI chaperones is still not clear (Yi and Dalbey, 2005; Herrmann and Funes, 2005; Pecina et al., 2004). Better understanding of the specific involvement of B17.2L and NDUFAF1 in the assembly process aids the identification of important stages in the assembly process and adds to the insight into their mechanism of action. For these purposes, we have investigated the presence of these chaperones in specific subcomplexes for a cohort of patients displaying various CI assembly disturbances.

Materials and methods

Patients and mutations

Table 1 lists the patient and cybrid cell lines investigated in this study (next page).

Table 1: Patients and mutations

Listed are the investigated control, cybrid and patient cell lines used in this study. Indicated are which gene harbors which mutation, the amount of heteroplasmy in the case of mtDNA mutation, CI activity relative to either CIV (COX) or citrate synthase, the activities of the other OXPHOS complexes and the references in which the mutations were previously described.

Cell line	Assembly group	Affected gene	Mutation	Heteropl. (%)	CI activity (%)	Other activities (%)	Previously described in
Control (C)	-	-	-	-	-	-	-
Patient 1	Early matrix	NDUFS2	R228Q	-	39a	All normal	(Loeffen et al., 2001)
Patient 2	Early matrix	NDUFS7	V122M	-	68a	All normal	(Triepels et al., 1999)
Patient 3	Early matrix	NDUFS8	R94C	-	18a	CIII 88a, CIV 229b	(Visch et al., 2006)
Patient 4	Late matrix	NDUFV1	R29X, T423M	-	64a	All normal	(Schuelke et al., 1999; Visch et al., 2006)
Patient 5	Late matrix	NDUFS4	K158fs (5-bp duplication)		-	75a	CIII 98a
Patient 6	Late matrix	NDUFS4	R106X	-	36a/30b	CIII 100a/88b	(Budde et al., 2003)
Patient 7	Late matrix	NDUFS4	VPEEHI67/VEKSIstop		-	53a	All normal
Patient 8	Membrane	ND3	S43P	75%	54a	All normal	Manuscript in preparation
Cybrid control (CC)	-	-	-	-	-	All normal	This publication
ND1 cybrid (9)	Membrane	ND1	A52T	100%	9b	All normal	This publication

A COX normalization B Citrate synthase normalization

Cell culture

Fibroblast cells were cultured in M199 medium (Life Technologies) supplemented with 10% fetal calf serum (v/v) and penicillin/streptomycin. Cells were harvested by trypsinisation, resuspended in PBS (Gibco) and solubilised using a final concentration of 2% digitonin (Calbiochem) for 10 minutes on ice. After centrifugation (10 minutes, 10 000 g, 4°C), the mitochondria enriched pellet was washed twice in PBS prior to its solubilisation by 10 minutes of incubation on ice using 2 % of n-Dodecyl β-D-maltoside (DDM) (Sigma-Aldrich) in solubilisation buffer (1.75 M 6-aminocaproic acid (Fluka), 75 mM bis-tris HCl (Fluka), pH 7.0). The protein concentration was determined using the MicroBCA protein assay kit (Pierce) and samples were processed for blue-native gel electrophoresis.

SDS-PAGE and BN-PAGE protein analysis and immunodetection

SDS-PAGE analysis was performed as described in (Ugalde et al., 2004b), using 10% SDS and loading 40 µg of mitochondrial protein per lane. One- and two-dimensional BN-PAGE was performed as described in (Klement et al., 1995; Nijtmans et al., 2002), using 5-15% gels and loading 80 µg of mitochondrial protein per lane. After Western blotting, proteins were detected using antibodies raised against NDUFAF1 (Vogel et al., 2005), the NDUFS3 (Invitrogen), NDUFA6 (Invitrogen), NDUFA9 (Invitrogen) and ND1 (Dr. A. Lombes (Inserm, Paris, France)) subunits of CI and B17.2L (kindly provided by Professor M. Tsuneoka (Kurume University School of Medicine, Japan)). Secondary antibodies used peroxidase-conjugated anti-mouse or anti-rabbit IgGs (Invitrogen). The signal was obtained using ECL® plus (Amersham Biosciences).

Results

CI activity and assembly

All studied patient cell lines exhibit a specifically impaired CI activity. Based on the structural relationship of the affected subunits during assembly, we have categorized the investigated cell lines into three “assembly groups”. Cell lines 1-3 (harboring mutations in NDUFS2, NDUFS7 and NDUFS8) form the “early matrix” group, as these affected subunits are proposed to be part of an early matrix arm assembly module (Ugalde et al., 2004b). Cell lines 4-7 (harboring mutations in NDUFV1 and NDUFS4) form the “late matrix” group, as these affected subunits are

proposed to be incorporated after a nuclear preassembly has been formed (Ugalde et al., 2004b). Finally, cell lines 8 and 9 (harboring mutations in ND3 and ND1) form the “membrane” group, as these affected subunits are part of the CI membrane arm.

In order to verify CI activity and subsequently investigate assembly, mitochondrial lysates were analyzed on blue-native gels for CI in-gel activity (IGA) assay and Western blotting (WB) followed by CI immunodetection of CI (figure 1A, IGA and WB). In general, the decrease in CI activity (figure 1A, IGA) matched the decrease in amount (figure 1A, WB). As judged from the CII signal, less protein was loaded in lanes 1 and 4, resulting in a slight underestimation of the amount of CI (figure 1A, lanes 1 and 4). For the “late matrix”” patients, the IGA does not correspond to the enzymatic measurements (materials and methods section). As IGA measurement assays the functionality of the dehydrogenase module of CI, it is very well possible that this “late assembly” module is unstable upon solubilisation for BN and subsequent IGA analysis but not during the enzymatic measurement.

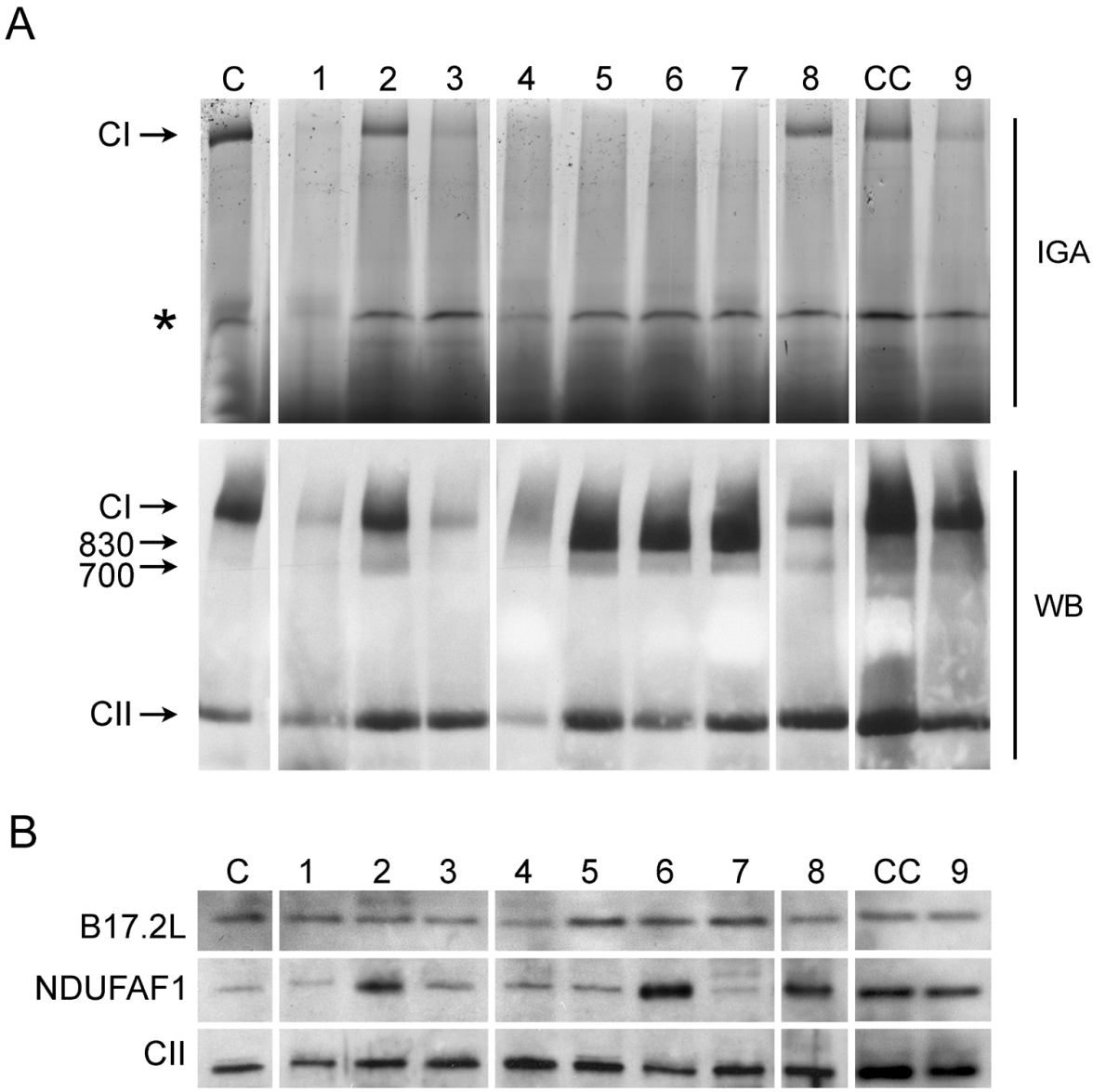

Figure 1: One-dimensional blue-native analysis of CI activity and assembly

(A) Analyzed for a cohort of CI deficient patients are CI activity via in-gel activity assay on blue-native gel (top panel) and CI assembly via immunodetection using an ND1 antibody on Western blot of a duplicate gel (bottom panel). "C": fibroblast control. "CC": cybrid control. "CI": complex I. An additional in-gel active low molecular weight band is indicated with an asterisk. On Western blot, the 830 kDa subcomplex often observed for NDUFS4 and NDUFV1 patients is indicated with "830" and a subcomplex present for most of the lanes is indicated at 700 kDa with "700". The CII signal was used as a loading control and is indicated with "CII". Samples C and 1-8 were run on one gel, CC and 9 on another. (B) Mitochondrial expression levels of assembly chaperones B17.2L and NDUFAF1 and of loading control CII were determined by SDS-PAGE followed by Western immunodetection using ECL and exposure to film. "C": fibroblast control. "CC": cybrid control. Again, samples C and 1-8 were run on one gel, CC and 9 on another.

The “early matrix” (lanes 1-3) and “membrane” (lanes 8, 9) patients displayed a variable CI amount and activity but no clear subcomplexes, except for a signal of about 700 kDa (figure 1A, ‘700’) that appeared in all lanes. In contrast, the “late matrix” group (NDUFV1 and NDUFS4, lanes 4-7) displayed a large subcomplex of about 850 kDa. Because this subcomplex almost certainly represents the 830 kDa assembly intermediate frequently observed for NDUFS4 and NDUFV1 patients (Ugalde et al., 2004a; Scacco et al., 2003; Ogilvie et al., 2005), it is indicated “830”.

Amounts of chaperones B17.2L and NDUFAF1

The amounts of the chaperones B17.2L and NDUFAF1 were assayed by Western detection after SDS-PAGE in figure 1B.

B17.2L - As can be seen in figure 1B, in the “late matrix” group, levels of B17.2L may be slightly elevated (cell lines 5, 6 and 7). No changes are observed for the other cell lines.

NDUFAF1 - NDUFAF1 levels are increased in cell lines 2, 6 and 8, and possibly slightly decreased in cell line 7 (figure 1B). In the other cell lines, NDUFAF1 levels are relatively comparable to the control level.

Two-dimensional BN SDS-PAGE analysis of CI assembly

SDS analysis of chaperone amounts only gives a partial impression of the effects of the different CI mutations on the involvement of the chaperones. Another aspect is the association of B17.2L and NDUFAF1 to subcomplexes, which was assayed by one-dimensional blue-native PAGE (as done for figure 1A) followed by two-dimensional SDS-PAGE, Western blotting and immunodetection. The presence of CI subcomplexes was assayed in figure 2A by immunodetections of CI peripheral arm subunit NDUFS3 and membrane arm subunit ND1. In all cell lines, three smaller previously described NDUFS3 subcomplexes (indicated with F, G and H) and the 700 kDa ND1 subcomplex (indicated with C) were observed with variable intensity (a previous description of these intermediates is given in (Ugalde et al., 2004b)). As expected, the ND1 signal was very low for the ND1 cybrid cell line (figure 2A, cell line 9), but the presence of NDUFS3 in fully assembled CI (figure 2A, cell line 9, “A”) illustrated that this minor amount is sufficient to allow CI assembly. In general, no patient specific accumulation of CI subcomplexes was observed except for the clear presence of the 830 kDa subcomplex in the “late matrix” group.

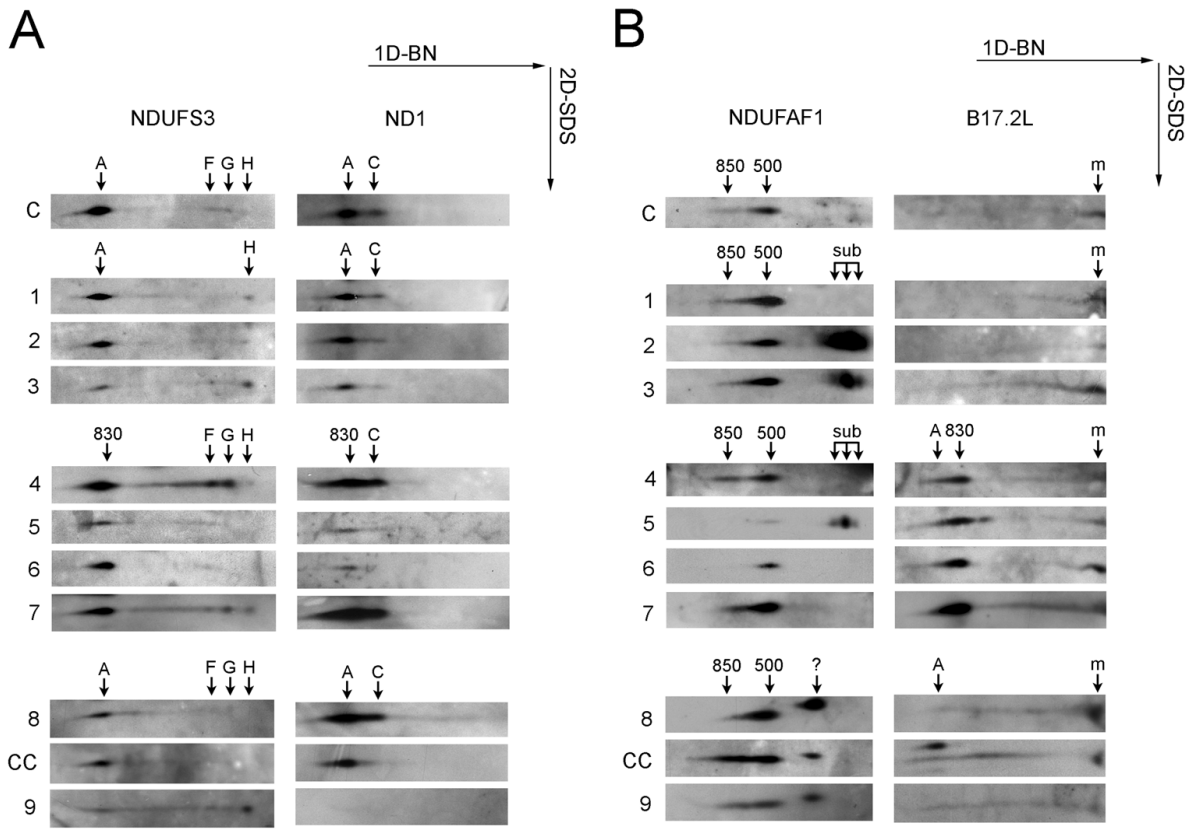

Figure 2: Two-dimensional SDS-PAGE analysis of CI assembly

Two-dimensional immunodetection of CI subunits NDUFS3 and ND1 (A) and assembly chaperones B17.2L and NDUFAF1 (B) is shown for each of the lanes from figure 1. For each panel, large protein complexes are to the left and smaller protein complexes to the right. Observed subcomplexes are indicated with arrows (indicated as in (Ugalde et al., 2004b)), CI is indicated with "A". The CI signal observed after B17.2L antibody incubation in the cybrid control cell line (panel CC) originates from a previous NDUFS3 antibody incubation.

High-molecular weight associations of chaperones B17.2L and NDUFAF1

Subsequently, high-molecular weight associations of NDUFAF1 and B17.2L were investigated by immunodetection shown in figure 2B.

B17.2L - Most apparent was the specific detection of B17.2L in the 830 kDa complex in the "late matrix" group (figure 2B, B17.2L, cell lines 4-7, indicated with '830'), whereas this complex was undetectable for the control and other patient cell lines. In those cases, the B17.2L protein migrated only at the front of the separating gradient, probably as a monomer (figure 2B, indicated with 'm'). In the "membrane" group, B17.2L was detected as a smear. These data show that

B17.2L is specifically associated to a high-molecular weight complex of 830 kDa in the NDUFV1 and three NDUFS4 patient cell lines.

NDUFAF1 - NDUFAF1 did not show dramatic differences in its association to the 500-850 kDa subcomplexes in the "early matrix" group (cell lines 1-3), whereas it did show association with previously observed smaller molecular weight complexes for one NDUFS4 patient and the NDUFS7 and NDUFS8 patients (panels NDUFAF1 for cell lines 2, 3 and 5) in the range of 100-150 kDa. The distribution of these three spots was similar for these three patients, suggesting that they are similar subcomplexes. Their origin remains enigmatic but they may represent early NDUFAF1 associations, a topic that requires further investigation. The "late matrix" group (cell lines 4-7) displayed variable NDUFAF1 abundance and distribution, and the "membrane" group (cell lines 8, 9) showed no great changes in NDUFAF1 association to the 500-850 kDa subcomplexes.

For the ND3 patient and both cybrid cell lines (control and ND1), a spot appeared in a complex slightly smaller than the NDUFAF1 subcomplexes (panels NDUFAF1 for cell lines 8, CC and 9, indicated with a question mark), which is possibly a non-specific signal or an aggregate that contains the NDUFAF1-epitope. In summary, changes in NDUFAF1 association were observed for patient 4 (NDUFV1), which showed a relative increase in intensity of the largest (~850 kDa) NDUFAF1 subcomplex, and for patient 5 (NDUFS4), which showed a decrease in NDUFAF1 signal and additional NDUFAF1 signal at 100-150 kDa. It thus seems that the observed increase in the amount of NDUFAF1 in cell lines 2, 6 and 8 (figure 1B) does not correlate with gross changes in the association of NDUFAF1 to subcomplexes.

High molecular weight association of NDUFAF1 in a patient deficient in mitochondrial translation

The relative independent abundance of NDUFAF1 from the CI assembly status is difficult to reconcile with its specific requirement in assembly (Vogel et al., 2005). To investigate this further, we analyzed a severely CI deficient patient cell line harboring a mutation in translation elongation factor G1 (EFG1) (Coenen et al., 2004). We confirmed the (near) absence of CI by blue-native Western immunodetection (figure 3A, CI signal) and investigated whether CI subcomplexes still occur by using a panel of CI subunit antibodies (figure 3B, NDUFS3, NDUFA9 and NDUFA6). In accordance with figure 3A, CI was virtually absent and only very long exposure times allowed visualization of a minor amount of NDUFA9 at the

migrating height of CI (panel "long exposure"). Confirmative of the disturbed mitochondrial translation was the absence of CIV signal (COXII subunit) and the strong decrease in CIII (when equal exposure times are used, core2 subunit). The effect of the absence of CI assembly intermediates on the associations of B17.2L and NDUFAF1 was investigated by immunodetection of B17.2L and NDUFAF1, this time with equal film exposure times to allow direct comparison (figure 3B, B17.2L and NDUFAF1 panels). B17.2L was equally absent on control and patient Western blots. On control fibroblast Western blots, NDUFAF1 displayed wild type association to complexes in the range of 500-850 kDa. To our surprise, although the signal was less intense, NDUFAF1 immunodetection on the EFG1 patient Western blot revealed NDUFAF1 association to the same high-molecular weight complexes as observed in the control, in spite of the absence of detected assembly intermediates. This indicates the NDUFAF1 subcomplexes of 500-850 kDa may not represent binding to assembly intermediates, but to (an)other protein complex(es).

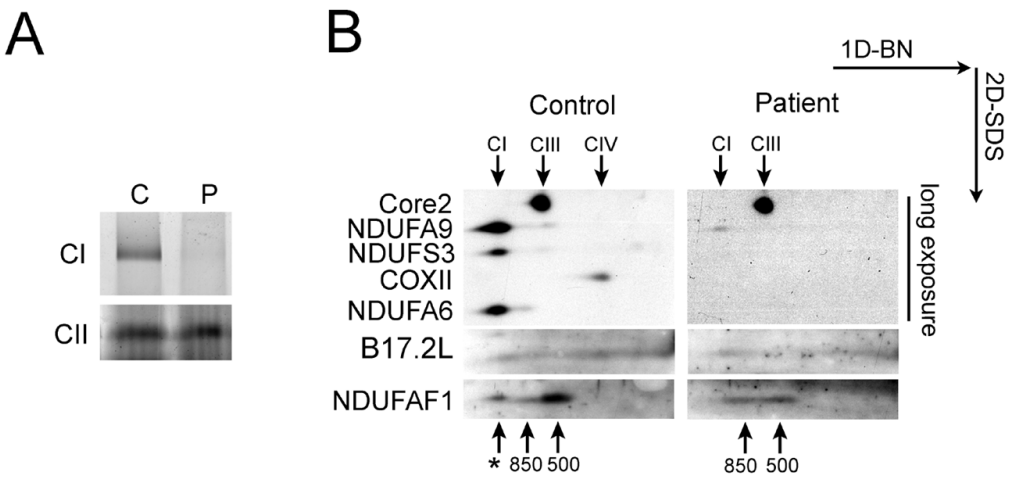

Figure 3: Blue-native analysis of CI assembly in EFG1-patient mitochondria

(A) One-dimensional blue-native Western blot analysis of CI amount using an anti-NDUFA9 antibody shown for control fibroblast mitochondria and EFG1-patient mitochondria. CII signal is shown as a loading control. (B) Two-dimensional immunodetection using a cocktail of antibodies against three CI subunits (NDUFS3, NDUFA9 and NDUFA6), CIV (COXII) and CIII (core2), and with B17.2L and NDUFAF1 antiserum, for control fibroblast mitochondria and EFG1-patient mitochondria. OXPHOS complexes I, III and IV are indicated where visible, as are the 500-850 kDa NDUFAF1 complexes. The cocktail incubation ECL signal (top panel) is exposed to film longer for the patient than for the control to demonstrate the minute presence of CI (NDUFA9 signal). This results a core2 signal much stronger than it would be for equal exposure time compared to the control. In this case, the core2 signal is severely reduced for the patient (data not shown). An asterisk indicates residual CI signal from a previous anti-NDUFA9 incubation.

Discussion

The assembly mechanism for human mitochondrial CI is far from understood. Analogous to the assembly of the other OXPHOS complexes, it is hypothesized to involve many assembly chaperones. So far, two human CI assembly factors have been identified, B17.2L (Ogilvie et al., 2005) and NDUFAF1 (Janssen et al., 2002; Vogel et al., 2005), of which mutations were only recently associated with CI deficiency (Ogilvie et al., 2005; Sugiana et al., 2006). To obtain insight under which conditions and in which assembly stages these chaperones are important, we have analyzed their high-molecular weight associations in a cohort of CI deficient patients displaying assembly disturbances of varying nature. To obtain the broadest possible overview of the involvement of the investigated chaperones in the assembly process we have chosen to include patient and cybrid cell lines with mutations in subunits present in membrane (ND1 and ND3) and in matrix arm subunits (NDUFV1, NDUFS2, NDUFS4, NDUFS7 and NDUFS8). In addition, an EFG1 patient was examined which does not display any assembly intermediates (Coenen et al., 2004).

Most apparent is the accumulation of an intermediate of about 830 kDa for the NDUFS4 patient cell lines and, to a lesser extent, for the NDUFV1 patient cell line. Finding of this large CI substructure in the three NDUFS4 and the NDUFV1 patient cell lines and the specific association of assembly chaperone B17.2L to this subcomplex, are in line with previously described data (Ogilvie et al., 2005; Ugalde et al., 2004a; Scacco et al., 2003). NDUFV1 and NDUFS4 are proposed to be incorporated late in assembly (Ugalde et al., 2004b; Antonicka et al., 2003). That different mutations in these two subunits result in the same assembly defect suggests participation of both subunits in a similar stage of the assembly process. The lack of in-gel activity of the 830 kDa complex shows that the dehydrogenase module, or at least an essential part of its functional structure, is missing in this subcomplex. It is yet unclear whether, in the absence of NDUFV1 and/or NDUFS4, B17.2L is specifically recruited to this intermediate for e.g. stabilization purposes or whether it accumulates because assembly is stalled and it does not dissociate from the intermediate.

Previously unobserved NDUFAF1 comigration with three smaller subcomplexes of 100-150 kDa is demonstrated for one of the NDUFS4 patients and especially for the NDUFS7 and NDUFS8 patients, of which the exact nature requires further investigation. In addition, the amount of the 500-850 kDa wild-type NDUFAF1

complexes does not differ strongly between different patients and the control. In two of the three NDUFS4 patients, NDUFAF1 presence in its protein complexes seems reduced, and the NDUFV1 patient shows a relative increase in intensity of the largest (~850 kDa) complex, but for the other patients no significant changes are observed. It is yet unclear why not all NDUFS4 patients show a similar decrease in NDUFAF1. Possibly, the nature of the mutation determines NDUFAF1 association to the 500-850 kDa subcomplexes, but this is yet speculative.

NDUFAF1 and B17.2L seem to play different roles in the CI assembly process. In summary, while B17.2L only seems to be recruited in specific assembly disturbances, NDUFAF1 generally appears unchanged in its association to the 500-850 kDa subcomplexes. Herewith, it seems that the presence of B17.2L in an 830 kDa subcomplex is a consistent marker for NDUFV1 and NDUFS4 mutations. On the contrary, NDUFAF1 may not be the most consistent biochemical marker for CI assembly disturbances. Even though CI and its assembly intermediates are almost completely absent, NDUFAF1 remains associated to the 500-850 kDa subcomplexes in the mitochondrial translation impaired EFG1 patient cell line (figure 3B). Also in the ND1 cybrid cell line, which hardly shows CI or its subcomplexes, NDUFAF1 remains associated to these high-molecular weight subcomplexes (figure 2B). A simple schematic of the observed difference in involvement between the two chaperones is shown in figure 4.

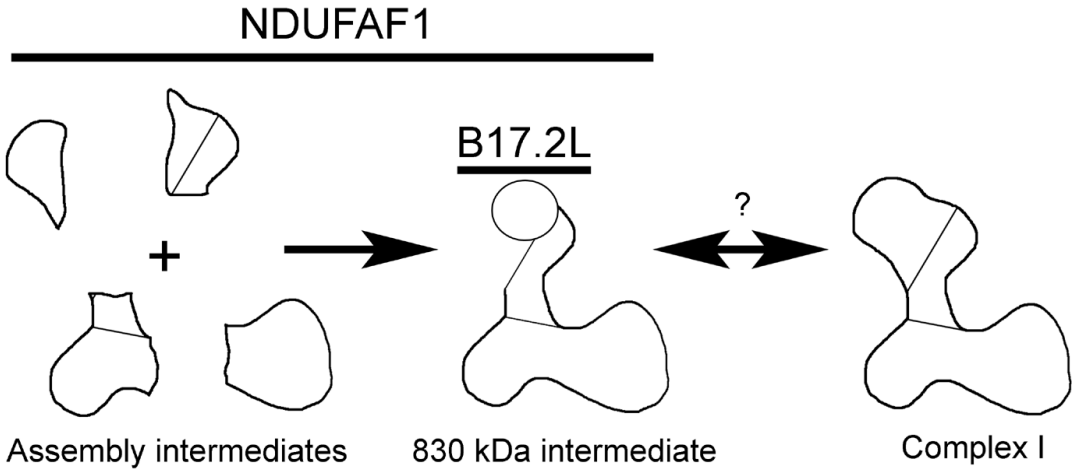

Figure 4: Different involvement of B17.2L and NDUFAF1 in assembly
This model shows the proposed difference in the involvement of B17.2L and NDUFAF1 in the assembly process of CI. B17.2L is specifically present in an 830 kDa intermediate, which originates either from assembly or CI instability (bidirectional arrow). NDUFAF1 generally shows only mild differences upon different mutations in different CI subunits, and hence may have a more general role in the assembly process.

How can the moderate change in NDUFAF1 abundance and association in the various assembly disturbances be reconciled with its absolute requirement for CI assembly (Vogel et al., 2005)? Possibly, NDUFAF1 exerts its function in CI assembly not via direct binding to assembly intermediates, but via an indirect route. Examples of such routes could be generic mitochondrial processes such as import, processing and translation, which indirectly have a great impact on OXPHOS complex assembly. Along these lines, previous investigations showed a shift in NDUFAF1 abundance in the 500-850 kDa complexes after releasing inhibition of translation, which hinted towards a role in mitochondrial translation (Vogel et al., 2005). However, evidence for this role is still circumstantial. Further research concerning the exact composition of the 500-850 kDa intermediates is required to help explain from which process they, and thus NDUFAF1, originate.

Acknowledgements

We are grateful to Dr. A. Lombes (Inserm, Paris, France) for providing the anti-ND1 antibody. This work was supported by 'Het Prinses Beatrix Fonds' to J.S. and L. v.d. H. (grant number 02-0104) and the European Community's sixth Framework Programme for Research, Priority 1 "Life sciences, genomics and biotechnology for health" (contract number LSHMCT-2004-503116). The Netherlands Organization for Scientific Research supported L.N. with a 'Vernieuwingsimpuls' grant.

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Chapter 7

Cytosolic signaling protein Ecsit also localizes to mitochondria where it interacts with chaperone NDUFAF1 and functions in complex I assembly

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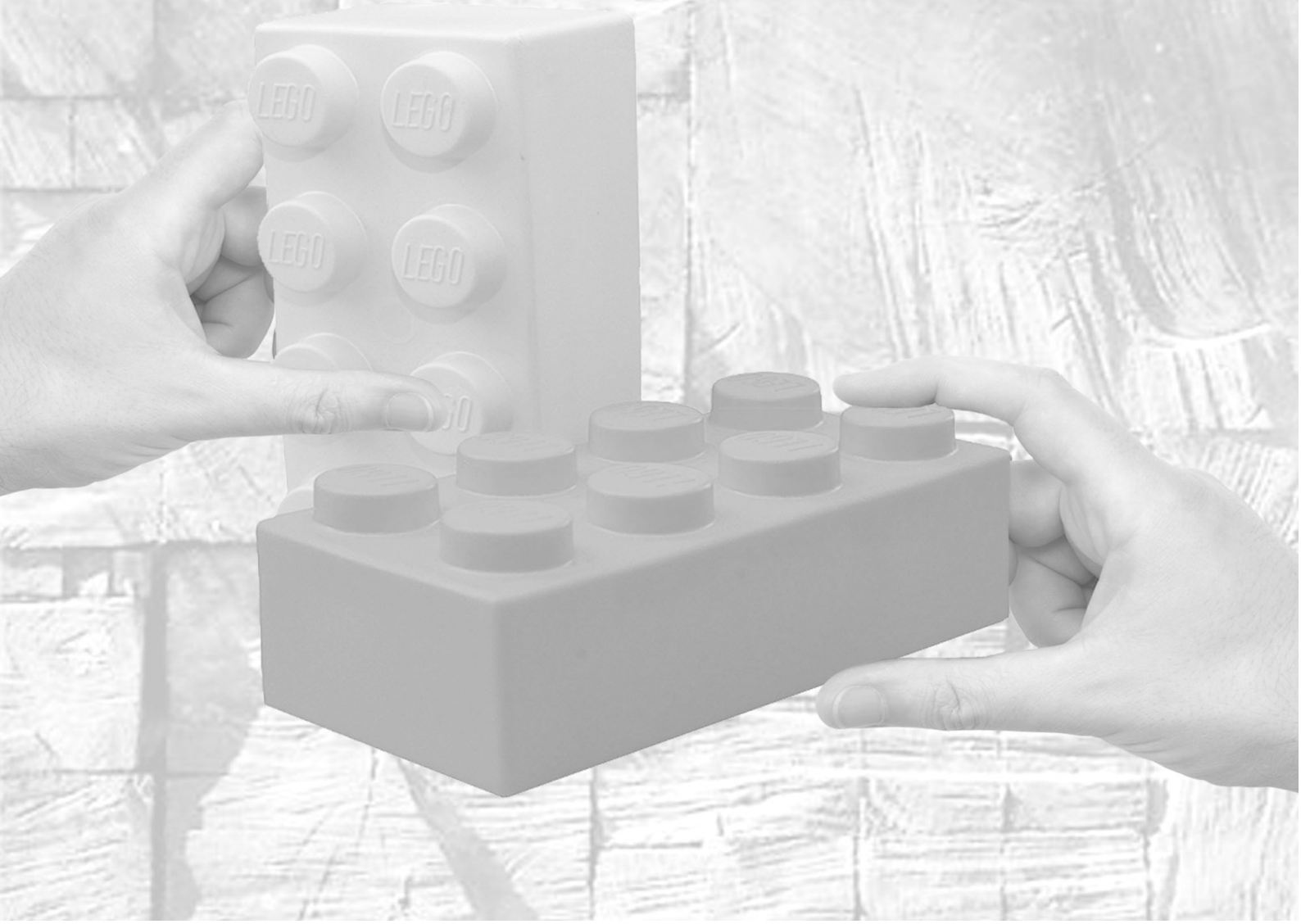
Genes & Development 2007; 21(5):615-24



Abstract

Ecsit is a cytosolic adaptor protein essential for inflammatory response and embryonic development via the Toll-like and BMP signal transduction pathways, respectively. Here, we demonstrate a mitochondrial function for Ecsit in the assembly of mitochondrial complex I (NADH:ubiquinone oxidoreductase). An N-terminal targeting signal directs Ecsit to mitochondria, where it interacts with assembly chaperone NDUFAF1 in 500-850 kDa complexes as demonstrated by affinity purification and vice versa RNAi knockdowns. In addition, Ecsit knockdown results in severely impaired complex I assembly and disturbed mitochondrial function. These findings support a function for Ecsit in the assembly or stability of mitochondrial complex I, possibly linking assembly of oxidative phosphorylation complexes to inflammatory response and embryonic development.

Introduction

Regulation of gene expression is one of the cornerstones of biological versatility. It is achieved by feedback mechanisms between different subcellular pathways, often mediated by regulatory adaptor proteins. In 1999, Kopp and colleagues described a prominent example of such a protein, termed Ecsit (an evolutionary conserved signaling intermediate in Toll pathways (Kopp et al., 1999)). Ecsit is a cytoplasmic signaling protein that constitutes a molecular link between two pathways: the Toll signaling pathway and the BMP pathway.

The family of Toll-like receptors (TLRs) likely consists of 10-15 members in most mammalian species, sharing a conserved Toll/IL-1 receptor (TIR) domain (Sims et al., 1988; Greenfeder et al., 1995; Medzhitov et al., 1997; Torigoe et al., 1997; Chaudhary et al., 1998; Rock et al., 1998; Iwasaki and Medzhitov, 2004). Toll-like receptors bind extracellular ligands (e.g. Gram-negative bacterial lipopolysaccharides) to activate expression of NF-κB and AP-1 transcription factors, which in turn activate expression of genes involved in the immune response (Medzhitov et al., 1997). Signal transduction can occur in two ways, either via TAK1 and regulators TAB1 and TAB2, or via MEKK1 and subsequent MKK activation (Moustakas and Heldin, 2003). Communication between the two cascades is possible as TAK1 can activate MKKs and their downstream effectors and MEKK1 can activate the TAK1 activated IKK complex. TRAF6 is an early component of both cascades and yeast two hybrid and immunoprecipitation studies have shown the association of Ecsit with TRAF6 (tumor necrosis factor [TNF] receptor-associated factor 6), thereby directly linking the protein to the Toll pathway (Kopp et al., 1999). In addition, Ecsit interacts with and may facilitate processing of MEKK-1, findings which have placed Ecsit in an immunological context.

In 2003, Xiao and colleagues found an additional role for this protein in the BMP pathway in mouse embryogenesis (Xiao et al., 2003). Null mutation of the Ecsit gene in mice resulted in embryonic lethality with phenotypes that mimic those of a BMP receptor gene (Bmpr1a) null mutant (reduced epiblast cell proliferation, block of mesoderm formation, and embryonic lethality at the beginning of gastrulation). Bmp4 is known to play an essential role in the gastrulation of the mouse embryo and signals through Bmpr1a, a type I Bmp receptor to induce upregulation of target genes including Tlx2, a homeobox gene (Tang et al., 1998). Ecsit2 associates constitutively with Smad4 and associates with Smad1 in a Bmp-inducible manner.

Furthermore, together with Smad1 and Smad4, it binds to the promoter of specific Bmp target genes (Xiao et al., 2003). Confirmative of its role in the Toll signaling pathway, shRNA inhibition of Ecsit in a macrophage cell line resulted in drastic inhibition of LPS-induced NF-κB activity (Xiao et al., 2003). This demonstrated that both the BMP and Toll signaling pathways require Ecsit, which therewith represents a link between immunity and embryonic development.

In this study, we show an unexpected additional function for Ecsit in mitochondria, to which it is targeted by an N-terminal targeting sequence and where it interacts with NDUFAF1, a chaperone involved in assembly of mitochondrial complex I (NADH:ubiquinone oxidoreductase) (Janssen et al., 2002; Vogel et al., 2005). Complex I is one of the five enzymatic complexes that comprise the oxidative phosphorylation (OXPHOS) system in the mitochondrial inner membrane, responsible for the generation of ATP from NADH and $FADH_2$ (Brandt, 2006; Janssen et al., 2006). Ecsit knockdown using RNA interference results in decreased NDUFAF1 and complex I protein levels, accumulation of complex I subcomplexes and disturbed mitochondrial function. Herewith, in addition to its cytoplasmic and nuclear functions, these findings point towards a mitochondrial function for Ecsit and could provide a link between mitochondrial OXPHOS system biogenesis and function, immune response, and mesoderm formation during embryogenesis.

Materials and methods

Generation of inducible cell lines and cell culture

NDUFAF1-TAP construct - NDUFAF1 was subcloned from the pcDNA4/TO/myc-His A construct (Invitrogen) (Vogel et al., 2005) into pDONR201 (Invitrogen). A Gateway® TAP Destination vector was produced by subcloning the TAP-tag of pCTAP-A (Stratagene) in frame behind Gateway® Reading Frame Cassette B (Invitrogen) in pcDNA5/FRT/TO (Invitrogen). To obtain an inducible NDUFAF1-TAP vector, the pDONR201-NDUFAF1 vector was recombined with the TAP-Destination vector using the Gateway® LR Clonase II Enzyme Mix (Invitrogen). *Ecsit-GFP construct* - The Ecsit open reading frame sequence (BC000193; without stopcodon) flanked by Gateway® AttB sites (Invitrogen) was created by PCR following manufacturer instruction and cloned into pDONR201 by using Gateway® BP Clonase II Enzyme Mix (Invitrogen). A Gateway® Destination vector was produced by subcloning the BamHI/NotI restriction fragment of pAcGFP1-N1 (Clontech) in frame behind Gateway® Reading Frame Cassette B (Invitrogen) in pcDNA5/FRT/TO (Invitrogen). To obtain an inducible Ecsit-GFP vector, the pDONR201-Ecsit vector was recombined with the AcGFP1-Destination vector using the Gateway® LR Clonase II Enzyme Mix (Invitrogen). *Ecsit-GFP –N terminus construct* – The Ecsit open reading frame was cloned as for the Ecsit-GFP construct, with the following modification. Ecsit was cloned with ATG start codon but without the following 141 base pairs, encoding 47 amino acids. Based on the open reading frame sequence BC000193, the 5' end of the sequence thus became 5' ATGAGCTCTGAA… 3'. All constructs were transfected into Flp-In T-REx293 cells (Invitrogen) using Superfect® Transfection Reagent (QIAGEN) following manufacturer protocols. The NDUFAF1-mycHIS inducible HEK293 T-REx^{tm} cell line is previously described in (Vogel et al., 2005). All inducible cell lines, HeLa and Human Embryonic Kidney (HEK) 293 cells were cultured in DMEM (Biowhitaker) supplemented with 10% fetal calf serum (v/v) and 1% penicillin/streptomycin (v/v) (Gibco). The inducible cell lines were treated with 1 µg/ml doxycycline (Sigma Aldrich) for expression of the transgene.

Preparation of mitochondria, cell fractionation and trypsin treatment of mitochondria

For blue-native and SDS-PAGE analysis, HEK293 and HeLa mitochondria were purified with the use of digitonin as previously described in (Ugalde et al., 2004).

Fractionation of HEK293 cells was performed by pottering as described in (Vogel et al., 2005). Trypsin treatment of mitochondria was performed by adding 30 U trypsin (Promega), 100 U trypsin, or 30 U trypsin plus 1% v/v triton X100 (Roche) to 50 µl mitochondrial lysate followed by incubation for 15 minutes at 37 ºC.

Blue-native and SDS PAGE analysis and CI in-gel activity assay

Blue-native gradient gels (5-15%) were cast as earlier described (Nijtmans et al., 2002) and run with 40 or 80 µg of solubilised mitochondrial protein. After electrophoresis, gels were further processed for in-gel activity assays, Western blotting or second dimension 10% SDS-PAGE as described in (Nijtmans et al., 2002). Proteins were transferred to a PROTAN® nitrocellulose membrane (Schleicher & Schuell). 1D 10% SDS-PAGE analysis was performed as described previously (Ugalde et al., 2004).

siRNA transfection

For transfection, HeLa cells were plated in 1,5 mL of DMEM supplemented with 10% FCS (without antibiotics) in 6-wells plates with a cell density of 2.0×10^5 cells per well. The next day, cells were transfected with siRNA duplex (control: Cyclophilin B (Dharmacon), Ecsit #1 antisense strand: 5'-UUGACGUUCAUGAAUCGAG dGdT -3', #2 antisense strand: 5'-AUUGAUGUCAAACUCGUAG dTdT -3') in the presence of oligofectamine (7,5 µl) (Invitrogen) and opti-MEM (Invitrogen) to achieve a final concentration of 100 nM siRNA in a total volume of 1,8 mL per well. Cells were incubated at 37°C in a CO_2 incubator for 72 hours prior to a second, identical, round of transfection for 72 hours.

Antibodies and ECL detection

Immunodetection was performed using the following primary antibodies. Complex I: NDUFS3 (Invitrogen), ND1 (a gift from Dr. Anne Lombes, Paris), NDUFA1 (a gift from Professor Immo Scheffler, San Diego). NDUFAF1 affinity purified serum is available from our laboratory (Vogel et al., 2005). Other antibodies used were raised against Ecsit (Abcam), TRAF6 (Abcam), GAPDH (Abcam), COXII (Invitrogen), Core2 (Invitrogen), ATPase α (Invitrogen), SDHA (Invitrogen), Tom20 (BD Biosciences) and GFP (a gift from Dr. Frank van Kuppeveld). Secondary antibodies that were used are peroxidase-conjugated anti-mouse or anti-rabbit IgGs (Invitrogen). For the detection of immunoprecipitated proteins, ReliaBLOT™

HRP Conjugate was used (Bethyl Laboratories). The signal was generated using ECL® plus (Amersham Biosciences).

Confocal imaging

HEK293 cells expressing inducible Ecsit-GFP (with and without N-terminus) were cultured on glass slides were washed with PBS and incubated with 1µM Mitotracker Red (Invitrogen) for 15 minutes and with 10 µM Hoechst 3342 (Invitrogen) for 30 minutes, both at 37 °C. After incubation, cells were washed with PBS and glass slides were mounted in an incubation chamber placed on the stage of an inverted microscope (Nikon Diaphot), attached to an Oz confocal microscope (Noran Instruments). Measurements were performed at 20° C in the dark. The light from an argon ion laser (488 nm; Omnichrome) was delivered to the cells via a x40 water immersion fluor objective (NA 1.2; Nikon). GFP and mitotracker fluorescence light was separated by a 565DRLPXR dichroic mirror, directed through 510AF23 and 630DF30 emission filters (all from Omega Optical Inc.) and quantified using separate photomultiplier tubes (PMTs) at 8-bit resolution (Hamamatsu Photonics). Hoechst 3342 was excited using 364 nm light generated by a high-power argon-ion laser (Coherent Enterprise) and its fluorescence emission was detected using a 400 nM long pass filter and PMT. Hardware and image acquisition were controlled by Intervision software (Version 1.5, Noran Instruments) running under IRIX 6.2 on an Indy workstation (Silicon Graphics Inc.). Images (512x480 pixels) were collected at 30 Hz with a pixel dwell time of 100 ns and averaged in real-time to optimize signal-to-noise ratio (Koopman et al., 2006b). Image processing and analysis was performed using Image Pro Plus 5.1 (Media Cybernetics).

Quantification of NAD(P)H, superoxide, oxidant levels and mitochondrial morphology

Mitochondrial NADH levels – NAD(P)H fluorescence intensity was measured using a CoolSNAP HQ CCD-camera (Roper Scientific) attached to an inverted microscope (Axiovert 200 M, Carl Zeiss). Prior to recordings, coverslips were washed with PBS, and placed into an incubation chamber containing HEPES-Tris medium (132 mM NaCl, 4.2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM D-glucose and 10 mM HEPES, pH 7.4). NAD(P)H was excited at 360 nm using a monochromator (Polychrome IV, TILL Photonics) and fluorescence emission was directed to the CCD-camera using a 415DCLP dichroic mirror and a 510WB40 emission filter (Omega Optical Inc.). Fields of view were recorded using an image capturing time of 1 s. Mean fluorescence intensity was determined in a region of

interest (ROI) containing a high density of mitochondrial structures, which was background corrected using an extracellular ROI of identical size. The imaging setup was controlled using Metafluor 6.0 software (Universal Imaging Corporation). Quantitative image analysis was performed using Metamorph 6.0 (Universal Imaging Corporation). *Cellular superoxide and oxidant levels* – Cellular superoxide and oxidant levels were quantified using hydroethidine (HEt) and 5-(and-6)-chloromethyl-2',7'- dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) as described previously (Koopman et al., 2005a; Koopman et al., 2006a). *Mitochondrial morphology* – Quantitative analysis of mitochondrial morphology in living cells was performed as described previously (Koopman et al., 2005b; Koopman et al., 2006b). *Statistics* – Numerical results were visualized with Origin Pro 7.5 software (OriginLab) and are presented as means ±SEM (standard error of the mean). Statistical differences were determined with an independent two-sample Student's t-test (Bonferroni corrected). P-values < 0.05 (*) were considered significant.

FT-MS analysis

The proteins were in-gel reduced with 10 mM dithiotreitol and alkylated with 50 mM iodoactamide before in-gel digestion with trypsin. Peptides were extracted from the gel and purified and desalted using Stage tips (Rappsilber et al., 2003). Peptide identification experiments were performed using a nano-HPLC Agilent 1100 nanoflow system connected online to a linear quadrupole ion trap-Fourier transform mass spectrometer (LTQ-FT, Thermo Electron). LC and MS settings are further explained in supplemental table 3. Peptides and proteins were identified using the Mascot (Matrix Science) algorithm to search a local version of the NCBInr database (http://www.ncbi.nlm.nih.gov). First ranked peptides were parsed from Mascot database search html files with MSQuant (www.msquant.sourceforge.net) to generate unique first ranked peptide lists. Protein identifications were evaluated against criteria described in supplemental table 3.

Results

Ecsit co-purifies with OXPHOS assembly chaperone NDUFAF1

Mitochondrial ATP production occurs via the oxidative phosphorylation (OXPHOS) system, which consists of five membrane embedded protein complexes (Janssen et al., 2006). The assembly process of the largest of these, complex I, is a challenging problem (Vogel et al., 2004). It is known to involve assembly chaperone NDUFAF1, but the mechanism of its action remains unclear (Janssen et al., 2002; Vogel et al., 2005). In order to find mitochondrial binding partners for NDUFAF1 we have performed Tandem Affinity Purification (TAP) of mitochondrial lysates of inducible HEK293 T-REx™ cells that express TAP-tagged NDUFAF1 protein. FT-MS analysis of the eluate revealed several proteins, which are listed in supplemental table 1. Amongst the peptides found, the analysis specifically identified several peptides corresponding to the Ecsit protein, a previously described cytoplasmic protein involved in immunity and embryonic development (Kopp et al., 1999; Xiao et al., 2003) (supplemental table 1). Ten Ecsit splice isoforms are predicted, of which only isoforms 1 and 2 have been annotated and encode proteins with predicted molecular masses of 50 and 33 kDa, respectively (figure 1A). Analysis of the TAP purified peptides reveals the presence of the peptide DSTGAADPPQPHIVGIQSPDQQAALAR, which crosses the boundary between exons 5 and 6 and thereby identifies the largest, 50 kDa Ecsit isoform 1 (figure 1B, supplemental table 2).

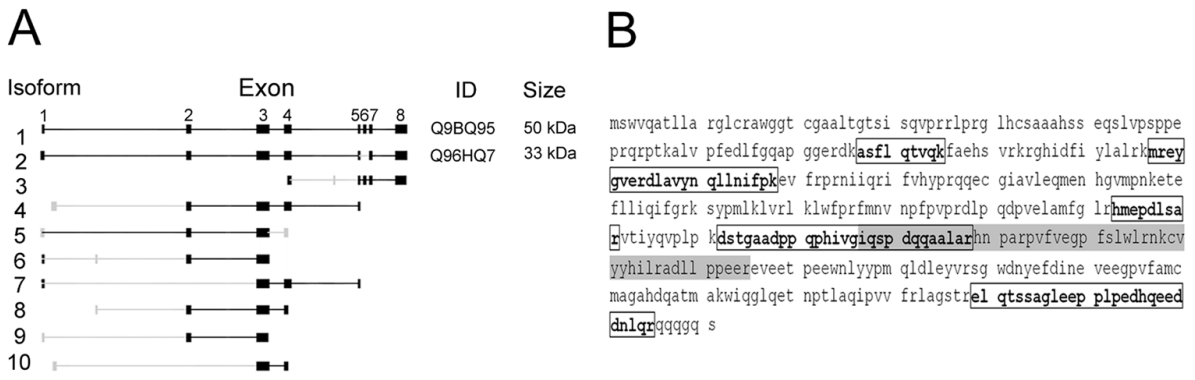


Figure 1: Ecsit isoform 1 co-purifies with tandem affinity purified NDUFAF1
(A) Ten Ecsit splice isoforms are predicted by the EBI alternative splicing database, of which two are identified in experimental studies (isoforms 1 and 2). Shown are the exons (vertical bars) and introns (horizontal lines) that comprise the complete transcript. The Swissprot annotation and predicted molecular mass are indicated when available. (B) FT-MS sequence coverage of Ecsit in eluates of tandem affinity purified NDUFAF1 in HEK293 mitochondria demonstrates the presence of Ecsit isoform 1. Identified peptides are indicated bold and in boxes, whereas exon 6, unique for Ecsit isoform 1, is indicated in grey.

Ecsit localizes to mitochondria

The interaction between NDUFAF1 and Ecsit is unexpected, since NDUFAF1 is described to be mitochondrial, whereas Ecsit is described as a cytoplasmic protein. However, although a mitochondrial function for Ecsit has not yet been described in literature, all tested mitochondrial targeting sequence prediction programs (MitoPROT2, TargetP, Predotar, SignalP) clearly indicate a mitochondrial targeting sequence with high probability. MitoPROT2 predicts a 99.3 % chance of a cleavable N-terminal target sequence of 49 amino acids, corresponding to approximately 5 kDa.

To verify these predictions, we have performed fractionations of HEK293 cells to separate mitochondria from other cellular compartments. Figure 2A shows predominant immunodetection of 50 kDa Ecsit isoform 1 in the cytoplasm/nucleus, in line with its previously described cytoplasmic function (Kopp et al., 1999; Xiao et al., 2003). In contrast to the cytoplasmic fraction, an approximately 45 kDa Ecsit signal is specifically observed in mitochondria (figure 2A). As controls, Ecsit binding partner TRAF6 and GAPDH do not show presence in mitochondria and are clearly cytoplasmic, and mitochondrial complex IV subunit COXII localizes in mitochondria.

To confirm the mitochondrial localization of the 45 kDa Ecsit, we have performed trypsin digestion of mitochondrial outer membrane proteins (figure 2B). After cell fractionation, performed as for figure 2A, mitochondria were treated for 15 minutes at 37 ºC with either 30 U of trypsin, 100 U of trypsin or 30 U of trypsin in the presence of 1% triton, which lyses the mitochondria and should thus result in complete degradation of all mitochondrial proteins. Increasingly stringent trypsin treatment led to the degradation of the 50 kDa Ecsit, whereas the 45 kDa Ecsit was only degraded upon mitochondrial lysis. This suggests that the 45 kDa Ecsit is shielded by the mitochondrial outer and inner membranes and most likely is imported into mitochondria. As controls, outer membrane protein Tom20 is rapidly degraded upon trypsin treatment and matrix protein NDUFS3 is only degraded upon mitochondrial lysis. After digestion using 100 U of trypsin, in addition to the 45 kDa band, smaller bands were detected with the Ecsit antibody. Due to their size, it is unlikely that these bands represent partial tryptic digestion products. However, at this stage, we cannot exclude processing by another (mitochondrial) protease. Both fractionations (figures 2A and 2B) demonstrated that a small fraction of the 50 kDa cytoplasmic Ecsit localizes to mitochondria. This signal may either represent minute cytoplasmic background after pottering or an intermediate in the import process.

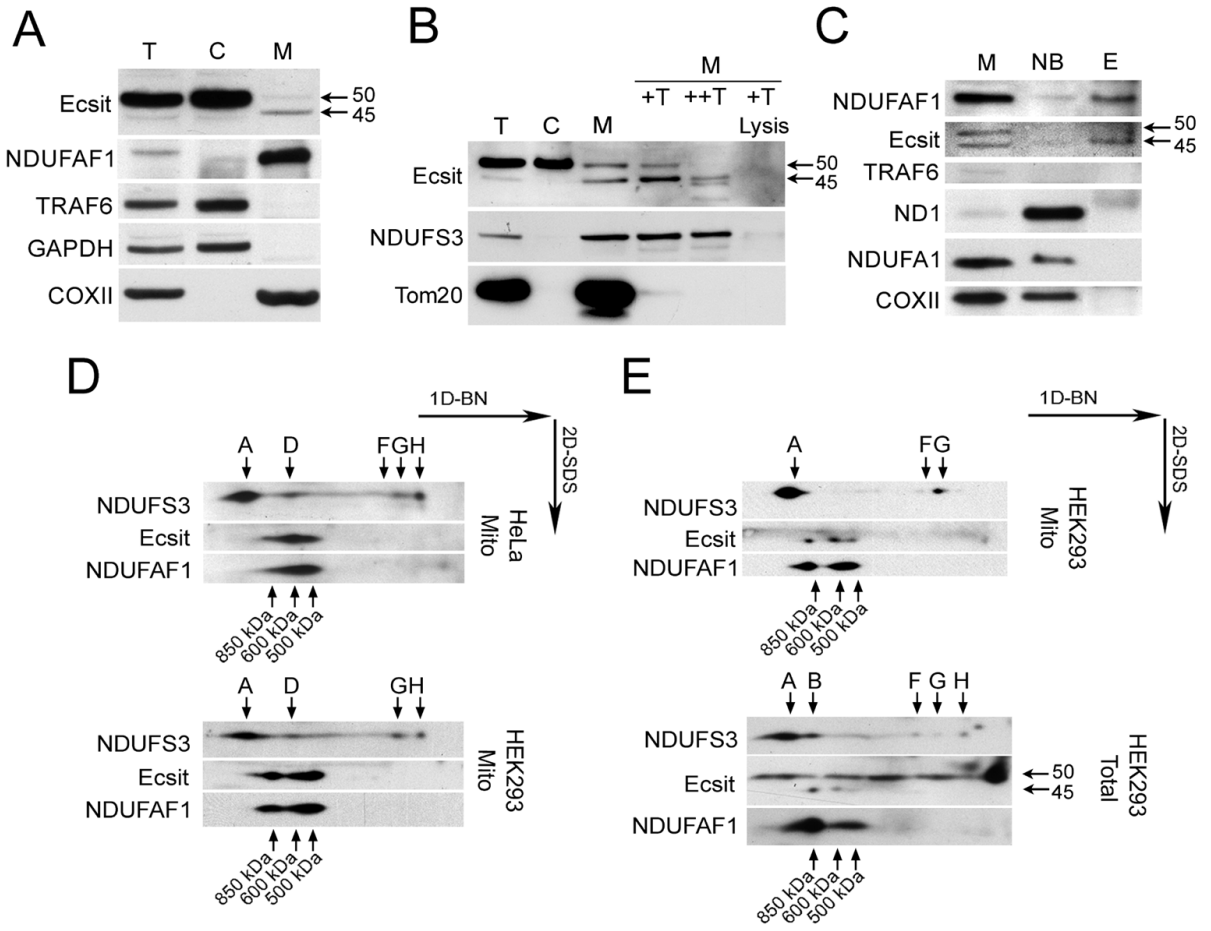


Figure 2: Ecsit localizes to mitochondria and interacts with NDUFAF1 in three high molecular weight complexes

(A) HEK293 cells were fractionated by pottering and lysates from total cell, mitochondria and cytoplasm were subsequently analysed by SDS-PAGE and Western blotting. T: Total cell. C: cytoplasm. M: mitochondria. All lanes were immunodecorated with antibodies targeted to Ecsit, NDUFAF1, TRAF6, cytoplasmic control GAPDH and mitochondrial control COXII. Ecsit is predominantly present in the cytoplasm, however, a smaller band of about 45 kDa is visible specifically in mitochondria. In contrast, cytoplasmic Ecsit binding partner TRAF6 is not detected in mitochondria. (B) Trypsin import assay. T: Total cell. C: cytoplasm. M: mitochondria. M +T: mitochondria + 30 U trypsin 15 minutes at 37 ºC. M ++T: as M +T but with 100 U trypsin. M +T Lysis: as M +T but with 1% triton to lyse the mitochondria. Increasingly stringent trypsin digestion of mitochondrial outer membrane proteins results in disappearance of the 50 kDa Ecsit, whereas the 45 kDa Ecsit remains intact. NDUFS3 is used as a mitochondrial matrix control, Tom20 is used as an outer membrane control. (C) Immunoprecipitation using an anti-myc antibody in mitochondria purified from an NDUFAF1-myc-HIS inducible HEK293 cell line. Shown are mitochondria (M), non-bound (NB) and eluate fractions (E). Myc-immunoprecipitation co-elutes the 45 kDa mitochondrial Ecsit together with NDUFAF1, as opposed to mitochondrial controls ND1, NDUFA1, COXII and cytoplasmic control TRAF6.

(Legend to figure 2, continued)
(D) Two-dimensional blue-native SDS-PAGE analysis of HeLa and HEK293 mitochondrial lysates demonstrates the co-localization of 45 kDa Ecsit and NDUFAF1 in three complexes of approximately 500, 600 and 850 kDa. Complex I subunit NDUFS3 is shown to demonstrate the position of complex I ("A", 1 MDa). Subcomplexes observed in our previous complex I assembly study (Ugalde et al., 2004) are indicated with A-H when visible. (E) HEK293 mitochondrial Ecsit signal was compared to a total cell preparation. "50" indicates the 50 kDa cytoplasmic Ecsit in the HEK293 total cell lysate, which is absent in HEK293 purified mitochondria. This demonstrates that only the 45 kDa, mitochondrial, Ecsit interacts with NDUFAF1 in complexes of 500-850 kDa.

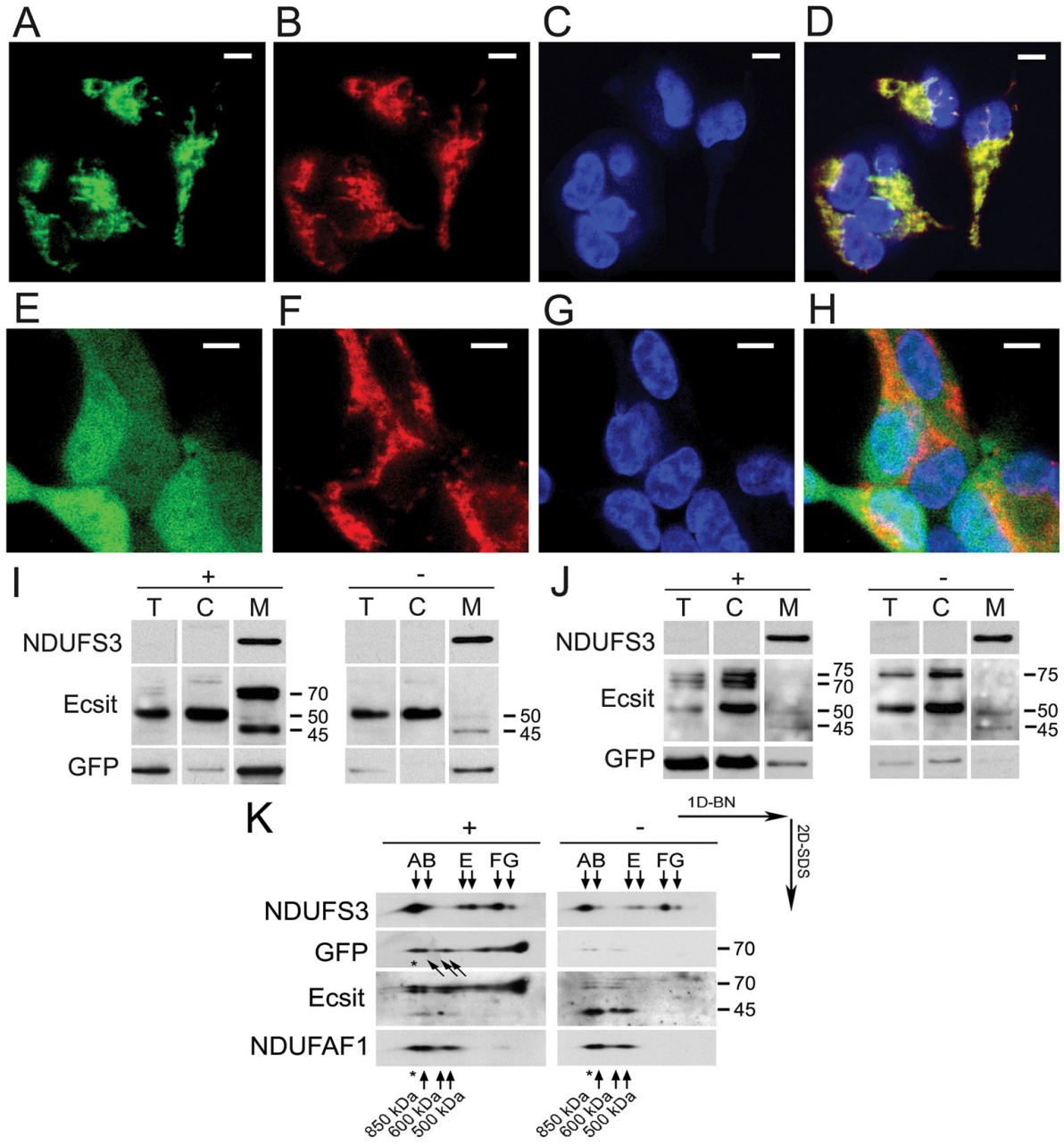
To establish whether it is the mitochondrial Ecsit that interacts with NDUFAF1, we performed anti-myc immunoprecipitations in mitochondrial lysates of inducible HEK293 T-REx™ cells that express myc-tagged NDUFAF1 protein (figure 2C). Two bands are visible for Ecsit in the total mitochondrial lysate, of 45 and 50 kDa in size, of which the 45 kDa form specifically co-purifies with NDUFAF1. In contrast, mitochondrial controls ND1, NDUFA1 and COXII do not co-purify. Cytosolic Ecsit binding partner TRAF6 is not found in the mitochondrial preparation, demonstrating that the NDUFAF1 interaction is specifically mitochondrial.

Mitochondrial Ecsit and NDUFAF1 co-localize in three high molecular weight complexes

To investigate whether Ecsit associates to mitochondrial high-molecular weight protein complexes, we have performed two-dimensional blue-native PAGE analysis of mitochondria enriched lysates of HEK293 and HeLa cells. Although slightly different between the two cell lines, this shows the specific presence of Ecsit in three complexes of approximately 500, 600 and 850 kDa. This pattern strongly resembles that of OXPHOS assembly chaperone NDUFAF1, which accurately comigrates with Ecsit (figure 2D). At least for the HEK293 lysates, the two-dimensional resolution shows that instead of the previously reported two NDUFAF1 containing complexes of approximately 600 and 700 kDa (Vogel et al., 2005), NDUFAF1 may in fact be present in three complexes of approximately 500, 600 and 850 kDa. To demonstrate the mitochondrial origin of these protein complexes and to show that this Ecsit signal represents the mitochondrial 45 kDa Ecsit, we have compared a mitochondrial HEK293 lysate to a total cell lysate in the same analysis (figure 2E). This analysis revealed the additional presence of the 50 kDa Ecsit in the total cell lysate, confirming that the 45 kDa mitochondrial Ecsit, but not the 50 kDa protein, is present in the three complexes.

Ecsit requires its N-terminal targeting sequence for mitochondrial localization

Although only a small percentage of the total cellular Ecsit pool is targeted to mitochondria (figure 2A), as discussed, Ecsit is predicted to have an N-terminal mitochondrial targeting sequence. To experimentally verify the requirement of the Ecsit N-terminus for mitochondrial targeting, we have analysed Ecsit subcellular localization by confocal microscopy of C-terminal-GFP-tagged Ecsit with and without the first 48 N-terminal amino acids predicted to be required for mitochondrial targeting by MitoProt II (figure 3A-H). In living cells, mitochondria (visualized by Mitotracker Red staining) clearly co-localized with the Ecsit-GFP signal, demonstrating mitochondrial targeting of Ecsit-GFP (figure 3D). In contrast, Ecsit-GFP lacking its N-terminus accumulated in the cytosol (figure 3H).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50
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J
75
K
1D-BN
2D-SDS
AB
E
FG
NDUFAF1
850 kDa
600 kDa
500 kDa

Figure 3: Ecsit requires its N-terminal targeting sequence for mitochondrial localization
(A-H) Confocal microscopy of HEK293 cells transiently transfected with an inducible Ecsit-GFP construct (A-D) and with an inducible Ecsit-GFP construct lacking the 48 amino acid Ecsit N-terminus (E-H). A and E show GFP signal. B and F show the mitochondrial network using Mitotracker Red. C and G show nuclear staining. D and H show the overlay between the three signals. Scale bars represent 10 µm. Without the N-terminus, Ecsit-GFP is no longer targeted to mitochondria. Thus, Ecsit-GFP requires its N-terminal targeting sequence for mitochondrial localization. (I) Cell fractionation of Ecsit-GFP inducible HEK293 cells. "+": induction of expression. "-": no induction of expression. T: total cell. C: cytoplasm. M: mitochondria. NDUFS3 is used as mitochondrial control. Other antibodies used are anti-Ecsit ("Ecsit" panel) and anti-GFP ("GFP" panel). The endogenous Ecsit signals are visible at 50 (total cell and cytoplasm) and 45 kDa (mitochondria). The induced Ecsit-GFP targets predominantly to mitochondria and is detected at approximately 70 kDa (45 kDa + 24 kDa) in two bands using the anti-Ecsit antibody. Only one of these anti-Ecsit stained bands can be made visible using the anti-GFP antibody ("GFP" panel). The sensitive anti-GFP antibody also shows minor Ecsit-GFP leakage expression (in the not induced situation). (J) Cell fractionation of inducible HEK293 cells expressing Ecsit-GFP without N-terminus, as performed in figure 3I. In the induced situation, Ecsit appears predominantly in total cell and cytoplasm as several bands migrating at approximately 70-75 kDa (Ecsit panel), of which only one is detected using the anti-GFP antibody (GFP panel). Again, minor leakage expression is observed in the not induced situation. (K) Two-dimensional analysis of Ecsit-GFP incorporation into high-molecular weight Ecsit/NDUFAF1 protein complexes of 500-850 kDa. NDUFS3 signal is used as a marker for previously observed CI subcomplexes A-H when visible (Ugalde et al., 2004). In accordance with figure A, two types of Ecsit-GFP are visible on the anti-Ecsit incubated blot ("Ecsit" panel) of which only one is detectable using anti-GFP antibody ("GFP" panel). The Ecsit-GFP complexes comigrate with the NDUFAF1 complexes (500-850 kDa). In addition, a larger complex matching the size of CI (1 MDa) is indicated with an asterisk.

The predominant mitochondrial targeting of Ecsit-GFP (figure 3A-D) contrasts with the mainly cytoplasmic localization of endogenous Ecsit (figure 2A). To further investigate the mitochondrial targeting of Ecsit-GFP, we have performed biochemical fractionation as done for endogenous Ecsit in figure 2A (figure 3I). In addition to the cytoplasmic localization of 50 kDa endogenous Ecsit (figure 3I, Ecsit panel, 50 kDa), this fractionation confirms the predominant targeting of Ecsit-GFP to mitochondria, in line with the confocal imaging (figure 3I, Ecsit panel, 70 kDa). Enriched in mitochondria are two 70 kDa Ecsit-GFP bands, likely representing mitochondrial Ecsit (45 kDa Ecsit + 24 kDa GFP), of which only one is stained using the anti-GFP antibody. Some leakage Ecsit-GFP expression is observed in the not induced situation using the anti-GFP antibody (figure 3I, GFP panel). This minor amount is also predominantly targeted to mitochondria. The same procedure was applied to the inducible HEK293 cells expressing Ecsit-GFP without its N-terminal sequence (figure 3J). Using the Ecsit antibody, multiple bands of approximately 75 kDa were detected in the induced situation in the total cell and cytoplasmic fractions, but not in the mitochondrial fraction (figure 3J, Ecsit panel,

70-75 kDa). Also the GFP-signal was predominantly cytosolic, in line with confocal imaging (figure 3J, GFP panel).

To verify whether the Ecsit-GFP (with N-terminus) is actually incorporated into mitochondrial protein complexes, we performed two-dimensional blue-native SDS-PAGE analysis of mitochondrial lysates of Ecsit-GFP inducible cells (figure 3K). This analysis shows that Ecsit-GFP is normally incorporated into the endogenous complexes of 500-850 kDa (figure 3K, panels Ecsit and NDUFAF1). As in figure 3I, two 70 kDa Ecsit-GFP signals can be discerned in figure 3K, of which only one is detected by the anti-GFP antibody, most likely due to incomplete unfolding of the GFP molecule in one of the two situations.

Ecsit knockdown in HeLa mitochondria results in NDUFAF1 decrease and impaired complex I assembly

The mitochondrial association with assembly chaperone NDUFAF1 allows the possibility that Ecsit is involved in the assembly of OXPHOS complexes. To address this hypothesis, we investigated the effect of Ecsit knockdown on OXPHOS complex assembly using two small interfering RNAs (siRNA) against Ecsit mRNA directed against exon 4 (target #1) and against exon 7 (target #2). The effects of knockdown were analysed by SDS-PAGE analysis (figure 4A). Ecsit RNAi effectively knocks down the 45 kDa Ecsit signal and has substantially reduced the amount of mitochondrial NDUFAF1 (Figure 4A). Apparently, Ecsit is required for stable presence of NDUFAF1 within the mitochondrion. To analyse the effects of Ecsit knockdown on OXPHOS assembly we performed one-dimensional blue-native PAGE analysis (Figure 4B). Complex I is severely reduced upon Ecsit knockdown, whereas the amounts of the other OXPHOS complexes appear relatively unchanged. Furthermore, immunodetection for complex I subunit NDUFS3 reveals the accumulation of intermediates of about 500 kDa in size. To further investigate the nature of these complexes, we performed two-dimensional SDS PAGE analysis (figure 4C). As shown using antibodies against complex I subunits NDUFS3 and ND1, RNAi results in accumulation of intermediates smaller than complex I, indicative of disturbed assembly or stability of the holo-complex (figure 4C, “sub”). Furthermore, Ecsit knockdown results in a strong decrease in the NDUFAF1 subcomplexes of 500-850 kDa, strongly suggesting that the stability of these complexes relies on the presence of Ecsit and NDUFAF1 in these subcomplexes.

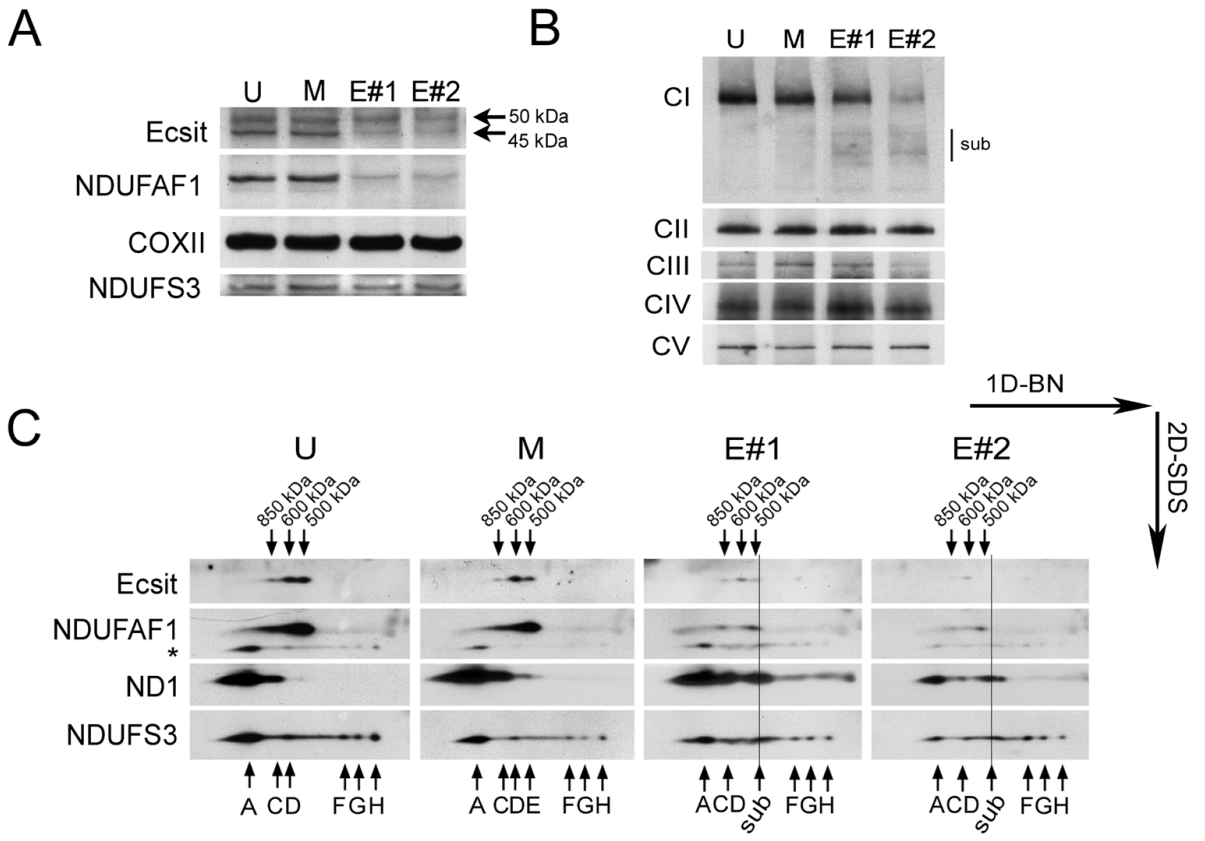

Figure 4: Ecsit knockdown using RNA interference results in disturbed complex I assembly
(A) RNA interference was performed using two siRNAs (#1 and #2) against Ecsit mRNA. Following SDS-PAGE and Western blotting, immunodetection was performed for Ecsit, NDUFAF1, COXII and NDUFS3 in untreated (U), mock transfected (M) and siRNA transfected (#1, #2) HeLa cells. The Ecsit signal is knocked down which correlates with a severe depression in NDUFAF1 protein. Monomeric COXII and NDUFS3 levels remain unchanged. (B) The effect of Ecsit knockdown on OXPHOS complex assembly was investigated by blue-native PAGE followed by Western blotting and immunodetection of complex I subunit NDUFS3 (CI), complex II subunit SDHA (CII), complex III subunit core2 (CIII), complex IV subunit COXII (CIV) and complex V subunit ATPase α (CV). Arrows indicate accumulated subcomplexes detected with the anti-NDUFS3 antibody. (C) Two-dimensional blue-native SDS-PAGE analysis of samples analysed in figures 5A and 5B. Shown are immunodetections of Ecsit, NDUFAF1 and complex I (NDUFS3 and ND1 signals), in untreated, mock transfected, Ecsit siRNA transfected (#1, #2) HeLa cells. Subcomplexes that correspond to previously described complex I subcomplexes (Ugalde et al., 2004) are indicated with A-H. "Sub" indicates accumulated subcomplexes after both Ecsit siRNA transfections. The Ecsit/NDUFAF1 complexes are indicated with 500-850 kDa. An asterisk indicates signal from a previous NDUFS3 detection.

Ecsit knockdown in HeLa mitochondria results in disturbed mitochondrial function

The impaired assembly or stability of complex I upon Ecsit knockdown may have serious consequences for mitochondrial function. For example, complex I deficiency is known to result in a broad spectrum of mitochondrial disorders (Janssen et al., 2006), increases cellular superoxide levels (Koopman et al., 2005a) and affects mitochondrial morphology (Koopman et al., 2005b). To investigate the effects of Ecsit knockdown on mitochondrial physiology and morphology we investigated several parameters. As demonstrated by complex I in-gel activity assay, complex I activity drops to 50-60% of the control value (figure 5A). Mitochondrial NAD(P)H levels were significantly increased in the siRNA treated cells compared to untreated and mock-treated controls (figure 5B), suggesting that NADH oxidation by complex I is decreased. Downstream effects of complex I dysfunction were assayed by measuring superoxide and cytosolic oxidant levels (Koopman et al., 2005a; Koopman et al., 2006a) and by determining the degree of mitochondrial branching (*F*) and number per cell (*Nc*) (Koopman et al., 2005a; Koopman et al., 2005b; Koopman et al., 2006b) (figures 5C and 5D). This analysis indicated that the level of cellular radical species was increased up to 150-200% of control values (figure 5C). Although the number of mitochondria per cell was not affected by siRNA treatment, mitochondrial branching was reduced (figure 5D). Taken together, these data support the notion that Ecsit is required for normal mitochondrial functioning and morphology.

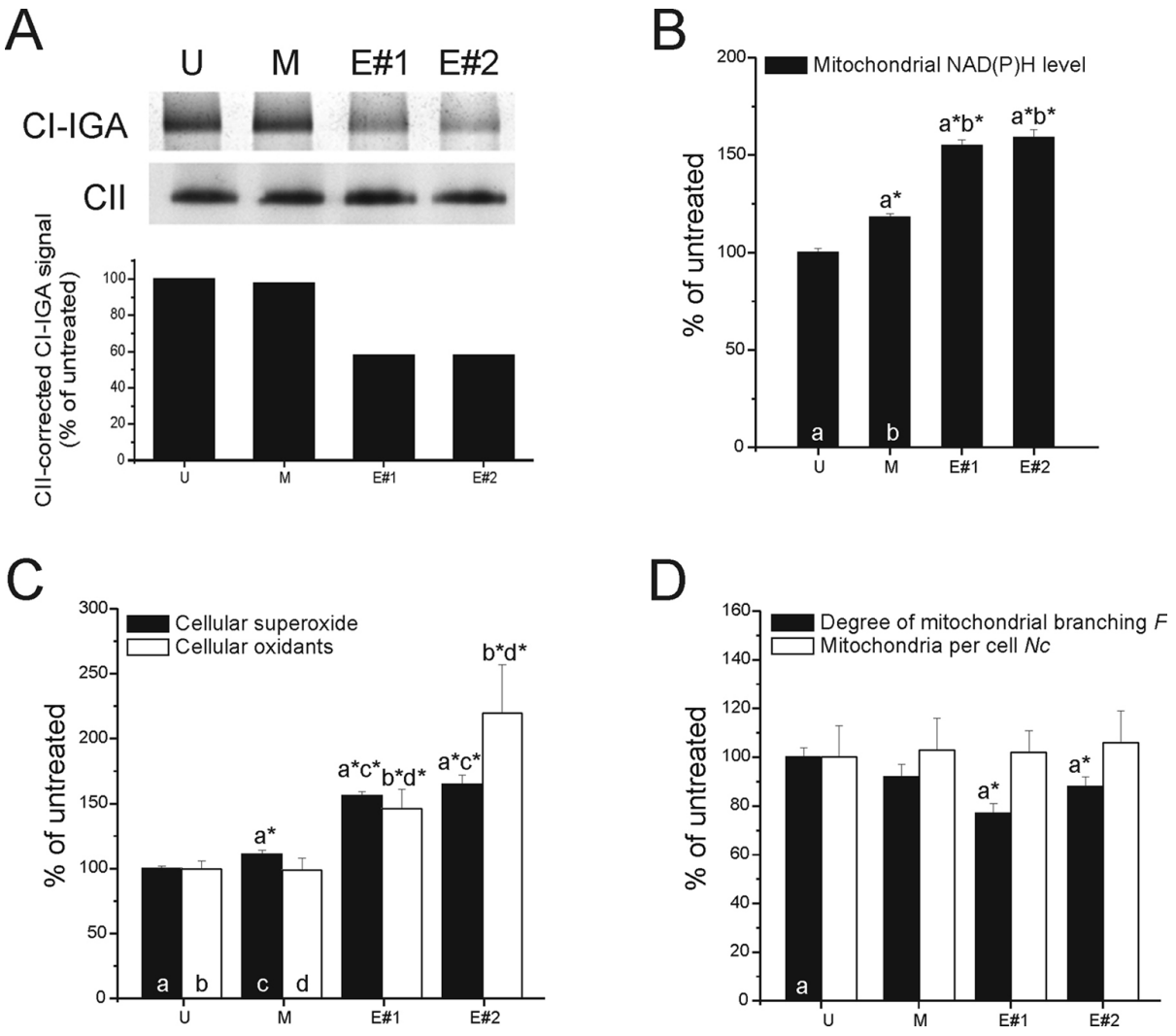

Figure 5: Ecsit knockdown using RNA interference affects mitochondrial and cellular physiology
(A) Complex I in-gel activity (CI-IGA) and complex II (CII) expression following native electrophoresis in (U)ntreated, M(ock)-treated and two Ecsit RNAi knockdowns (E#1, E#2). The lower panel depicts the CI-IGA signals corrected for CII expression (expressed as % of the value in untreated cells) determined by integrated optical density analysis. (B) NAD(P)H levels in (U)ntreated, (M)ock treated and siRNA-treated (E#1, E#2) cells. Bars represent the average of 262 (U), 293 (M), 234 (E#1) and 180 (E#2) cells. (C) Cellular superoxide (filled bars) and oxidant levels (open bars) in (U)ntreated, (M)ock treated and siRNA-treated (E#1, E#2) cells. Bars represent the average of 294 (U), 284 (M), 335 (E#1) and 93 (E#2) cells for superoxide levels, and 47 (U), 33 (M), 41 (E#1) and 25 (E#2) for oxidant levels. (D) Degree of mitochondrial branching (F, black bars) and number of mitochondria per cell (Nc, open bars) in (U)ntreated, (M)ock treated and siRNA-treated (E#1, E#2) cells. Bars represent the average of 50 (U), 56 (M), 70 (E#1) and 50 (E#2) cells. In panel B, C and D numerals (a,b,c,d,) represent statistically significant differences with the indicated columns. Data was obtained during 2 independent experiments from multiple cells (N).

Discussion

In previous studies, Ecsit has been described as a cytosolic signaling protein essential for the Toll pathway of innate immunity and the BMP pathway of embryonic development (Kopp et al., 1999; Xiao et al., 2003). In this study, we show N-terminal targeting of Ecsit to mitochondria, where it interacts with mitochondrial complex I specific assembly chaperone NDUFAF1 in three high molecular weight protein complexes of 500-850 kDa. Ecsit is required for correct complex I assembly or stability in particular, and mitochondrial function in general, and may thus represent a link between mitochondrial function, immune response and embryonic development.

Cytosolic Ecsit associates with TRAF6 and MEKK-1 and is described to facilitate the processing of MEKK-1 (Kopp et al., 1999). MEKK-1, in turn, is able to activate two different pathways leading to transcriptional activation of inflammatory genes (Moustakas and Heldin, 2003). It now seems that, while Ecsit may facilitate the processing of another protein such as MEKK-1, Ecsit itself can be processed in the mitochondrion at its N-terminus, after which it associates with mitochondrial complexes including complex I assembly chaperone NDUFAF1.

It is yet unclear under which factors determine whether Ecsit is targeted to either the mitochondrion or the cytosol. Whichever the underlying mechanism, we show, at least in HEK293 cells, that only a fraction of the total Ecsit amount is recruited to mitochondria and that this targeting depends on an N-terminal mitochondrial targeting sequence. In addition, we show that addition of a C-terminal GFP tag alters the Ecsit distribution from mainly cytoplasmic to predominantly mitochondrial. This shift in localization is most likely not caused by the overexpression itself, as the (low level) leakage expression of Ecsit-GFP in the not induced cells is also targeted to the mitochondrion.

Even though, compared to its cytosolic counterpart, only a fraction of the Ecsit pool is present in mitochondria, Ecsit knockdown results in severely depressed NDUFAF1 amounts, demonstrating its requirement for stable mitochondrial presence of a complex I specific chaperone. Furthermore, Ecsit knockdown results in specifically disturbed complex I assembly or stability and subsequently impaired mitochondrial function. Similar to complex I deficient patient and rotenone-treated control cells, NAD(P)H, superoxide (Koopman et al., 2005a) and cytosolic oxidant levels (Koopman et al., 2006a) are increased in Ecsit knockdown cells.

Conversely, NDUFAF1 knockdown is known to result in impaired complex I assembly (Vogel et al., 2005) but only results in a minor decrease in the amount of Ecsit in the 500 kDa subcomplex (data not shown). In contrast to Ecsit knockdown, no accumulation of complex I intermediates can be observed after NDUFAF1 knockdown (unpublished results), which indicates that although both proteins are present in the 500-850 kDa complexes their mechanism of action may be different. Elucidation of the exact composition of these intermediates may clarify the specific significance of the presence of Ecsit in these complexes for complex I assembly or stability.

In conclusion, as Ecsit is required for stabilisation of complex I assembly chaperone NDUFAF1 and its absence results in impaired complex I assembly, accumulation of intermediates and mitochondrial dysfunction, it seems that Ecsit is involved in additional processes apart from its functions in immune response and embryonic development. This putative link between mitochondria and the immune response has recently gained much attention with the discovery of MAVS (mitochondrial antiviral signaling) (Seth et al., 2005; McWhirter et al., 2005). This protein is associated to the mitochondrial outer membrane via a hydrophobic C-terminus and acts as a signaling molecule in the immune response via association to TRAF6, similar to what has been described for the Ecsit protein. As Ecsit is now also found inside the mitochondrion, Ecsit may extend the influence of this cascade to the inner-mitochondrial level. Possibly, Ecsit modulates the energetic requirements upon inflammatory response by regulating the rate of complex I synthesis. Alternatively, Ecsit may induce other mitochondrial processes, such as apoptosis, upon microbial or viral infection. Future research will have to verify which of these possibilities is reality.

Acknowledgements

This work was supported by ‘Het Prinses Beatrix Fonds’ to J.S. and L. v.d. H. (grant number 02-0104) and the European Community’s sixth Framework Programme for Research, Priority 1 “Life sciences, genomics and biotechnology for health” (contract number LSHMCT-2004-503116). The Netherlands Organisation for Scientific Research supported L.N. with a ‘Vernieuwingsimpuls’ grant. Imaging equipment was financed by grants of ZON (Netherlands Organization for Health Research and Development, No: 903-46-176) and NWO (Netherlands Organization for Scientific Research, No: 911-02-008).

Supplemental data

Supplemental table 1: Number of peptide hits for eluted proteins in control and doxycycline treated NDUFAF1-TAP inducible HEK293 cells

Accession No.	Protein description	Control	DOX
gi|12653969	**NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 1 [Homo sapiens**	**0**	**18**
gi|12803087	Tubulin, beta polypeptide [Homo sapiens]	0	12
gi|12653815	Tubulin, alpha, ubiquitous [Homo sapiens]	0	7
gi|12652877	**Evolutionarily conserved signaling intermediate in Toll pathway [Homo sapiens]**	**0**	**6**
gi|10436258	unnamed protein product [Homo sapiens]	0	6
gi|12803681	Chaperonin [Homo sapiens]	0	4
gi|1706611	EFTU_HUMAN Elongation factor Tu, mitochondrial precursor (EF-Tu) (P43)	0	3
gi|123648	HSP7C_HUMAN Heat shock cognate 71 kDa protein (Heat shock 70 kDa protein 8)	0	3
gi|1090507	2019238A ATP synthase:SUBUNIT=alpha	0	2
gi|12803275	Heat shock 70kDa protein 1A [Homo sapiens]	0	2
gi|10279705	OTTHUMP00000030560 [Homo sapiens]	0	2
gi|113950	ANXA2_HUMAN Annexin A2 (Annexin II) (Lipocortin II) (Calpactin I heavy chain) (Chromobindin 8) (p36) (Protein I)	0	2
gi|16306717	Alpha isoform of regulatory subunit A, protein phosphatase 2 [Homo sapiens]	0	1
gi|14250065	Calmodulin 2 [Homo sapiens]	0	1
gi|13904998	MYL6 protein [Homo sapiens]	0	1
gi|11228051	unnamed protein product [Homo sapiens]	0	1
gi|11228055	unnamed protein product [Homo sapiens]	0	1
gi|1346345	K2C6B_HUMAN Keratin, type II cytoskeletal 6B (Cytokeratin 6B) (CK 6B) (K6b keratin)	0	1
gi|1064990	A Chain A, Trypsin (E.C.3.4.21.4) Complexed With The Inhibitor Diisopropyl-Fluorophosphofluoridate (Dfp)	0	1

Supplemental table 2: Specification of the peptide hits found for the Ecsit protein

Accession number	Peptide sequence	Charge	Peptide score
gi|12652877	ASFLQTVQK	2	29
gi|12652877	ASFLQTVQK	2	50
gi|12652877	DLAVYNQLLNIFPK	2	40
gi|12652877	DLAVYNQLLNIFPK	2	28
gi|12652877	DSTGAADPPQPHIVGIQSPDQQAALAR	3	62
gi|12652877	ELQTSSAGLEEPPLPEDHQEEDDNLQR	3	62
gi|12652877	FAEHSVR	2	35
gi|12652877	HMEPDLSAR	2	20
gi|12652877	MREYGVER	2	27

Supplemental table 3: Applied FT-MS settings

NLC settings	
Column	100 µm ID, packed with 3 µm Reprosil C18 beads (Dr. Maisch GmbH, Ammerbuch, Germany)
Gradient	60 min gradient from 3% buffer A to 30% buffer B
Buffer A	0.5% Acetic acid
Buffer B	80% Acetonitrile in 0.5% acetic acid
Flowrate	300 nl.min
MS settings	
Data-dependent mode	Sequencing of four most abundant ions
Dynamic exclusion	180 sec
Mass range FTMS	300-2,000 m/z
Resolution FTMS	100,000
Charge state rejection	Singly charged and unassigned charge states
Mascot settings	
Database	NCBInr, downloaded 2005-05-05
Enzymatic cleavage	Trypsin, 1 miscleavage allowed
Parent ion mass tolerance	20 ppm
Fragment ion mass tolerance	0.8 Da
Fixed modification	Carbamidomethyl (C)
Variable modification	Oxidation (M), Deamidation (N,Q)
Validation criteria	
Mass accuracy	<3.5 ppm
Proteins identified >1 peptide	Average peptide score >30
Single peptide hits	Peptide score >50
	Delta score >10

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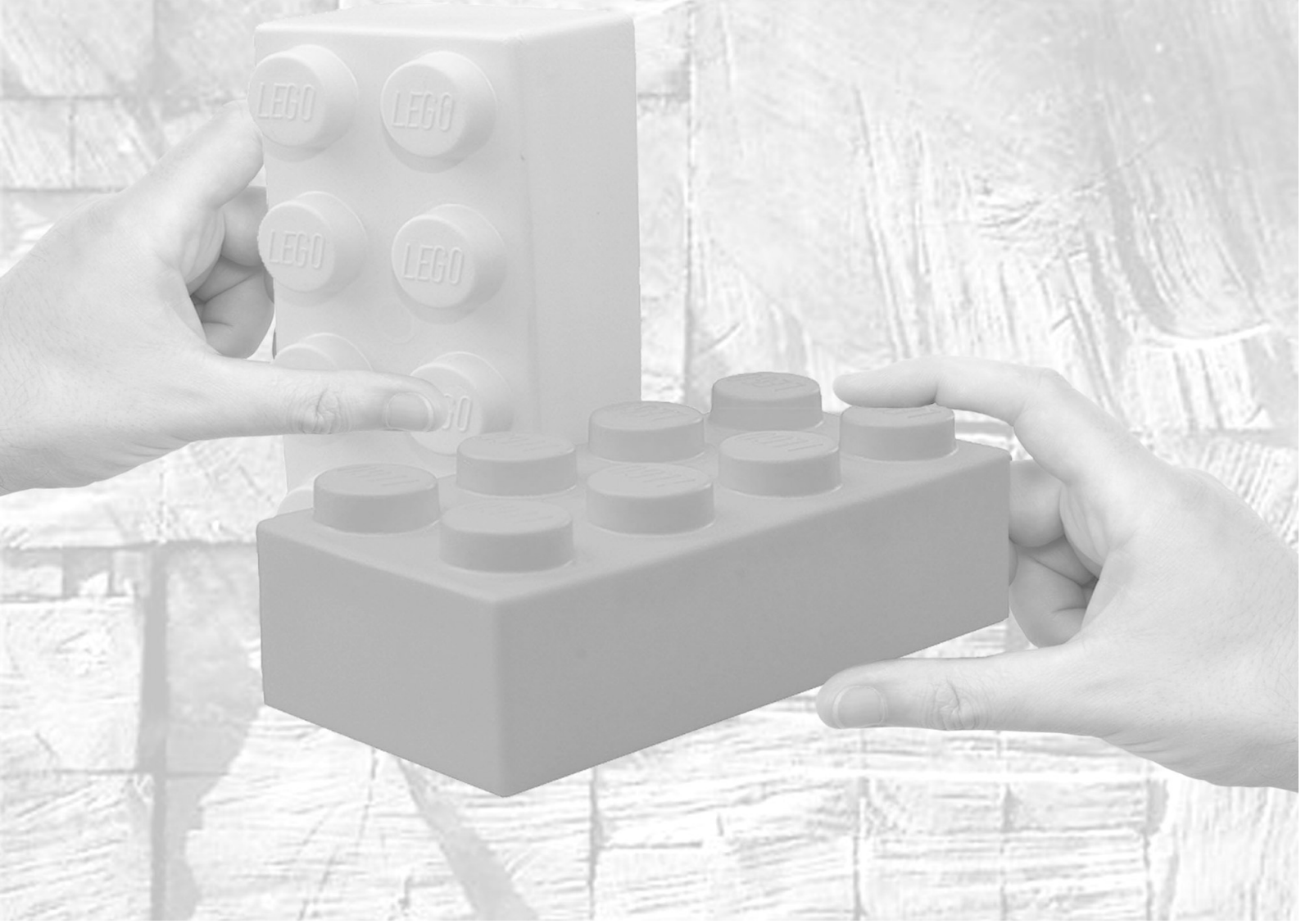
Chapter 8

General discussion

The previous chapters have described the development of two subsequent models for complex I assembly and research into the function of complex I assembly chaperones in this process, leading to the discovery of the Ecsit protein in mitochondria. By reflecting on these data in the context of previously performed studies, this chapter will debate the implications of both parts of this thesis. For part I, a general assembly mechanism is proposed based on a comparison of complex I assembly between different organisms. In addition, part II debates the versatile nature of several assembly chaperones and discusses the possible entanglement of complex I assembly with processes such as apoptosis and immunity.

Published in adapted form in Biochimica et Biophysica Acta 2007; doi: 10.1016/j.bbabio.2007.07.008.



Introduction

Mitochondrial ATP production via the OXPHOS system is one of the cornerstones of cellular metabolism. A prerequisite for this is a stable and properly functioning mitochondrial complex I (CI). Studying the assembly of this approximately 1 MDa complex is important. If not for elucidation of the molecular basis of CI deficiency, then for understanding how large mitochondrial protein complexes are assembled from mitochondrial and nuclear DNA-encoded subunits at the mitochondrial inner membrane. This thesis aims to contribute to the understanding of both. Upon proposal of a theoretical assembly mechanism, the preceding chapters have added two consecutive CI assembly models to the model proposed by Antonicka and colleagues (Antonicka et al., 2003). In addition, NDUFAF1 was shown to be an assembly chaperone for human CI and the occurrence of chaperones NDUFAF1 and B17.2L was studied in a cohort of CI deficient patients. Description of the immune system protein Ecsit in mitochondria where it functions as a CI assembly chaperone has concluded this thesis. This chapter will place these findings into the context of existing studies. Consistent with the structure of this thesis, this chapter will discuss the two main topics separately (Part I: CI assembly; Part II: CI assembly chaperones).

Part I: CI assembly

CI assembly studies: an introduction

Although (bovine heart) CI stability, subunit composition and topology has been extensively studied using various methods to fractionate the complex (Hatefi, 1976; Galante and Hatefi, 1978; Han et al., 1988; Han et al., 1989; Finel et al., 1992; Finel et al., 1994; Finel, 1998; Sazanov et al., 2000; Carroll et al., 2003; Carroll et al., 2006), the assembly process of eukaryotic CI has long remained enigmatic. By using compounds that inhibit either translation of nuclear DNA-encoded or mitochondrial DNA-(mtDNA)encoded transcripts, it became clear that subassemblies of nuclear DNA-encoded CI subunits can be formed in the absence of mtDNA-encoded subunits (Hall and Hare, 1990; Hofhaus and Attardi, 1993; Hofhaus and Attardi, 1995; Bai and Attardi, 1998; Potluri et al., 2004). These findings had the important implication that CI subunits are not incorporated during assembly one by one (in a sequential manner), but that discrete assembly intermediates consisting of several subunits occur which are combined during assembly (in a semi-sequential manner).

Additional insights were obtained with the systematic introduction systematic introduction of mutations in CI genes of the fungus *N. crassa* (for extensive reviews see (Schulte, 2001; Videira, 1998; Weidner et al., 1992; Schulte et al., 1994; Videira and Duarte, 2001; Videira and Duarte 2002)). In this organism, peripheral and membrane assembly intermediates are formed independently (Tuschen et al., 1990). In turn, the membrane arm of *N. crassa* CI is assembled from a large and small intermediate. The small intermediate contains the ND2 and ND5 subunits (NuoN and NuoL homologues), whereas the large intermediate contains ND1, 3, 4, 4L and 6 (NuoH, A, M, K and J homologues)(Videira, 1998; Kuffner et al., 1998). Generally, mutations in subunits of the one arm do not adversely affect the assembly of the other (Tuschen et al., 1990; Nehls et al., 1992; Duarte et al., 1995). Exceptions to this rule are loss of the acyl carrier protein (NDUFAB1 or SDAP homologue), which disturbs formation of both peripheral and membrane arms, and loss of the 11.5 kDa protein (NDUFS5 homologue), which results in accumulation of membrane arm intermediates and failure to detect an active peripheral arm (Schneider et al., 1995; Schneider et al., 1997; Marques et al., 2005). Altogether, these studies have resulted in one of the first models of CI assembly in a eukaryote (Schulte, 2001; Schulte et al., 1994).

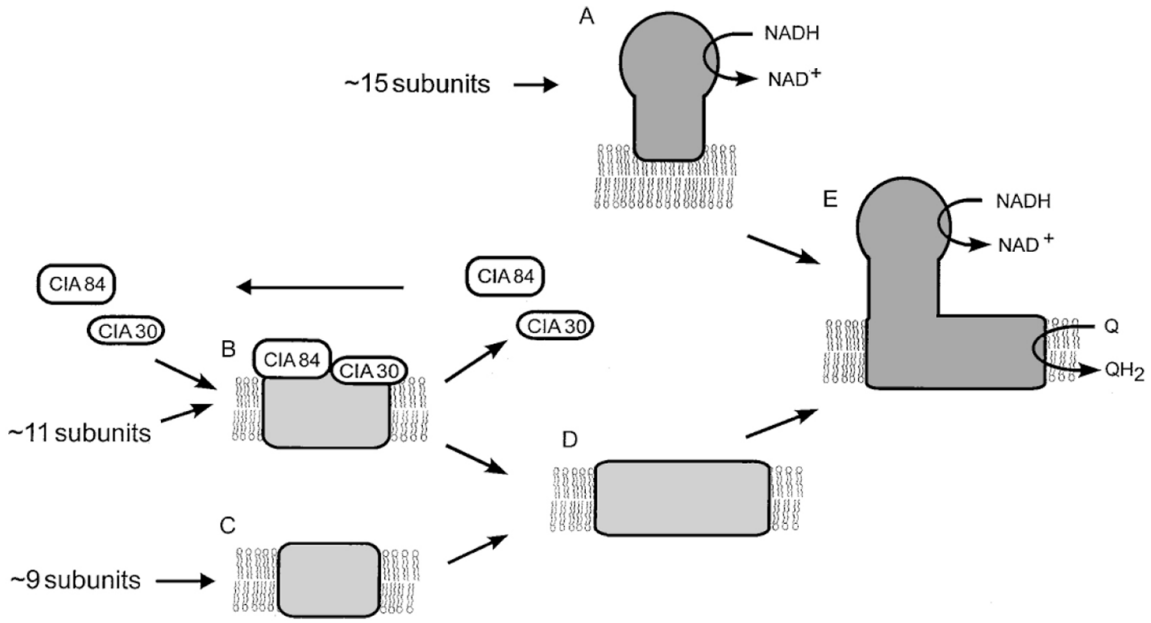


***Figure 1. CI assembly in* Neurospora crassa**
In this model, first proposed in 1994 by Ulrich Schulte and colleagues, small and large membrane arm intermediates (C and B) are combined via the CIA proteins CIA30 and CIA84 to form the CI membrane arm (D). This arm is combined with a pre-assembled peripheral arm (A) to form mature CI (E) (Schulte et al., 1994; Schulte, 2001).

Human CI assembly

Initial investigations for CI assembly in humans and other higher eukaryotes have mainly contributed data concerning the requirement of mtDNA-encoded subunits (Chomyn, 2001). Immunoprecipitation studies in an ND4 cybrid cell line demonstrated that the membrane arm was not assembled when ND4 is disrupted (Hofhaus and Attardi, 1993). Five years later a similar study in a mouse ND6 frameshift mutant cell line demonstrated the requirement of ND6 (Bai and Attardi, 1998), later confirmed for human CI assembly in a CI deficient patient cell line carrying an ND6 mutation (Kirby et al., 2003). The ND5 subunit seems to be more important for activity than for assembly (Hofhaus and Attardi, 1995; Bai et al., 2000). Cells lacking ND4 or ND5 did show small amounts of the 24 kDa subunit (NDUFV2) in membrane fractions, indicating that assembly is not severely disrupted (Bourges et al., 2004). In addition, loss of ND3 does not lead to a great disturbance of assembly (Kirby et al., 2004a; McFarland et al., 2004), whereas ND2 disruption results in disturbed assembly with accumulated intermediates

(Antonicka et al., 2003; Ugalde et al., 2007). Finally, ND1 mutation results in a severe reduction of fully assembled CI (Kirby et al., 2004b).

Valuable information about the assembly of peripheral arm subunits has predominantly come from assembly studies in CI deficient patients. In 2001, Triepels and colleagues classified CI deficient patients by their assembly profiles by comparison of immunodetected amounts of CI subunits, suggesting an important role for subunits NDUFA9 and NDUFS3 (Triepels et al., 2001). In two patients harbouring a mutation in the NDUFS6 subunit, CI assembly was severely impaired and accumulation of a large 750 kDa subcomplex was observed using an anti-NDUFA9 antibody (Kirby et al., 2004a). In addition, mutations in subunits NDUFS1, NDUFS4 and NDUFV1 lead to accumulation of a subcomplex slightly smaller than the fully assembled complex (Scacco et al., 2003; Ugalde et al., 2004a; Ogilvie et al., 2005; Iuso et al., 2006). Whether this subcomplex is the same in all of these studied cell lines is unclear, but the (near) absence of CI activity and the hypothesized co-localization in the tip of the peripheral arm of the complex support that their mutation may result in similar assembly/stability defects. Recently, Ogilvie and colleagues demonstrated association of assembly chaperone B17.2L with this complex and performed immunoprecipitation using an antibody raised against the B17.2L chaperone to identify associated proteins in wild type mitochondria (Ogilvie et al., 2005). Although the 830 kDa intermediate was not detected in wild type mitochondria, co-elution was observed of the ND1, NDUFS1-4, NDUFV1 and NDUFA13 subunits, suggesting the existence of (an) assembly intermediate(s) with this composition. Finally, of note, NDUFS8 mutation results in a severe impairment of CI assembly (Ugalde et al., 2004a; Procaccio and Wallace, 2004), and two NDUFA1 mutations have been implicated in disturbed assembly and inability to detect a 20 kDa subunit (presumably the NDUFB8 subunit) in the holo-complex (Fernandez-Moreira et al., 2007).

An attempt to coherently incorporate the available assembly data into a model was first made in 2003, when Antonicka and colleagues published their human CI assembly model, based on the occurrence of CI subcomplexes in a cohort of CI deficient patients (Antonicka et al., 2003). Different genetic defects resulting in CI deficiency can result in the accumulation of similar subcomplexes, which were argued to be illustrative of assembly intermediates. In the model, membrane and peripheral arm assembly does not occur independently as described for *N. crassa* but rather via membrane anchoring of preformed nuclear DNA-encoded scaffold of subunits. This provided a useful framework for future assembly studies, although

the observed subcomplexes were derived from CI deficient patient cell lines and hence no distinction could be made between products of assembly, disturbed assembly, CI instability or degradation. In 2004, this study was followed by another based on a conditional assembly system (Ugalde et al., 2004b). In this system, the dynamics of assembly intermediate formation could be followed in time by removing a block in mitochondrial translation. Although this system is artificial and nuclear DNA-encoded pre-assemblies may still have pre-formed, it allowed studying of the dynamics of subcomplex formation in time. In contrast to the 2003 study, it showed several similarities with the *N. crassa* model, in that membrane and peripheral arms could seemingly be assembled independently via distinct substructures.

In an additional study in 2007, NDUFS3 containing subcomplexes were made visible at high resolution using leakage expression of an inducible NDUFS3-GFP expression system in HEK293 cells. It confirmed several of the detected subcomplexes and demonstrated the presumable entry-point of mitochondrial DNA-encoded subunits into the assembly process (Vogel et al., 2007a). This study led to the model shown in figure 2. In this model, an early peripheral arm assembly intermediate is membrane anchored prior to expansion with additional peripheral and membrane arm assembly modules. In the proposed assembly model, the distinction between membrane and peripheral arm assembly was no longer as black and white as described in the previous study and for *N. crassa*. In fact, apart from the absence of NDUFA9 in early assembly intermediates, the model is generally similar to the 2003 study by Antonicka and colleagues. In both studies, an early peripheral arm assembly intermediate is membrane anchored prior to expansion with additional membrane arm and peripheral arm assembly modules.

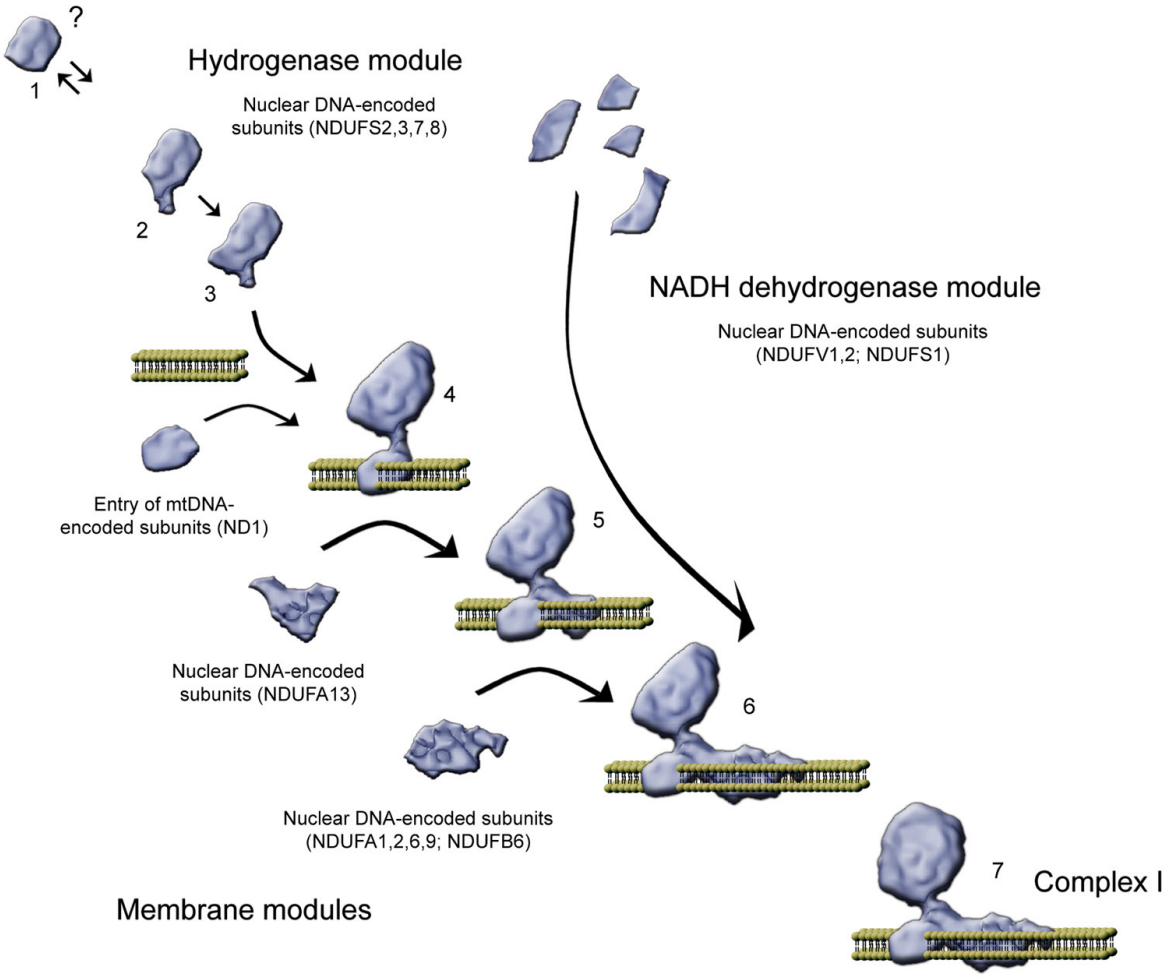


Figure 2. Current model for human complex I assembly
An evolutionary conserved core structure consisting of at least the NDUFS2 and NDUFS3 subunits is formed, expanded and anchored to the mitochondrial inner membrane (1-4). Upon membrane insertion, the complex is further assembled via the addition of pre-formed membrane and dehydrogenase modules (5-7).

Comparison of existing complex I assembly models

Homology searches have revealed a high degree of conservation of certain modules of the complex between various organisms. Archaeal, cyanobacterial, bacterial and mammalian CI share great structural resemblance, although the electron input device (NADH dehydrogenase in mammalian CI) varies. Such phylogenetic studies have led to models describing the modular evolution of CI (Friedrich et al., 1993; Friedrich and Weiss, 1997; Finel, 1998; Friedrich and Scheide, 2000; Mathiesen and Hagerhall, 2003; Friedrich and Bottcher, 2004). In these models, CI is proposed to have originated by fusion of pre-existing protein

assemblies constituting modules for electron transfer and proton transport. In more detail, CI is proposed to have originated from an ancestral soluble nickel-iron hydrogenase (sharing homology with the NDUFS2 and NDUFS7 subunits). This hydrogenase has gained a quinone binding site and has become membrane bound upon acquisition of a protein of unknown function (NDUFS3) and ferrodoxin-type (NDUFS8), ion translocating (ND5) and quinone-binding (ND1) subunits. This structure was subsequently expanded by triplication of proton translocating subunits (ND2 and ND4). After the complex has lost its nickel-iron active site and its ability to react with molecular hydrogen, finally, membrane subunits (ND3, ND4L and ND6) and the NADH dehydrogenase module (NDUFS1, NDUFV1 and NDUFV2) are acquired.

It has been suggested that the co-evolutionary structural relationship between CI subunits may be reflected by the order of assembly and composition of assembly intermediates (Videira 1998; Vogel et al., 2004). If so, how does the current model for CI assembly in humans relate to assembly studies performed in other organisms? If co-evolution of groups of subunits is partially mirrored in the human assembly system, would one not expect to see similarities in other organisms? Can a general assembly mechanism be extracted from these studies?

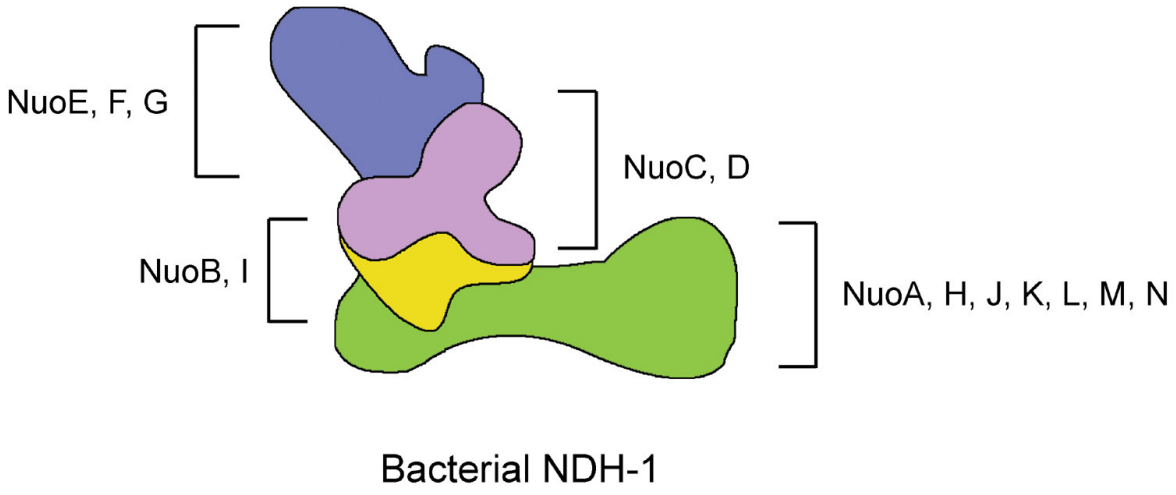


Figure 3: Topology of the bacterial NDH-1 subunits
Bacterial NDH-1 is composed of the 14 most conserved CI subunits, which are termed NuoA-N (see also chapter 1, table 1).

Through the years, the assembly process of CI has been investigated in various organisms, such as *Chlamydomonas reinhardtii, E. coli, N. crassa, Cricetulus griseus* and *Homo sapiens* (Tuschen et al., 1990; Braun et al., 1998; Cardol et al., 2002; Antonicka et al., 2003; Ugalde et al., 2004; Yadava et al., 2004; Cardol et al., 2006). Comparison between the CI assembly mechanisms in different organisms may result in a consensus that allows understanding of the general mechanism of CI assembly. A useful starting point for the comparison between CI assembly mechanisms in different organisms is the structure of bacterial CI (NDH-1) (figure 3) (Leif et al., 1993). The underlying assumption is that the basic framework for the CI assembly process in all organisms is represented by the combination of its most conserved structural components. Figure 4 (on page 224) summarizes the assembly models proposed for the various organisms, simplified by only showing assembly of the 14 'minimal' CI subunits. Details of each assembly scheme are given below, always referring to the NDH-1 homologue of each subunit.

In *E. coli* CI assembly, incorporation of the NuoE, F and G subunits (forming the NADH dehydrogenase module) requires the presence of NuoB, C and D (forming the hydrogenase module) (Braun et al., 1998). It is yet unclear whether this combination takes place before or after the addition of membrane arm subunits. Regarding membrane arm assembly, certain point mutations in the NuoH subunit of *E. coli* and *Paracoccus denitrificans* CI result in severely disturbed assembly (Kervinen et al., 2006). In addition, disruption of the NuoJ gene did not result in disturbed assembly, suggesting that this membrane subunit is added to the complex at a final stage (Kao et al., 2005). Finally, the distal location of the NuoM and NuoL subunits makes it likely that these subunits are added at a late stage in assembly (Baranova et al., 2007a; Holt et al., 2003; Baranova et al., 2007b).

Another organism in which CI assembly has been studied is *C. reinhardtii*. In this organism, frameshift mutations for ND1/ND6 resulted in a failure to detect the 850 kDa holo-CI (Remacle et al., 2001; Cardol et al., 2002). Using the same strategy, absence of ND4 or ND4/5 resulted in accumulation of a 650 kDa subcomplex. Also, ND5 deletion did not abolish CI assembly but rather seemed to destabilize the complex. Consequently, it was proposed that, as opposed to the ND1 and ND6 subunits, the ND4 and ND5 subunits (NuoM and L homologues) are incorporated at a late stage in assembly (Cardol et al., 2002). Recent investigation of the role of ND3 and ND4L (NuoA and K homologues) in assembly resulted in the first assembly model for this organism, in which a nuclear encoded precomplex of 200

kDa, containing the 76 kDa (NuoG homologue) and 49 kDa (NuoD homologue) subunits, is membrane anchored by combining with ND1 (NuoH homologue), ND3, ND4L and ND6 (NuoJ homologue) and subsequently expanded to result in holo-CI with the addition of ND4 and ND5 (Cardol et al., 2006).

As discussed, CI assembly was extensively studied in the fungus *N. crassa*. In brief, peripheral and membrane assembly intermediates were shown to be formed independently (Tuschen et al., 1990). Furthermore, the assembly of the membrane arm occurs via the combination of a small and large intermediate (Nehls et al., 1992).

A useful model system for subsequent CI assembly studies in higher eukaryotes proved to be *C. griseus* (Chinese hamster)(Au et al., 1999; Scheffler and Yadava, 2001; Potluri et al., 2004; Yadava et al., 2004; Yadava and Scheffler, 2004). By using (inducible) complementation of the MWFE and ESSS subunits (homologues of the human CI NDUFA1 and NDUFB11 subunits) it was demonstrated that the stability of Chinese hamster homologues of peripheral arm subunits NDUFS1, 2, 3, 7, 8 and NDUFV1, 2 (NuoB, C, D, E, F, G, I homologues) was unaffected by the absence of MWFE, although holo-CI was not assembled. These data strongly suggest that the peripheral arm can be assembled without the presence of a membrane arm, analogous to assembly in *N. crassa*. Furthermore, incorporation of MWFE is proposed to require membrane arm subunits and the subunit may serve as a membrane anchor to which membrane subunits are attached during CI assembly. Likewise, the ESSS subunit was shown only to be incorporated into CI when membrane subunits are available (Potluri et al., 2004).

Finally, human CI assembly has been studied and three models were proposed (Antonicka et al., 2003; Ugalde et al., 2004b; Vogel et al., 2007a). Although different at points, the models agree in that a peripheral scaffold containing NDUFS2 and NDUFS3 (NuoD and C homologues) is first anchored to the membrane by ND1 (NuoH homologue) prior to addition of the NADH dehydrogenase module subunits (NuoE, F and G) and remaining membrane subunits.

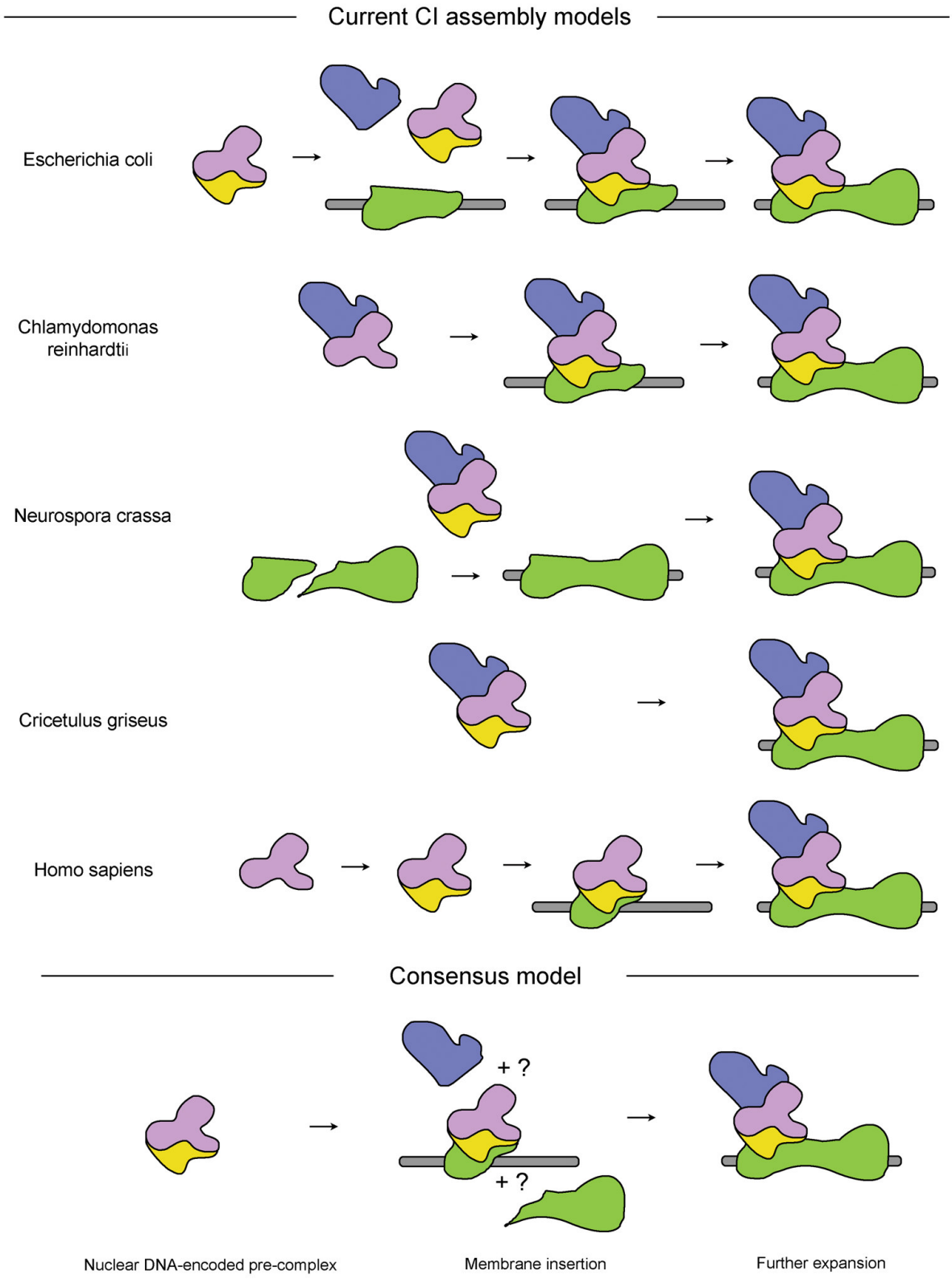

Current CI assembly models
Escherichia coli
Chlamydomonas reinhardtii
Neurospora crassa
Cricetulus griseus
Homo sapiens
Consensus model
+ ?
+ ?
Nuclear DNA-encoded pre-complex
Membrane insertion
Further expansion

Figure 4: Schematic representation of existing CI assembly models
A consensus assembly model is proposed based on what is known about CI assembly in different organisms. Assembly of the basic building blocks by analogy to bacterial NDH-1 is shown for Escherichia coli, Chlamydomonas reinhardtti, Neurospora crassa, Cricetulus griseus *(Chinese hamster) and* Homo sapiens*. Based on these schemes, a consensus can be extracted in which a core of nuclear DNA-encoded subunits is anchored to the mitochondrial inner membrane and expanded with membrane modules and the electron input (dehydrogenase) module. It is yet unclear in which exact order the modules are added (indicated with question marks).*

A general concept for complex I assembly

It is clear that there are differences between the various models. The investigated organisms are part of different evolutionary lineages and assembly of e.g. the membrane or peripheral CI arm has been studied more extensively in the one organism than in the other. Nevertheless, a general concept can be extracted. It seems that a nuclear scaffold formed by the NuoC and NuoD subunits forms the starting point of peripheral arm assembly, which is subsequently anchored to the mitochondrial inner membrane after the addition of NuoB and NuoI and early ND homologue subunits such as NuoH and NuoA. Whether or not more membrane subunits are present and whether this subassembly also includes the NADH dehydrogenase fragment (NuoE, F, G) is not clear. This subassembly is expanded with additional membrane subunits (NuoJ, K, L, M, N) to result in holo-CI. This model fits very well with theoretical data predicting subunit topology by evolutionary conservation (Friedrich and Weiss, 1997), and is compatible with the first described crystal structure of a CI peripheral arm for *Thermus thermophilus* (Sazanov and Hinchliffe, 2006).

Future perspectives

Investigations for the assembly process of human CI have demonstrated that many different intermediates are formed along the way, some of which may be breakdown products of larger assemblies, some of which may be true assembly intermediates (Antonicka et al., 2003; Ugalde et al., 2004b; Vogel et al., 2007a). The most recent assembly study at some points suggests that assembly is not necessarily a static process in which subassemblies are sequentially combined, but rather a dynamic process in which subunits or subcomplexes may be recycled during assembly. For example, the origin of assembly intermediate 1 is rather enigmatic, as it appears both after breakdown and during assembly (see figure 2). In addition, subcomplexes 2 and 3 appear in an equal ratio during assembly, suggesting that the formation of these subcomplexes is tightly linked. The

possibility of recycling during assembly is an interesting subject for future assembly studies, in which turnover rates of subunits and kinetics of the assembly process should be investigated.

Another topic for future studies is the step in which a preformed nuclear DNA-encoded subassembly is anchored to the mitochondrial inner membrane. Identification of which of the complex I subcomplexes are membrane bound can be aided by fractionation of the mitochondrial subcompartments (matrix, inner and outer membranes and inter membrane space proteins), followed by BN-PAGE analysis of each fraction. Along these lines, also demanding future attention is that although several assembly intermediates were observed for nuclear-DNA encoded subunits during human CI assembly, no clear membrane arm assembly intermediates have been observed e.g. like for *N. crassa* CI assembly (Tuschen et al., 1990; Nehls et al., 1992). One of the technical limitations is the absence of proper antibodies for detection of the ND subunits during assembly. In this case, studying disturbed assembly upon ND subunit mutation may indirectly provide the solution (Ugalde et al., 2007).

Possibly, CI assembly of the membrane arm occurs co-translationally, e.g. like CIV in yeast mitochondria. A search for factors such as Oxa1 that link mitochondrial translation to the membrane insertion of mtDNA-encoded CI subunits is a promising endeavour. In addition, one can imagine that both iron-sulfur cluster containing preformed subassemblies and hydrophobic membrane subassemblies would require chaperones for stabilization and combination without aggregation or production of radical species. An ongoing search for these chaperones is required for a better understanding of the assembly process, which brings us to the next paragraph.

Part II: Assembly chaperones

To assemble a fully functional mitochondrial CI is to combine 38 subunits encoded by the nuclear genome and seven subunits encoded by the mitochondrial genome. Chapter 1 has demonstrated that assembly, in addition to just the combination of these subunits, encompasses nuclear and mitochondrial transcription, translation, processing, export, import, membrane insertion, stabilization and activation, including numerous feedback mechanisms required to coordinate the process. When viewed from this perspective, the absolute requirement of chaperone proteins in the CI assembly process becomes evident.

The classical definition of a chaperone

What exactly is a chaperone? Chaperones were first discovered when several genes, conserved from bacteria to mammals, were found activated upon transient heat stress in *Drosophila*, encoding the later termed heat-shock proteins (HSPs) (Ritossa, 1962; Ritossa, 1996). Later, a solid ground for the definition of chaperone function was provided with the demonstration that BiP, a protein interacting with proteins transiting through the ER prior to their assembly into macromolecular structures, is also a member of the HSP70 family (Pelham, 1984; Pelham, 1986) (for a review see (Morange, 2005)). In general, a chaperone is any protein that binds to an unfolded or partially folded target protein to prevent misfolding, aggregation, and/or degradation of it. Chaperones also facilitate the target protein's proper folding. A more detailed definition was proposed by John Ellis: *"molecular chaperones are currently defined in functional terms as a class of unrelated families of protein that assist the correct non-covalent assembly of other polypeptide-containing structures in vivo, but which are not components of these assembled structures when they are performing their normal biological functions. The term assembly in this definition embraces not only the folding of newly synthesized polypeptides and any association into oligomers that may occur, but also includes any changes in the degree of either folding or association that may take place when proteins carry out their functions, are transported across membranes, or are repaired or destroyed after stresses such as heat shock."* (Ellis, 1987; Ellis et al., 1989; Ellis and van der Vlies, 1991; Ellis, 1993).

Human CI chaperones

In 1998, Kuffner and colleagues used essentially the same definition (Ellis and van der Vlies, 1991) to term Complex I Intermediate Associated proteins CIA30 and CIA84 chaperones for *N. crassa* CI assembly (Kuffner et al., 1998). Disruption of the 21.3 kDa nuclear DNA-encoded subunit (homologue of the human NDUFS8 subunit) resulted in accumulation of a large membrane arm intermediate, to which CIA30 and CIA84 were found associated. Metabolic labeling experiments demonstrated that CIA84 cycles between a bound and unbound state to this intermediate. Additionally, knockout of the *cia* genes resulted in a membrane arm subunit knockout phenotype. In conclusion, the authors stated that the CIA proteins are involved in the assembly of the larger complex without being a component of the final functional structure, hence fitting the definition of chaperone (Kuffner et al., 1998).

Both human orthologues of the CIA proteins have been found. CIA84 orthologue PTCD1 was identified in a bioinformatics screen, but its function in human CI assembly remains uninvestigated (Gabaldon et al., 2005). In line with what is described for *N. crassa*, CIA30 orthologue NDUFAF1 seems to have an important role in human CI assembly as its knockdown using RNA interference resulted in impaired CI assembly/stability (Janssen et al., 2002; Vogel et al., 2005). Surprisingly, NDUFAF1 association with high-molecular weight protein complexes was only moderately decreased in a translation deficient patient cell line harbouring a mutation in mitochondrial Elongation Factor G1 (EFG1) and in an ND1 patient cybrid cell line, both displaying little or no CI and assembly intermediates (Vogel et al., 2007c). At present, in contrast to its *N. crassa* counterpart CIA30, it is still uncertain whether NDUFAF1 directly associates to CI subunits, with which its mechanism of action remains rather enigmatic.

A second assembly chaperone for human CI was predicted and confirmed in 2005 (Gabaldon et al., 2005; Ogilvie et al., 2005). In line with the above description of a chaperone, B17.2L was found associated to a large CI subcomplex, is absolutely required for assembly but not part of the final structure (Ogilvie et al., 2005). As opposed to NDUFAF1, which consistently appears associated to protein complexes of 500-850 kDa, B17.2L was found associated to a high-molecular weight CI subcomplex only after mutation of NDUFV1 or NDUFS4 subunits of CI. With the use of immunoprecipitation, in control cells, B17.2L was found associated to CI subunits ND1, NDUFS2, NDUFS3, NDUFV1, NDUFV2, NDUFS4 and GRIM-19.

The most recently identified protein demonstrated to be essential for CI assembly is Ecsit (Evolutionary Conserved Signaling Intermediate of the Toll pathway)(Vogel et al., 2007b). Ecsit is predominantly cytosolic, but a small amount is recruited to the mitochondrion via its N-terminal targeting sequence. Once imported, this mitochondrial Ecsit is incorporated into the same three high-molecular weight chaperone complexes of 500-850 kDa as NDUFAF1. Although only a relatively small amount is mitochondrial, as demonstrated by siRNA knockdown, Ecsit is required for stable mitochondrial presence of NDUFAF1, CI assembly/stability and normal mitochondrial physiology (Vogel et al., 2007b). Hence, being required for CI assembly but not a component of the final functional structure, also Ecsit fits the definition of a CI chaperone.

Complex I assembly chaperones, versatile proteins

Only a few chaperones found for an approximately 1 MDa enzyme complex is a rather meagre score. By analogy to the other OXPHOS complexes, many more must surely exist, but why have they not been detected? One obvious answer is that the *Saccharomyces cerevisiae* toolbox of genetics that has proven so fruitful for studying e.g. CIV assembly is not available for CI, as it does not have CI. Another answer may lie in the versatile nature of assembly chaperones. First of all, many yet undiscovered chaperones that can associate to CI assembly intermediates (such as B17.2L) may not be detectable in their high-molecular weight associations under normal circumstances as their binding is transient. For example, the CIA proteins in *N. crassa* were only found after introduction of a mutation that resulted in accumulation of a large membrane arm assembly intermediate (Kuffner et al., 1998). Second, most chaperones may fit the broadest possible interpretation of the classical definition of a chaperone, in the sense that

chaperones may not need to associate directly to assembly intermediates in order to have a strong influence on CI assembly or stability. A protein that influences CI assembly or stability may do so indirectly, for example by facilitating the processing or maturation of another chaperone. Third, when chaperones function in additional processes rather than being confined to a function in CI assembly, this subsequently makes them harder to find from a CI-limited perspective. Several examples of this scenario exist.

For example, B17.2L was initially described as Mimitin, the so-called myc induced mitochondrial protein (Tsuneoka et al., 2005). Its transcription was shown to be directly stimulated by c-myc and its levels were found elevated in esophageal squamous cell carcinoma (ESCC) tumors. As its suppression using RNA interference led to decreased cell proliferation in several tissue types, a role in c-myc mediated cell proliferation was proposed. It was shortly later that Ogilvie and colleagues described its requirement for human CI assembly (Ogilvie et al., 2005).

Another example is the finding of specifically impaired CI assembly/stability upon knockdown of Apoptosis Inducing Factor (AIF) in mice (Vahsen et al., 2004; Joza et al., 2005). The AIF protein is a signaling molecule in apoptosis and has an N-terminal mitochondrial localization sequence. Upon apoptosis-induced mitochondrial outer membrane permeabilisation it translocates to the nucleus, chaperoned by HSP70. Once in the nucleus, it performs a role in chromatin condensation (Modjtahedi et al., 2006). Loss of AIF leads to increased ROS production and AIF knockdown desensitises different cell types to different apoptotic stimulants. In addition to this role in apoptosis, AIF knockout results in a drop in the mouse homologues of CI subunits NDUFA9, NDUFB6, NDUFS7 and GRIM19, CI activity and embryonic lethality, demonstrating its requirement for CI integrity (Vahsen et al., 2004). Whether the effect of AIF is confined to OXPHOS complex CI alone is debated, as its *Saccharomyces cerevisiae* homologue knockout exhibit reduced growth on non-fermentable carbon sources, and siRNA for AIF additionally resulted in a slight CIII defect. Whichever the exact mechanism, a protein such as AIF shows that a protein can be a signaling molecule in apoptosis *and* be specifically required for CI assembly or stability. Such chaperones may represent signaling nodes between various subcellular processes and the assembly of the OXPHOS system.

Finally, a recent example is described in chapter 7 of this thesis, with the finding of Ecsit (Evolutionary Conserved Signaling Intermediate of the Toll pathway) inside the mitochondrion (Vogel et al., 2007b). The Ecsit protein is a signaling protein in the (cytosolic) Toll-pathway and mediates communication between ligand binding at the plasma membrane to activation of transcription of pro-inflammatory genes (Kopp et al., 1999; Kopp and Medzhitov, 1999). Ecsit is predominantly present in the cytosol, but a small amount is recruited to the mitochondrion via its N-terminal targeting sequence. Once imported, this mitochondrial Ecsit is incorporated together with CI assembly chaperone NDUFAF1 into three high-molecular weight complexes of 500-850 kDa. Although only a relatively small amount is mitochondrial, as demonstrated by siRNA knockdown, Ecsit is required for CI assembly or stabilization, normal mitochondrial physiology and for stable mitochondrial presence of NDUFAF1 in its wild type complexes (Vogel et al., 2007b). As the NDUFAF1 protein complexes exist even when CI assembly is severely impaired (chapter 6), but rely on a signaling protein for stable presence in the mitochondrion, this suggests that they may represent regulatory complexes rather than CI assembly intermediates. Although yet speculative, Ecsit may extend the cascade of the immune response to the inner-mitochondrial level, e.g. to control the amount of ATP production or to induce apoptosis upon inflammation.

CI assembly, a prominent example of mitochondrial integration with the rest of the cell?

That assembly chaperones may have additional functions apart from their requirement for CI assembly is in line with the growing awareness that mitochondria are more than just a powerhouse that provides ATP. They are plastic organelles, entangled with various (sub) cellular processes such as the cell cycle, apoptosis and development, via signaling cascades that include kinases and phosphatases that ultimately regulate mitochondrial metabolic activity (McBride et al., 2006).

Although the link between immunity and mitochondrial function of Ecsit has yet to be established, the finding of the Ecsit protein in mitochondria may represent another connection between mitochondria and non-mitochondrial process. Could mitochondria be a key link between the immune cascade and the decision to either go into apoptosis (via loss of membrane potential, release of inner membrane proteins and induction of caspases) or to activate interferons and cytokine production and promote cell survival? The recent finding of the mitochondrial antiviral signaling protein MAVS seems to support this idea. MAVS (also termed

VISA, Cardif and IPS-1) is the first described direct link between immune signaling cascades and mitochondria (Seth et al., 2005; Xu et al., 2005; Kawai et al., 2005; Meylan et al., 2005). It is bound to the mitochondrial outer membrane via its C-terminal transmembrane sequence and is required to activate pathways that lead to interferon production. Interestingly, its knockdown by siRNA leads to an increase in (mitochondria mediated) apoptosis, suggesting that MAVS could protect cells from apoptosis during the early stages of viral infection, maximizing the production of cytokines from the infected cell (McWhirter et al., 2005). Another argument in favour of a central mitochondrial role in the switch to apoptosis comes from the large body of evidence demonstrating that the release of any of several mitochondrial intermembrane proteins such as cytochrome *c*, AIF, endonuclease G and SMAC/Diablo can either initiate or amplify cell death cascades (Yi et al., 2006). Taken together, mitochondria may be at the center of a delicate balance between the host immune response and virus-induced apoptosis (Lin et al., 2006).

Is it possible that this balance is somehow controlled via maintenance of CI integrity and reactive oxygen species (ROS) production, or is this a bridge too far? Studies for the composition and function of CI have demonstrated that several CI subunits may have multiple functions in addition to their structural presence in CI. In the case of apoptosis, a prominent example is NDUFS1, for which an apoptotic function is described as a substrate of caspases, as its cleavage is a requirement for the mitochondrial changes associated with apoptosis (Ricci et al., 2004). Another example is NDUFA13 (GRIM-19, or Gene associated with Retinoid-interferon-Induced Mortality-19 in bovine CI) which is also a cell death regulatory protein induced by interferon-beta and retinoic acid and released from the mitochondrion upon apoptosis (Fearnley et al., 2001; Huang et al., 2004; Huang et al., 2007). A recent study performed by Huang and colleagues demonstrated that IFN-β/RA stimulation resulted in upregulation of the GRIM-19 and NDUFS3 subunits of CI, elevated mitochondrial reactive oxygen species (ROS) levels and induction of apoptosis (Huang et al., 2007). RNA interference (RNAi) of GRIM-19 and NDUFS3 prevented this IFN-β/RA stimulated apoptosis. Other death stimuli such as UV, CPT and staurosporine caused cell death without upregulation of GRIM-19 and NDUFS3, and RNAi for these subunits together with these stimuli did not prevent cell death (as with IFN-β/RA). Therefore, it seems that IFN-β/RA stimulation can specifically induce apoptosis via CI and ROS upregulation, hence providing another link between cytokine-induced immune pathways and mitochondrial integrity (Fearnley et al., 2001; Huang et al., 2004; Huang et al., 2007).

A final argument in favour of a prominent role for CI in mitochondrial integrity in immunity and apoptosis comes from tumor necrosis factor (TNF)-α stimulated ROS production. TNF-α is a cytokine, secreted upon inflammation, which has widespread physiological functions in immune regulation and apoptosis. Stimulation of cells with TNF-α, interleukin (IL)-1β or pathogens leads to the translocation of nuclear factor (NF)-kB to the nucleus and the activation of inflammatory response genes. In addition, signal transduction triggered by TNF-α induces an increase in intracellular ROS via the TRAF2 protein (Chandel et al., 2001). Interestingly, TNF-α induction of ROS does not occur when respiratory chain activity is obstructed using rotenone, and transfection of rho-0 cells with TRAF2 did not result in activation of NF-kB (whereas it did in control cells). It thus seems that mitochondrial ROS generation is required for NF-kB activation via TRAF2. The significant increase of ROS levels detected by DCFH-DA in response to TRAF2-mediated signaling triggered by TNF receptor-related proteins may play a regulatory role in apoptosis (Chandel et al., 2001). In addition, stimulation of human articular chondrocytes with TNF-α and IL-1β significantly decreased the activity of CI and the production of ATP, suggesting that the primary source of TNF-α induced ROS production may be CI (Lopez-Armada et al., 2006).

To control the integrity of the mitochondrial inner membrane is to control cell fate. If a stable CI is requirement for this integrity, this adds a new perspective to the investigations for CI assembly and or stability (figure 5). Putative chaperones involved in its assembly or stability may represent extensions of various cellular, cytoplasmic pathways into the mitochondrion. With this said, research for CI assembly chaperones has gained a new dimension, hopefully aiding the understanding of a broad spectrum of clinical phenotypes associated with mitochondrial disorders.

Future perspectives

Although NDUFAF1 and Ecsit are a requirement for CI assembly/stability, their function is yet unknown. Analysis of the composition of the NDUFAF1 containing protein complexes may elucidate the function of these complexes, e.g. by finding proteins involved in a well described pathway. In addition, it will be interesting to study whether mitochondrial Ecsit is indeed an extension of the cytosolic immune cascade. This could be done by analysis of CI function and stability and the

recruitment of Ecsit to mitochondria, e.g. upon microbial infection or stimulation of the Toll-like receptor pathway.

How to find more chaperones for complex I assembly? An obvious approach is to affinity purify previously described assembly intermediates and to analyse their composition e.g. by mass spectrometry. For example, by labeling the NDUFS3 subunit with an affinity purification tag and by using the proper separation technique (sucrose gradient ultracentrifugation, BN-PAGE) one should be able to affinity purify the six observed NDUFS3 containing subcomplexes. However, as assembly chaperones may only transiently bind to assembly intermediates, another sensible method is to analyse an accumulated intermediate in a disturbed assembly system, either using drugs such as doxycycline or cycloheximide or by investigating CI deficient patient cell lines which display an accumulated assembly intermediate. Other strategies are to analyze the mitochondrial proteome for proteins that are yet unannotated but are predicted to contain protein-protein interaction motifs or to perform phylogenetic analyses for genes that have co-evolved with CI subunits. Whichever the exact strategy may be, future studies will surely deliver more than the currently known chaperones B17.2L, NDUFAF1 and Ecsit.

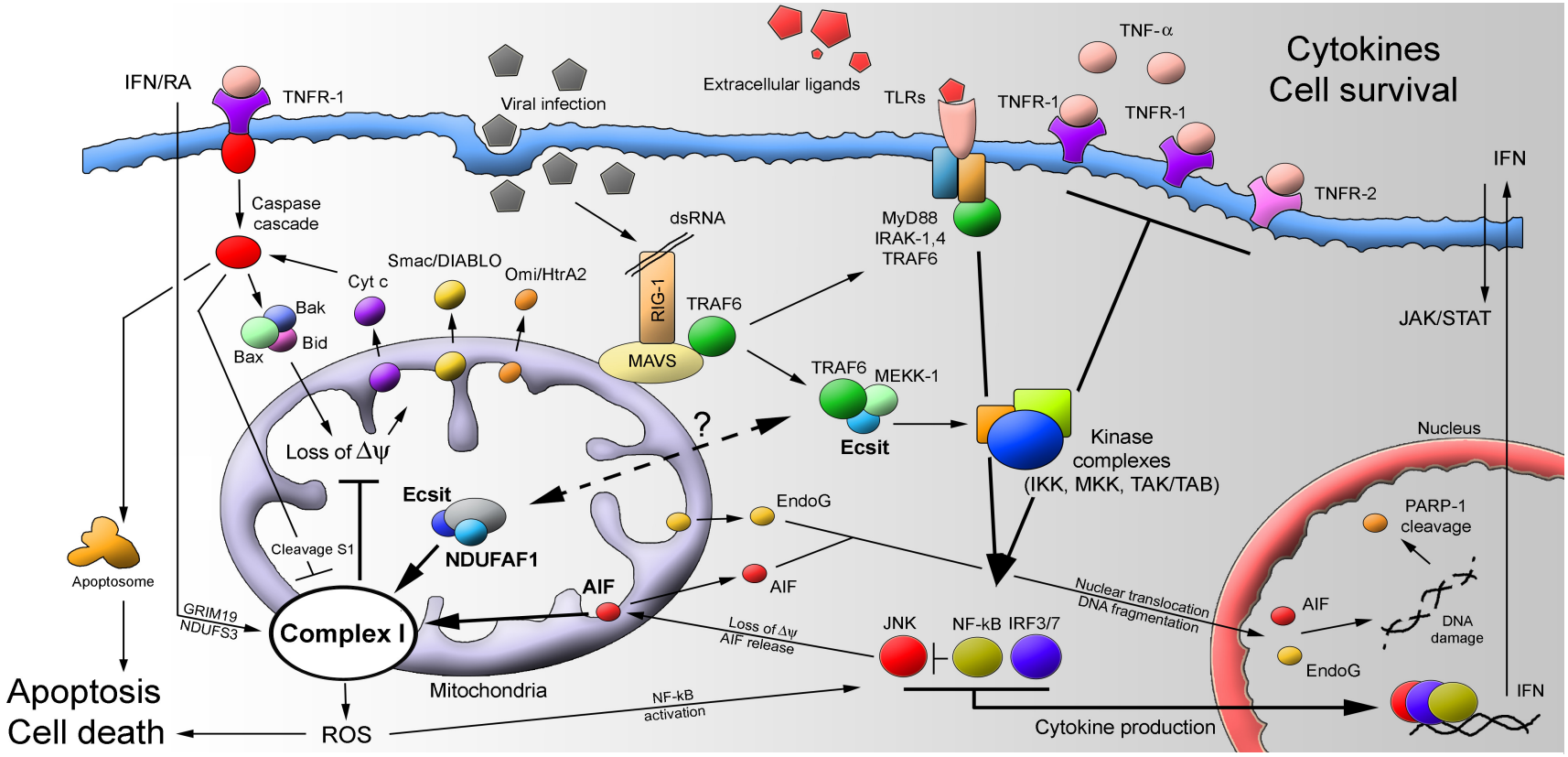


Figure 5. Mitochondria on the crossroads between immune response and apoptosis

Extracellular ligands and cytokines such as TNF are recognized at the plasma membrane via Toll-like receptors (TLRs) and TNF receptors 1 and 2 (TNFR-1, 2) respectively, which trigger cascades that stimulate the production of cytokines such as interferons (IFN) via nuclear translocation of transcription factors such as NF-kB. Alternatively, the cell can switch on apoptotic pathways via DNA caspase cleavage, disturbance of the mitochondrial membrane potential via Bid, Bax and Bak and subsequent release of pro-apoptotic factors such as cytochrome c, Smac/DIABLO and Omi/HtrA2. In addition, caspase independent cell death can be induced with the release of apoptosis inducing factor (AIF) and Endonuclease G (EndoG) from the mitochondrion. Mitochondria, and CI in particular, may constitute an important factor in the choice between cytokine production and apoptosis. Apoptosis is linked to CI integrity via NDUFS1 cleavage, ROS production and release of AIF, required for CI stability, from the mitochondrion. Immune pathways have recently been linked to mitochondria via the finding of mitochondrial antiviral signaling protein (MAVS) at the mitochondrial surface, which links recogition of dsRNA (an intermediate of viral replication) to production of interferons. Cytosolic signaling molecule Ecsit, part of the Toll-like receptor cascade, is found inside the mitochondrion. As Ecsit is required for CI stability/assembly, Ecsit may represent a mitochondrial extension of the immune pathway, controlling cell fate by controlling mitochondrial integrity via CI (question marks).

Final remarks

In conclusion, the complicated sequence of events eventually resulting in combination of the 38 nuclear DNA-encoded and seven mtDNA-encoded CI subunits requires the action of many proteins that are not part of the final functional structure. The entire process from translation to assembly encompasses a multitude of processes ensuring the proper import, processing and stabilization of subunits and subcomplexes. Once at the level of subcomplexes, based on assembly studies performed in different organisms, this most likely results in the membrane anchoring of a scaffold of highly conserved nuclear DNA-encoded CI subunits and subsequent expansion with preassembled dehydrogenase and proton translocation modules. Regarding the human CI assembly model, whether membrane insertion truly occurs upon addition of mtDNA-encoded subunit ND1 is yet to be demonstrated, as is the existence of intermediates for membrane arm subunits and the dehydrogenase (flavoprotein) assembly module. In any case, the proposed model serves as a solid framework for future studies for CI assembly.

The role of assembly chaperones in this process could be more diverse than previously thought. On the one hand, the direct association of B17.2L to CI subunits shows that chaperones can directly affect assembly or stability of CI substructures. On the other, the association of assembly chaperone NDUFAF1 with cytosolic signaling molecule Ecsit in structures that may not represent assembly intermediates suggests that some chaperone proteins may excert their function via regulatory complexes. Regulation could e.g. take place via direct feedback between assembly and activity of mitochondrial respiration and the particular energy requirements of the cell. If such a system exists, this opens up a new field in mitochondrial research in which not the assembly process itself, but a mechanism regulating the activity of the process is the focus of attention. Further investigation of this possibility aids the elucidation of both the mechanism of CI assembly and the understanding of many yet poorly understood CI deficiencies.

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Summary

Complex I (CI) assembly is a prominent example of how communication between the mitochondrion and the nucleus is required to make mitochondrial protein complexes, and investigation of the process will deliver insight into how the initial symbiosis between proto-mitochondrion and eukaryotic cell has developed into evolutionary integration. In a more applied context, in the case of mutation in one of the 45 CI subunits, studying disturbed CI assembly can aid the understanding and diagnosis of the many yet unexplained CI deficiencies. These are the major cause of mitochondrial disorders, which often result in severe multi-system disorders and ultimately in early childhood death.

To understand the assembly of a macromolecular mitochondrial complex such as CI we should study both the intermediate assembly steps and the function of proteins proposed to coordinate and chaperone its assembly.

Chapter 1 serves as an introduction to the research field of CI and provides an overview of the multiple processes involved in its assembly. Following this introduction, the thesis is divided into two parts. Part I describes investigations for the specific subassemblies that occur during the course of CI assembly. Based on these data, the first CI assembly models have been established. Part II discusses the existence and function of CI assembly chaperones.

PART I: The complex I assembly scheme

Theoretical studies have indicated that groups of CI subunits have co-evolved as modular structures in different organisms. **Chapter 2** discusses the possibility that CI assembly reflects this modular build-up in the formation of modular subassemblies. Experimental support for this is presented in **Chapter 3**, in which CI assembly is investigated in a conditional assembly system. In this system, mitochondrial translation was first inhibited resulting in depletion of mtDNA encoded CI subunits, hence severely hampering CI assembly. Upon removal of this inhibition, the formation of specific subassemblies, indicative of assembly intermediates, could be studied. Composition analysis of the subassemblies demonstrated that the two structural arms of CI, the peripheral and membrane arms, assemble via distinct subassemblies corresponding to the modular evolution scheme. A different strategy to study CI assembly is described in **Chapter 4**. By

using leakage expression of an inducible NDUFS3-GFP construct, steady state labelling of six subassemblies was observed, of which two clearly accumulated after inhibition of mitochondrial translation. Removal of the inhibition resulted in the reversal of this effect and formation of larger subcomplexes, strongly suggesting that these two subcomplexes are true assembly intermediates which require mtDNA-encoded subunits for progression in assembly. By analysis of the stability of the observed subcomplexes, several breakdown products could be distinguished that can confuse (future) assembly analyses. Altogether, these studies have contributed to our insight into which assembly intermediates occur and which are of particular importance, providing a framework model for complex I assembly.

PART II: Complex I assembly chaperones

By analogy to the other oxidative phosphorylation complexes, many CI assembly chaperones are speculated to exist, but only two were found during the course of this project: B17.2L and NDUFAF1. Although NDUFAF1 is a homologue of *Neurospora crassa* CI assembly chaperone CIA30, it was yet unclear whether it is also an orthologue. Therefore, in **Chapter 5**, we investigated its subcellular localization and the effect of its knockdown using RNA interference. NDUFAF1 was specifically targeted to mitochondria and appeared present in several high-molecular weight complexes. The specifically impaired CI activity and assembly after RNA interference demonstrated that NDUFAF1 is absolutely required for CI assembly in human mitochondria, supporting orthology between CIA30 and NDUFAF1. **Chapter 6** describes the occurrence of chaperones B17.2L and NDUFAF1 in a set of CI deficient patients displaying varying assembly disturbances. B17.2L specifically appeared in a 830 kDa subcomplex in patients that carry a mutation in CI subunits NDUFS4 or NDUFV1, whereas NDUFAF1 association to high-molecular weight protein complexes was only mildly different between the patients. Furthermore, even though a patient harbouring a mutation in mitochondrial elongation factor G1 displayed no CI assembly intermediates, NDUFAF1 was nevertheless present in its high-molecular weight associations. This suggests that the NDUFAF1-containing protein complexes may not represent assembly intermediates and that B17.2L and NDUFAF1 may operate differently in assembly. A novel CI chaperone, Ecsit, is described in **Chapter 7**. Ecsit is known as a cytosolic signaling intermediate in the Toll pathway, a cascade that mediates transcription of pro-inflammatory genes in response to binding of antigens at the plasma membrane. This chapter describes the unexpected finding of Ecsit in mitochondria, in the same mitochondrial high-molecular weight complexes as

NDUFAF1. Its knockdown results in decreased NDUFAF1 amount, specifically impaired CI assembly or stability and mitochondrial dysfunction. Ecsit may constitute a link between immunity and mitochondrial function, e.g. by regulation of the energy requirement or induction of apoptosis upon microbial or viral infection, via CI.

In conclusion, **Chapter 8** provides the general discussion, reflecting on both parts of this thesis. In part I, CI assembly is compared between different organisms in order to extract a general mechanism. It seems that assembly initiates with membrane anchoring of a highly conserved core of nuclear DNA-encoded CI subunits, which is subsequently expanded with membrane and peripheral arm modules. Part II discusses the function of molecular chaperones in CI assembly. The classical definition of a chaperone is discussed in relation to the function of currently known CI chaperones. Finding of the novel chaperone Ecsit in mitochondria signifies that CI-specific assembly chaperones may have an additional, regulatory function and are not simply the 'glue' that sticks assembly subcomplexes together. Discovery of mitochondrial Ecsit is in line with recent findings demonstrating that mitochondrial and immune function are closely related. If mitochondrial Ecsit is truly an extension of the immune cascade into mitochondria, the effects of disturbed CI assembly could be greater than previously anticipated which may help in explaining a broader spectrum of the clinical consequences of disturbed CI assembly.

Samenvatting

Complex I (CI) assemblage is een vooraanstaand voorbeeld van hoe communicatie tussen het mitochondrion en de celkern nodig is voor de vorming van mitochondriële eiwitcomplexen. Door het bestuderen van het assemblageproces kan dus waardevolle informatie worden vergaard over hoe de symbiose tussen het proto-mitochondrion en de eukaryote cel zich heeft ontwikkeld tot evolutionaire integratie. In een meer toegepaste context, in gevallen van mutatie in één der 45 eiwitonderdelen (de zogenaamde subunits) van CI, kan het bestuderen van verstoorde CI assemblage leiden tot een beter begrip en betere diagnose van tot op heden onverklaarde CI deficiënties. Deze zijn de belangrijkste oorzaak van mitochondriële ziekten, welke vaak resulteren in ernstige multi-systeem defecten en uiteindelijk in dood op jonge leeftijd.

Voorwaarde voor het begrijpen van de assemblage van een macromoleculair mitochondrieel eiwitcomplex als CI is het bestuderen van zowel de tussenstappen van het assemblageproces (de zogenaamde intermediairen) als de eiwitten die het proces coördineren en vergemakkelijken (de zogenaamde chaperones).

Hoofdstuk 1 dient ter inleiding op het CI onderzoek en geeft een overzicht van de verschillende processen die leiden tot haar uiteindelijke assemblage. Na deze introductie is dit proefschrift opgedeeld in twee delen. Deel I beschrijft onderzoek naar welke intermediairen worden gevormd tijdens het assemblageproces, wat uiteindelijk heeft geleid tot het opstellen van de eerste assemblagemodellen. Deel II beschrijft het bestaan en de functie van CI assemblage chaperones.

Deel I: Het complex I assemblage model

Theoretische studies hebben aangetoond dat groepen van CI subunits zijn geco-evolueerd als modulaire structuren in verschillende organismen. **Hoofdstuk 2** bespreekt de mogelijke overeenkomsten tussen CI assemblage en deze modulaire opbouw. Experimentele aanwijzingen voor dit idee worden besproken in **Hoofdstuk 3**, waarin CI assemblage wordt bestudeerd in een conditioneel assemblage systeem. In dit systeem wordt mitochondriële eiwitsynthese eerst geremd waardoor CI assemblage ernstig wordt verstoord. Vervolgens wordt deze remming verwijderd waardoor CI assemblage weer kan plaatsvinden en assemblage intermediairen ontstaan. Analyse van de samenstelling van deze

intermediairen toonde aan dat de twee structurele armen van CI, de perifere en de membraan arm, worden opgebouwd uit intermediairen die overeenkomen met de modulaire opbouw van CI. Een andere strategie om CI assemblage te onderzoeken wordt besproken in **Hoofdstuk 4**. Door het gebruik van zeer milde constitutieve expressie van een induceerbaar NDUFS3-GFP construct kan de vorming van CI assemblage intermediairen die NDUFS3-GFP bevatten direct worden gevolgd in de tijd. Op deze manier zijn zes intermediairen geïdentificeerd, van welke er twee specifiek ophopen na remming van mitochondriële eiwitsynthese. Het verwijderen van de remming doet deze ophoping verdwijnen en leidt tot het ontstaan van grotere intermediairen en CI. Dit toont aan dat deze intermediairen zeer waarschijnlijk echte assemblage intermediairen zijn welke mitochondrieel DNA gecodeerde CI subunits nodig hebben om voort te gaan tijdens assemblage. Analyse van de stabiliteit van de onderzochte intermediairen toonde bovendien aan welke intermediairen mogelijk afbraakproducten van CI zijn, wat verwarring in toekomstige studies naar CI assemblage kan voorkomen. Tesamen hebben genoemde studies bijgedragen aan het inzicht van welke assemblage intermediairen voorkomen en welke van cruciaal belang zijn.

Deel II: Complex I assemblage chaperones

Analoog aan de andere oxidatieve phosphorylering complexen wordt vermoed dat er ook voor CI veel assemblage chaperones moeten bestaan. Er zijn er echter maar twee gevonden tijdens de looptijd van dit project: B17.2L en NDUFAF1. Hoewel NDUFAF1 bekend is homoloog te zijn aan *Neurospora crassa* CI assemblage chaperone CIA30, was onbekend of het ook een ortholoog is. Daarom is in **Hoofdstuk 5** onderzocht of NDUFAF1 ook mitochondrieel is en wat het effect is van zijn afwezigheid middels RNA interferentie. NDUFAF1 bleek mitochondrieel en RNA interferentie leidde tot een sterke afname in CI assemblage en activiteit, wat ondersteunt dat NDUFAF1 net als CIA30 in *Neurospora crassa* nodig is voor CI assemblage. In **Hoofdstuk 6** wordt onderzocht hoe B17.2L en NDUFAF1 associëren met hoogmoleculair gewicht eiwitcomplexen in een groep CI deficiënte patiënten. Het bleek dat waar B17.2L specifiek associeerde met een 830 kDa groot complex in patiënten met een NDUFS4 of NDUFV1 mutatie, NDUFAF1 juist weinig tot geen variatie vertoonde in zijn associatie met eiwitcomplexen tussen de patiënten. Zelfs in een patient met een mutatie in mitochondrieel elongatie factor G1, welke geen CI intermediairen vertoonde, was NDUFAF1 nog aanwezig in de eiwitcomplexen. Dit steunt de gedachte dat de NDUFAF1 eiwitcomplexen mogelijk geen assemblage intermediairen zijn en dat B17.2L en NDUFAF1 op een

verschillende manier betrokken zijn in het assemblageproces. Een nieuwe CI assemblage chaperone, Ecsit, wordt beschreven in **Hoofdstuk 7**. Ecsit is bekend als een cytosolisch eiwit betrokken bij de Toll-receptor gemedieerde immuuncascade, welke ligandbinding aan het plasmamembraan koppelt aan de productie van cytokines. Dit hoofdstuk beschrijft de verrassende aanwezigheid van Ecsit in het mitochondrion, waar het in dezelfde eiwitcomplexen aanwezig is als NDUFAF1. RNA interferentie van Ecsit leidde tot een afname in NDUFAF1, verstoorde CI assemblage of stabiliteit en mitochondriële disfunctie. Mogelijk is Ecsit een link tussen de immuunrespons en mitochondriële functie, bijvoorbeeld door regulatie van de energiebehoefte of inductie van apoptose na infectie, via CI.

Tenslotte levert **Hoofdstuk 8** de algemene discussie, waarin wordt teruggekeken naar beide delen van dit proefschrift. In deel I wordt CI assemblage tussen verschillende organismen vergeleken om een algemeen assemblage mechanisme op te stellen. Het blijkt dat assemblage begint met het koppelen van een deel van CI aan het membraan, waarna het deelcomplex verder wordt uitgebreid met membraan en perifere arm modules. Deel II bespreekt de functie van bekende CI assemblage chaperones en spiegelt deze aan de klassieke definitie van een chaperone. De vondst van de nieuwe chaperone Ecsit in het mitochondrion toont aan dat CI specifieke assemblage chaperones mogelijk additionele, regulatieve functies kunnen hebben en niet simpelweg de 'lijm' zijn die intermediairen aan elkaar plakt. Mitochondrieel Ecsit past goed in data uit recente publicaties die een hechte relatie tussen immuunrespons en mitochondrion suggereren. Als mitochondrieel Ecsit inderdaad een extensie is van de immuunrespons tot in mitochondrion, zou dit kunnen helpen in het verklaren van een breder spectrum van de klinische consequenties van verstoorde CI assemblage.

Samenvatting voor niet-ingewijden

Ons lichaam bruist van activiteit. Of we nu rustig op de bank zitten, een marathon lopen, of een appeltje eten, we worden continu van energie voorzien door onze stofwisseling. Die stofwisseling zorgt er onder meer voor dat het pas verorberde appeltje wordt afgebroken tot moleculaire bouwstenen, welke worden ingezet voor lichaamsonderhoud, groei en, als voornaamste, aanmaak van energie in de vorm van adenosine trifosfaat (ATP). Een volwassen man zet per dag wel 65 kg van deze energiedrager om. Dat is leuk om te weten, maar in het dagelijkse leven is energie zo vanzelfsprekend dat we er niet bij stilstaan. Behalve als iemand in onze naaste omgeving wordt getroffen door een erfelijke stofwisselingsziekte.

Erfelijke stofwisselingsziekten zijn aangeboren stoornissen in de stofwisseling. Deze kennen vele verschijningsvormen, waarbij het Nijmegen Center for Mitochondrial Disorders (NCMD) zich richt op mitochondriële ziekten. Deze ziekten worden veroorzaakt door stoornissen in de energiefabriek van de cel: het mitochondrion. Binnen het NCMD verdiept onze onderzoeksgroep zich specifiek in een der mitochondriële ziekten genaamd complex I deficiëntie. Complex I is één van de vijf enzymcomplexen uit het oxidatieve fosforyleringssysteem, dat het grootste aandeel van de mitochondriële ATP voorziening voor zijn rekening neemt. Zoals de naam al doet vermoeden, is bij complex I deficiëntie de primaire oorzaak van de stofwisselingsziekte een tekort aan of slecht functionerend complex I. Vaak zijn meerdere, veel energie verbruikende organen tegelijk aangedaan zoals de hersenen, ogen, hart- en skeletspieren, lever en nieren. Deze zogenaamde multisysteme defecten hebben een incidentie van 1:10.000 levende geboorten en resulteren meestal in een dood op jonge leeftijd. Helaas is er nog weinig inzicht in de moleculaire oorzaak van deze deficiënties, waardoor het opzetten van diagnostische en therapeutische strategieën tot op heden problematisch is gebleken.

In de meeste gevallen van complex I deficiëntie speelt verstoorde aanmaak van complex I een prominente rol. Deze aanmaak vereist communicatie tussen de celkern en het mitochondrion, waarbij de 38 kerngecodeerde en 7 door het mitochondrieel DNA gecodeerde eiwitten worden gecombineerd tot een functioneel enzymcomplex. Mijn promotieonderzoek heeft zich bezig gehouden met dit zogenaamde assemblageproces. Het bestuderen hiervan dient om inzicht te verkrijgen in de moleculaire achtergrond van complex I deficiëntie ten behoeve van

het ontwikkelen van diagnostiek, en ter bevordering van fundamenteel inzicht in de aanmaak van oxidatieve phosphoryleringscomplexen.

Dit proefschrift beschrijft in twee delen de uitkomst van vier jaar onderzoek naar het assemblageproces van complex I. Deel I beschrijft drie studies naar hoe de afzonderlijke bouwstenen van complex I worden gecombineerd tot een volwassen enzymcomplex. Allereerst is een hypothetisch assemblagemodel opgesteld naar aanleiding van onderzoeken naar de structuur van het complex in verschillende organismen. Vervolgens zijn twee studies beschreven met tot doel het vinden van tussenstappen in het assemblageproces. Deze studies hebben geleid tot de identificatie van enkele belangrijke tussenstappen en uiteindelijk tot een basismodel voor complex I assemblage. In deel II van dit proefschrift zijn hulpeiwitten bij assemblage, de zogenaamde chaperone eiwitten, onderzocht. Deze zijn noodzakelijk voor het juist verlopen van het proces, maar maken geen onderdeel uit van het gevormde enzymcomplex. In de drie beschreven studies is achtereenvolgens het belang van één van die hulpeiwitten aangetoond, is de verschijningsvorm van hulpeiwitten in een groep patienten met verschillende stoornissen in complex I assemblage onderzocht en is een nieuw hulpeiwit ontdekt, genaamd Ecsit. Verrassend genoeg was Ecsit voorheen bekend van zijn rol in de immuunrespons, een proces waarvan lang werd gedacht dat het niets met het mitochondrion te maken heeft.

Dit laatste ondersteunt de gedachte dat het mitochondrion geen autonome energiecentrale binnen de cel is, maar juist vervlochten met cellulaire processen zoals gereguleerde celdood (apoptose) en de immuunrespons. De mogelijke koppeling tussen complex I assemblage en andere processen blijkt hiervan een uitstekend voorbeeld te zijn. Ten eerste zijn verscheidene complex I subunits betrokken bij additionele processen zoals vetzuursynthese en apoptose. Ten tweede blijken assemblage chaperones ook betrokken bij respectievelijk myc-geinduceerde celproliferatie, de immuunrespons en gereguleerde celdood.

Hopelijk leiden toekomstige studies binnen deze veelbelovende onderzoekslijn tot een verdere bevestiging van het belang van complex I assemblage voor mitochondriële en cellulaire functie. Implicaties in celdood en immuunrespons zouden bijvoorbeeld het begrip kunnen vergroten van waarom complex I deficiënte patiënten zo snel achteruit gaan na een virale infectie. In een bredere context kan de bijdrage van mitochondriën aan erfelijke stofwisselingsziekten en processen als Parkinson, kanker en veroudering beter worden verklaard. In al deze gevallen

speelt onze energievoorziening een belangrijke rol. Opdat u daaraan denkt bij uw volgende appeltje.

Dankwoord

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* Joint first authorship

This thesis was laboratory tested