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Supernumerary subunits NDUFA3, NDUFA5 and NDUFA12 are required for the formation of the extramembrane arm of human mitochondrial complex I

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ABSTRACT

Mammalian complex I is composed of fourteen highly conserved core subunits and additional thirty subunits acquired in the course of evolution. At present, the function of the majority of these supernumerary subunits is poorly understood. In this work, we have studied NDUFA3, NDUFA5 and NDUFA12 supernumerary subunits to gain insight into their role in CI activity and biogenesis. Using human cell lines in which the expression of these subunits was knocked down with miRNAs, we showed that they are necessary for the formation of a functional holoenzyme. Analysis of the assembly intermediates in mitochondria depleted for these subunits further suggested that they are required for assembly and/or stability of the electron transferring Q module in the peripheral arm of the CI.

1. Introduction

Mitochondrial respiratory chain complex I (CI; NADH ubiquinone oxidoreductase; E.C. 1.6.5.3) transfers electrons from reduced NADH to ubiquinone coupled to proton pumping which creates an electrochemical gradient across the inner mitochondrial membrane thereby providing about 40% of the proton-motive force used by ATP synthase to produce ATP [1,2]. In accordance with its major role in cell bioenergetics, isolated CI deficiency is the most common cause of mitochondrial disease in infancy and childhood [3,4]. Importantly, the genetic cause of a half of these deficiencies is still unknown. Mammalian CI, with a molecular weight of 1 MDa, is also one of the largest multiprotein complexes in nature. Unlike its bacterial ancestor which has been crystalized recently [5], the structure of mammalian complex is not available. However, the architecture and the subunit compositions of the complex have been determined [6,7,8,9]. In humans it is composed of 44 subunits: 14 highly conserved subunits building up the L-shaped catalytic core sufficient for activity and found in all organisms expressing CI, and 30 so-called supernumerary subunits acquired in the course of evolution. Notably, the function(s) of a majority of these supernumerary subunits is as yet mostly unknown, but they are often suggested to be involved in CI assembly, stability, regulation of its activity or protection against oxidative stress damage. Indeed, mutations in several supernumerary subunits (NDUFA1, NDUFA2, NDUFA10, NDUFS4, and NDUFS6) have been reported to affect CI assembly and/or stability [10,11,12,13,14]. Numerous supernumerary subunits (NDUFS4, NDUFB11, NDUFA1, NDUFA7, and NDUFA10) have been found to be targets for phosphorylation by cAMP-dependent protein kinase (mtPKA) and thus they are likely to play a part in the regulation of CI activity and biogenesis [15]. The supernumerary proteins may also participate in other cellular pathway(s). For instance, NDUFAB1 has an acyl carrier protein domain therefore it could participate in fatty acid synthesis. NDUFA9 which comprises NADPH binding site and belongs to the short-chain dehydrogenases/reductases (SDR) superfamily could be involved in the metabolism of a number of compounds [16]. Finally, NDUFA13, is known to be involved in cell death and was originally identified as an interferon- and retinoic acid-inducible proapoptotic gene, GRIM-19 (Genes associated with Retinoid-IFN-induced Mortality-19) [17] (see [18,19] for detailed review on supernumerary subunits function).

In the present study we have sought to gain insight into the role of three supernumerary CI subunits: NDUFA3, NDUFA5 and NDUFA12. Mutation in NDUFA12 was previously shown to cause Leigh syndrome in one patient [20] and NDUFA5 knock out was shown to elicit mild chronic encephalopathy in mice [21]. To date, NDUFA3

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has not been linked to any human pathology and the function of this protein is entirely unknown. Structural information on individual supernumerary subunits is very limited. However, based on their protein sequence and proteomic analysis of different subcomplexes [22], NDUFA5 and NDUFA12 subunits are predicted to be localized in the extramembrane arm (Iλ subcomplex) or at the junction of extramembrane and membrane arms of the complex (Iα subcomplex). NDUFA3, which is predicted to contain a single transmembrane helix domain, is likely to be anchored in the membrane at the junction of both arms and has been found in the Iα subcomplex. In this study, we present evidence that NDUFA3 and NDUFA5 are required for assembly and/or stability of the matrix arm of CI in human mitochondria while NDUFA12 is more likely to facilitate or stabilize the interaction of this arm with the membrane module during CI assembly. Because of their requirement for the formation of a functional CI, these subunits should also be considered as potential targets for human CI-related pathologies where any genetic cause could not be identified so far.

2. Materials and methods

2.1. Flp recombinase-mediated RNA interference in HEK293 cells

Human Embryonic Kidney (HEK293) Flp-In cells (Invitrogen, St. Quentin en Yvelines, France) were cultured in DMEM medium containing 4 g/L glucose, supplemented by 2 mM glutamine, 10% fetal calf serum, 1 mM pyruvate, 100 μg/ml penicillin, streptomycin and zeocin (Invitrogen). HEK293 Flp-In cells were seeded in 12-well plates to reach 70% confluence on the following day, then transfected using Lipofectamin 2000 (Invitrogen) according to manufacturer procedure. Cells were co-transfected with 1.2 μg of pOG44 Flp recombinase expression plasmid, and 0.15 μg pEF5FRT/V5-GW-miR plasmid with miRNA previously cloned using miR pol II RNAi kit, LR clonase II kit, and BP clonase II kit (Invitrogen). Two days following transfection, 150 μg/ml hygromycin B were added in order to select for appropriate targeting of constructs. Recombined cells were subsequently cloned by dilution.

2.2. Quantitative RT-PCR

Gene silencing was analyzed by quantitative RT-PCR using the iQ SYBR Green Supermix and CFX-96 (Bio-Rad, Marnes-la-Coquette, France). Total mRNAs were extracted from cell pellets using RNeasy and QIAshredder kits (Qiagen, Courtaboeuf, France), reverse-transcribed using Superscript II kit (Invitrogen, St. Quentin en Yvelines, France) and amplified (45 cycles of a three-step procedure: 20 s denaturation at 96 °C; 20 s annealing at 56 °C; 20 s extension at 72 °C). HPRT was used as an internal control.

2.3. Isolation of mitochondria from HEK293 cells

Cells were collected by pipetting, washed twice with cold PBS and resuspended in five volumes of MSTE buffer (225 mM mannitol, 75 mM sucrose, 0.1 mM EGTA, 30 mM Tris–HCl, pH 7.4, and 1 mM PMSF). Cells were homogenized in a glass potter with 30 strokes and large debris were spun down twice at 500*g* for 5 min. The clarified homogenate was then centrifuged at 21 000*g* for 15 min to pellet mitochondria. The pellet was suspended in MHE buffer (250 mM mannitol, 5 mM HEPES (pH 7.4) and 0.5 mM EGTA) and the concentration of protein was determined by the Bradford method.

2.4. Gel electrophoresis

For Western blot analysis of CI subunits levels in the silenced cell lines, mitochondrial proteins (50 μg) were separated by SDS–PAGE on a 12% polyacrylamide gel [23], transferred to a nitrocellulose membrane and probed with antibodies against the subunit of interest. The antibody complexes were visualized with Western Lightning Ultra Chemiluminescent substrate kit (Perkin Elmer). For the analysis of respiratory chain complexes, mitochondrial proteins (100 μg) were extracted with 6% digitonin and separated by BN-PAGE according to Wittig et al. [24] or hrCN-PAGE according to Wittig et al. [25], on a 3.5–12% polyacrylamide gel. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and probed with antibodies against CI–CV. A separate set of gels were stained by in gel activity assay (IGA) detecting CI, CII and CIV activity as described in Wittig et al. [25].

2.5. Respiratory chain enzyme assays

Cells were collected by pipetting, washed twice with cold PBS and frozen as dry pellets (10^6 cells). Cells were subsequently thawed using 1 ml of ice-cold solution consisting of 0.25 M sucrose, 20 mM Tris–HCl (pH 7.2), 2 mM EGTA, 40 mM KCl, 1 mg/ml bovine serum albumin, 0.01% digitonin (w/v). After 5 min incubation on ice, cells were centrifuged (2500*g*, 5 min, 4 °C), and the pellets resuspended in 50 μl of the same solution devoid of digitonin. Activities of respiratory chain complexes were spectrophotometrically measured using a Cary 50 UV–visible spectrophotometer (Varian Inc., Les Ulis, France) as previously described [26,27]. All chemicals were from Sigma–Aldrich (St. Quentin, Falavier, France).

3. Results

3.1. Stable and uniform depletion of CI subunits

The functions of supernumerary CI subunits were examined in human cell lines in which their expression was knocked down with miRNAs designed to induce degradation of the target mRNA (Table 1). The main drawback of the vectors commonly used for stable expression of transgenes, is the risk of their random integration into sites spread over the entire host genome. To avoid this problem, we targeted insertion of miRNAs by homologous recombination using the Flp-In system. This approach was successfully used in our previous study of the CI supernumerary subunit, NDUFB6 [28]. Expression of three supernumerary CI subunits NDUFA3, NDUFA5 and NDUFA12, and one core subunit NDUFV1, used as a control, was knocked down in HEK293 Flp-In cells containing a unique FRT site in their genome. Analysis of the target transcripts by quantitative RT-PCR confirmed that miRNAs mediated gene silencing from the FRT site was efficient (70–90% decrease in targeted mRNA; Fig. 1A) and a residual amount of mRNA correlated with the protein level in the mitochondria as assessed by Western blot analysis (Figs. 1B and 1S).

3.2. The supernumerary subunits: NDUFA3, NDUFA5 and NDUFA12 are required for CI activity assuring its assembly and/or stability

Cell lines depleted of NDUFA3, NDUFA5 and NDUFA12 showed a significant decrease in CI activity (between 50% and 80%; Fig. 2),

Table 1
Sequences of miRNA expressed in HEK293 Flp-In cells.

Scrambled	AAATGTACTGCGCGTGGAGAC
NDUFV1 miR 1	CATTCTTGCCAATCAGACCTG
NDUFV1 miR 2	TTCAAGGGCACAGACATCTCC
NDUFA3 miR 1	TCATGACGGAGTACTTGAAGT
NDUFA3 miR 2	TTGTTGATCATGACGGAGTAC
NDUFA5 miR 1	AACCATAGCCAGCTTCTCATT
NDUFA5 miR 2	ACAATATTCTTAGCCTCTCGT
NDUFA12 miR 1	TGTACCAACCTTCGCATCATT
NDUFA12 miR 2	AATGTACCAACCTTCGCATCA

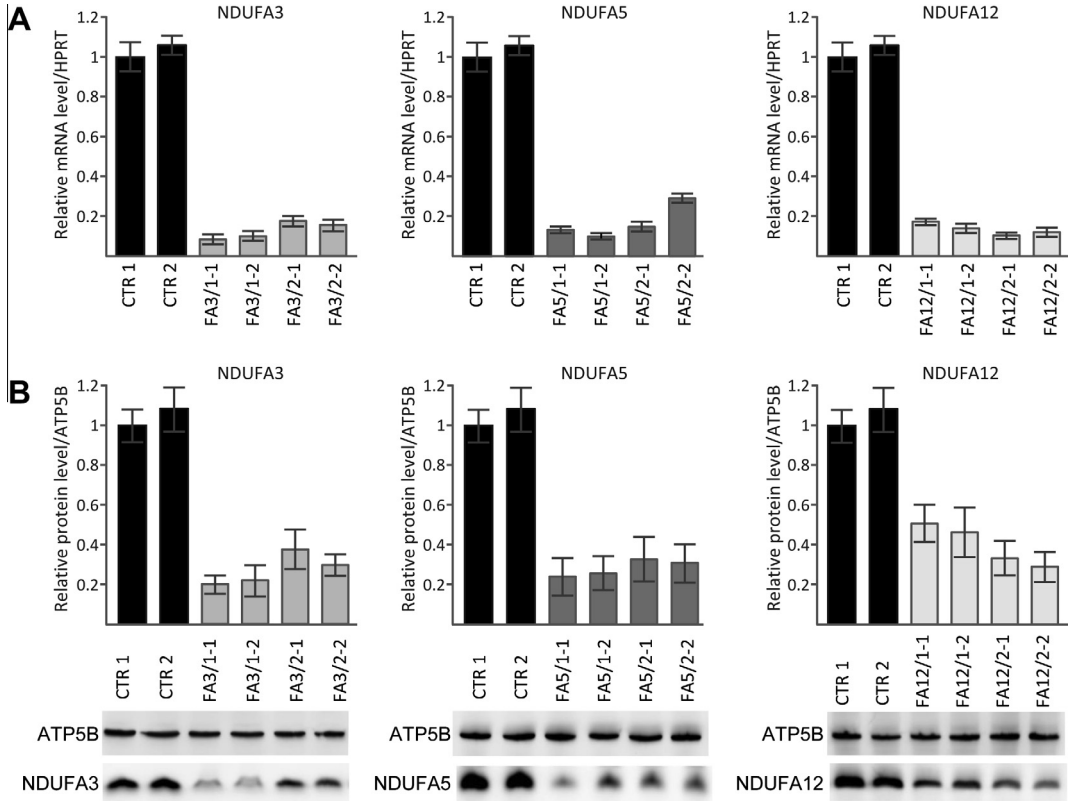

Fig. 1. Efficiency of CI supernumerary subunits silencing. (A) Levels of NDUFA3, NDUFA5 and NDUFA12 mRNAs in control cells (CTR1, CTR2; scrambled miRNAs) and miRNA-silenced cell lines (two different miRNAs for each gene: FA3/1 and FA3/2; FA5/1 and FA5/2; FA12/1 and FA12/2; Two clones for each miRNA: FA3/1-1, 1-2; FA3/2-1, 2-2 *etc.*) were analyzed by quantitative PCR (q-PCR) and normalized to housekeeping gene HPRT. (B) Accumulation of corresponding subunits in mitochondria was visualized by Western blot and quantified after normalization to ATP synthase β subunit ATP5B (lower panel). Values are means ± SD.

correlating with levels of targeted subunits in mitochondria. In contrast, activities of other respiratory chain complexes were not significantly affected in the depleted cell lines (Fig. 2, lower panels). These results indicate that all three studied proteins are specifically required for CI function. In order to better characterize their involvement, we have assessed the assembly state of the respiratory chain in these cells (Fig. 3). We isolated mitochondria from the most efficiently silenced cell lines (two cell lines for each studied gene). Mitochondrial proteins were extracted with 6% digitonin and separated by blue native (BN) or clear native (CN) polyacrylamide gel electrophoresis. The complexes were then analyzed either by Western blot, after their transfer to PVDF membrane or directly, by *in-gel* activity assay (IGA). Western blot with antibody directed against NDUFA9 and staining CI (Fig. 3A, upper panel), as well as CI-IGA assay (Fig. 3B and C), showed a significant decrease of assembled CI in all cell lines depleted for supernumerary subunits. This decrease correlated with residual levels of targeted subunits and ranged from 29% to 35% of the control (CI-IGA; Fig. 3C) or 20% to 44% (CI-Western blot; Fig. S2). Reduced expression of the core subunit NDUFV1 also resulted in the significant reduction of the holoenzyme. In contrast, the remaining subunits of CI could still assemble to form a large subcomplex (CI*), which presumably corresponds to the 830 kDa complex described in cells from NDUFV1, NDUFS4, NDUFS6 and NDUFA2 patients [12,29,30,31,14,11]. As shown in Fig. 3A and B (band marked with asterisk), this complex was able to interact with CIII and CIV to form a large complex that migrated slightly ahead of the normal supercomplex (see below).

Together, these analyses show that silencing of any of the three studied supernumerary subunits results in loss of CI activity due to the decreased accumulation of assembled enzyme thus pointing to a role of these subunits in CI assembly and/or stability.

3.3. Impact of the supernumerary subunits knock-down on respirasome formation

CI is known to interact with other respiratory chain complexes, CIII and CIV, to form a respirasome comprising different supercomplexes ($CI/CIII_2$ and $CI/CIII_2/CIV_{1-n}$) [32,33]. This supramolecular structure has been suggested to have structural or functional advantages, such as stabilization of individual complexes [34], increased efficiency of electron transfer by means of channeling or prevention of excessive superoxides formation [35,36].

Thus, we examined the impact of decreased CI levels on supercomplexes formation, as well as on accumulation of CII and CV which are not part of the respirasome. We have performed Western blot and IGA analyses, as described above. In control mitochondria, using anti-NDUFA9 antibody we have observed a single band corresponding to one CI interacting with a CIII dimer and a CIV monomer ($CI/CIII_2/CIV$ supercomplex) (Fig. 3A). As expected, the same supercomplex could be detected with antibodies against Cyc1 and Cox4 which label CIII and CIV, respectively. With anti-Cox4 antibody we were also able to detect a larger form of complex, containing additional copies of CIV ($CI/CIII_2/CIV_n$). Finally, anti-Cyc1 and Cox4 antibodies allowed seeing individual forms of CIII and CIV.

As expected, the loss of assembled CI in mitochondria from NDUFA3-, NDUFA5- and NDUFA12-depleted cells resulted in decreased levels of $CI/CIII_2/CIV_n$ supercomplexes but not individual CIII and CIV, as evidenced by Western blot with anti-Cyc1 and anti-Cox4 antibodies as well as CIV-IGA (Figs. 3A, B and S2). As mentioned above, the absence of NDUFV1 core subunit, did not affect the assembly of the rest of CI and this truncated 830 kDa complex (CI*) was able to interact with respiratory chain complexes III

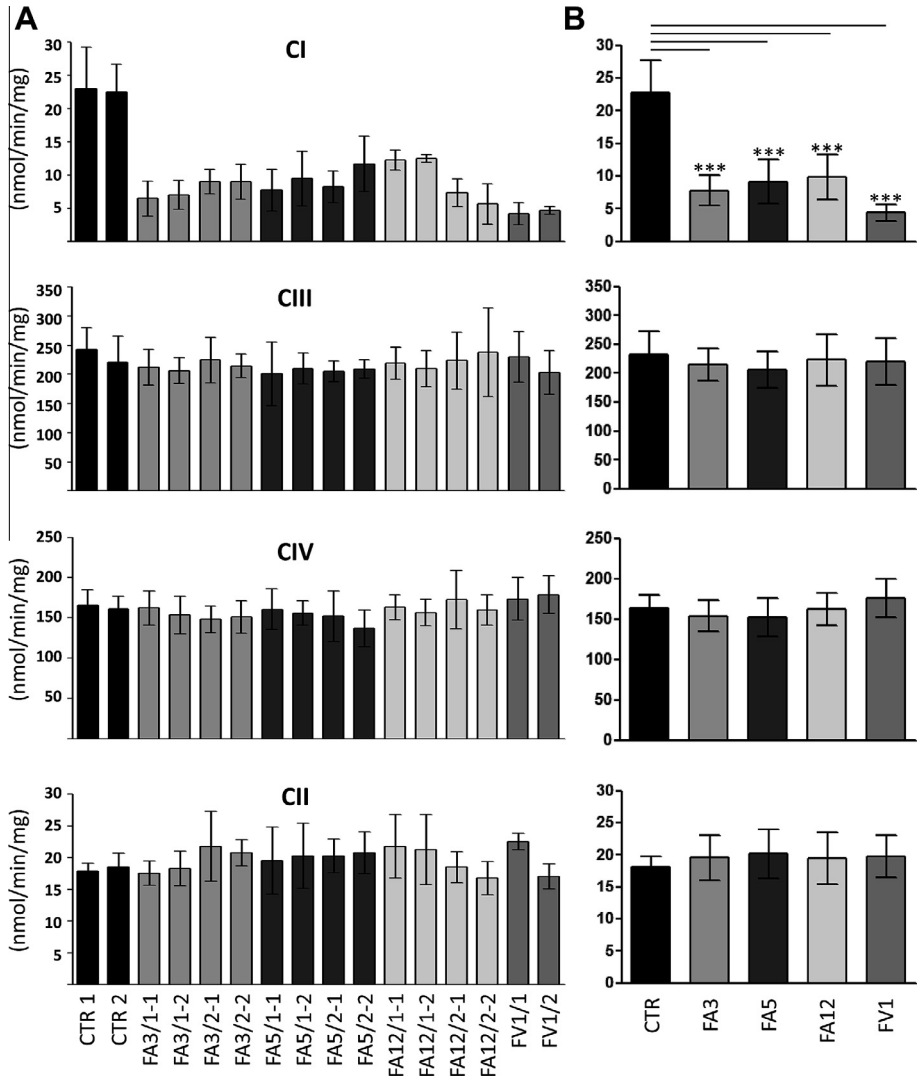

Fig. 2. Impact of CI supernumerary subunits knock-down on the respiratory chain activities. (A) Activities of mitochondrial respiratory chain complexes I–IV were analyzed spectrophotometrically; $n = 3$ (CI, CIII, CIV), $n = 4$ (CII); values are means ± SD. (B) Average respiratory chain activities obtained for each silenced subunit (from (A)) and compared to control cell lines. Data were analyzed using Student's two-tailed t-test, $P < 0.05$ $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

and IV, to form CI*/CIII/CIV supercomplexes (Fig. 3A and B). This result is in agreement with recently proposed model for respirasome formation in which the assembly of CIII and CIV precedes their subsequent interaction with the 830 kDa CI intermediate. The association of the NADH dehydrogenase (N) module with the 830 kDa complex is proposed to be the last step of respirasome formation [37].

Notably, CIV in these partial supercomplexes retained its enzymatic activity as shown by CIV-IGA with NDUFV1-silenced cells (Fig. 3B). Because of a lack of an IGA assay for CIII we could not determine its enzymatic activity by this technique.

3.4. Supernumerary subunits NDUFA3, NDUFA5 and NDUFA12 are required for the formation of the matrix arm of CI

Results of the native gels analyses indicated a role of three supernumerary subunits in CI assembly and/or stability. Since assembly defective mutants often accumulate subcomplexes that are helpful in determining the stage of assembly at which a given subunit can intervene, we have looked for such subcomplexes in mitochondria of the CI-silenced cell lines. Mitochondrial proteins extracted with digitonin were separated by 2D-BN/SDS–PAGE and probed with different CI subunit-specific antibodies (Fig. 4). We used antibodies directed against: (1) NDUFS2, a subunit of the extramembrane electron transfer module (Q module); (2) NDUFA9, located at the junction between the matrix and membrane arms and present in a 400 kDa subcomplex containing the Q module connected to the membrane subunit ND1; and (3) NDUFB6, a constituent of the 460 kDa membrane subcomplex. According to current assembly models, the interaction of the 460 kDa and 400 kDa subcomplexes results in the formation of the 650 kDa complex to which other membrane proteins are added to form the 830 kDa complex. Assembly of the 980 kDa CI holoenzyme culminates with the attachment of the NADH dehydrogenase module (N module) to the 830 kDa complex [38,39] (Fig. 4B).

The immunoblots of the 2D-BN/SDS–PAGE analysis confirmed the accumulation of fully assembled CI in control mitochondria where, except for Q module subcomplexes, no other intermediates are detected (Fig. 4A/CTR). In contrast, the major product detected in NDUFA3 and NDUFA5 depleted mitochondria was the 460 kDa membrane subcomplex (Fig. 4A/NDUFA3 and NDUFA5). Interestingly, antibodies against the Q module subunits, NDUFS2 an NDUFA9, revealed only background levels of their constituent subassemblies. These results constitute compelling evidence that

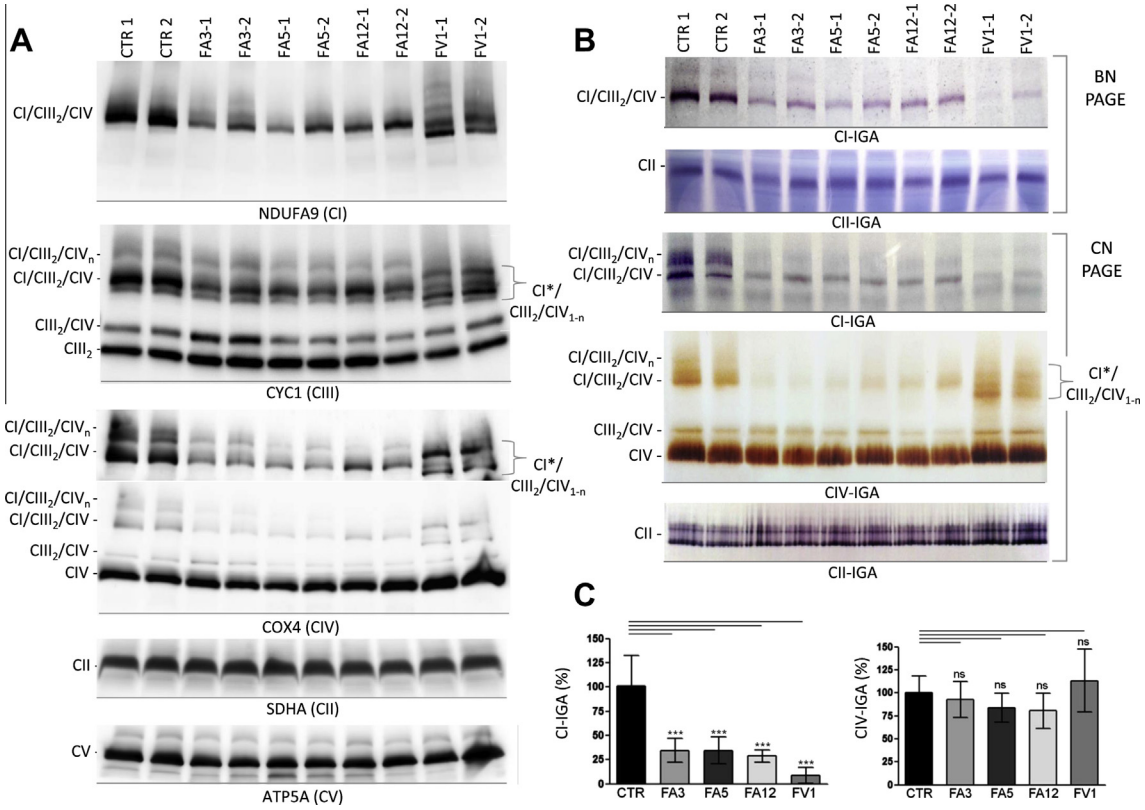

Fig. 3. Respiratory chain organization in CI supernumerary subunit-depleted cell lines. (A) BN-PAGE analysis of mitochondrial proteins from CI depleted cell lines, extracted with digitonin and probed with antibodies against CI–CV subunits. Different forms of supercomplexes and used antibodies are indicated. Two different exposures are shown for COX4 to better visualize CIV supercomplexes (1 min for CIV monomer and 10 min for supercomplexes). (B) *In-gel* activity (IGA) assay for CI, CII and CIV, performed after BN-PAGE (upper panel) and CN-PAGE (lower panel) on the same extracts. (C) Average CI and CIV activities in mitochondria from CI-depleted cell lines, normalized to CII and expressed as percentages relative to controls ($n = 6$; three measurement for two cell lines). Values are means ± SD. Data were analyzed using Student's two-tailed *t*-test, $P < 0.05$ $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

both subunits are involved in the assembly of the extramembrane Q module of CI. Moreover, such an assembly block would be expected to result in accumulation of the 460 kDa complex and the absence of the larger assembly intermediates (650 and 830 kDa), as observed in both cell lines. The cells knocked down for NDUFA12 subunit also accumulated the 460 kDa complex and lacked the 650 and 830 kDa subassemblies. In contrast, they contained the intermediates of the Q module (Fig. 4A/NDUFA12) which suggests that the NDUFA12 subunit does not intervene in Q module formation, but rather participates in the interaction of matrix and membrane arms of CI during its assembly.

The result of the immunochemical analyses also confirmed the expected accumulation of the 830 kDa complex in cells depleted of NDUFV1, a major component of N module (Fig. 4A/NDUFV1). In addition to the 830 kDa complex, we also detected substantial amounts of the 460 and 650 kDa subassemblies, suggesting that the truncated enzyme may be somewhat unstable.

4. Discussion

Mitochondrial CI plays a major role in bioenergetics and cell physiology; thus, not surprisingly, its dysfunctions are associated with many human pathologies, being the most frequent cause (>30%) of mitochondrial diseases [3,4,40]. CI disorders are genetically and clinically heterogeneous and although major progress has been made in recent years in identifying and describing genes mutated in CI diseases, the genetic cause for about a half of these disorders is still unknown. At present, pathogenic mutations have been found in only a half of CI subunits: all 14 conserved core subunits and one third (10/30) of the supernumerary subunits (NDUFA1, NDUFA2, NDUFA9, NDUFA10, NDUFA11, NDUFA12, NDUFB3, NDUFB9, NDUFS4, and NDUFS6) [41,42,43,44,11,12,14,20,45,46]. However, among remaining twenty supernumerary subunits, poorly characterized and not related to any CI deficit, some are likely to be important for CI function or/and assembly and thus could also be targets for human CI-based pathologies. This is supported by the results of this study, showing the importance of NDUFA3, NDUFA5 and NDUFA12, three supernumerary CI subunits, for the formation of a functional CI in human cells. Indeed, silencing of any of these subunits had a deleterious effect on CI activity as a result of blocked assembly and/or increased instability of the enzyme, pointing to their disease causing potential. In agreement with this conclusion, one pathogenic mutation in NDUFA12 has been recently associated with human disease [20] and NDUFA5 knock out was shown to elicit mild chronic encephalopathy in mice [21]. To date, no pathogenic mutation has been found in NDUFA3 and NDUFA5 subunits but in view of these results, screening for mutations in both genes should be considered for patients with isolated CI deficits.

Despite the complexity of CI biogenesis and lack of a crystal structure for the mammalian enzyme, numerous studies in fungi, rodents and CI-deficient patient cells, have led to substantial progress in clarifying the order in which different components of CI are assembled. This is especially true for the conserved core subunits. According to most recent assembly models, CI formation involves putting together its 44 structural subunits from preassembled modules with unique subunit compositions (for rev [38]). This process depends on the assistance of at least 9

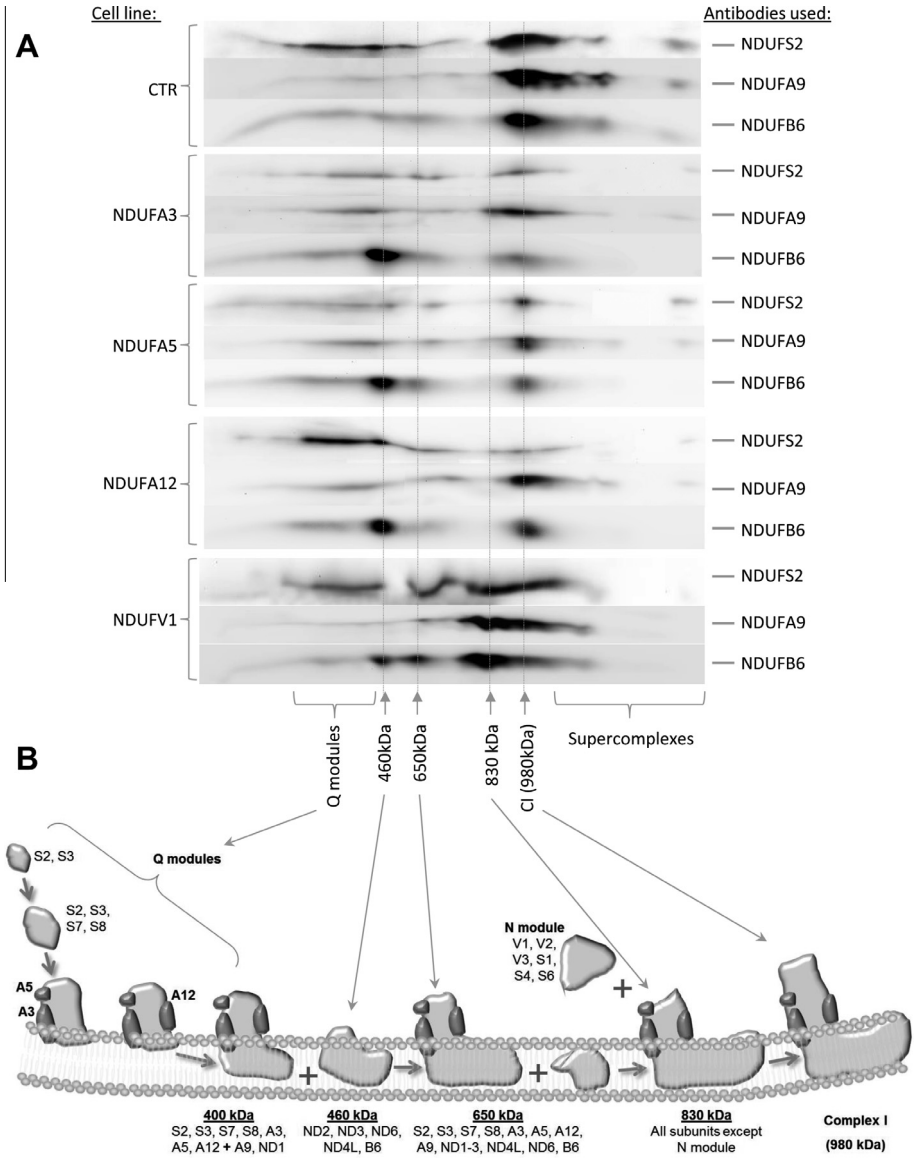

Fig. 4. CI assembly intermediates in supernumerary subunit-depleted cell lines. (A) 2D-BN-PAGE Western blot analysis of digitonin extracts from mitochondria of the CI subunits knocked-down cell lines. CI holoenzyme and its subassemblies were detected using antibodies against subunits representatives for different assembly intermediates according to current CI assembly model. (B) Model of CI assembly including stage at which NDUFA3, NDUFA5 and NDUFA12 subunits may interact with known assembly intermediates (adapted from [38]).

chaperons but is likely to also require some of the 30 supernumerary subunits. Indeed, mutations in supernumerary subunits NDUFA2, NDUFA10, NDUFS4, and NDUFS6 have been reported to affect CI assembly and/or stability [10,11,12,13,14]. The reduced level of CI observed in a patient with NDUFA12 associated Leigh syndrome as well as in NDUFA5 KO mouse, indicate that these two supernumerary subunits are also required for CI biogenesis. Our study also underlines the importance of these two subunits in CI formation. Moreover, we show, for the first time, the requirement of a new subunit, NDUFA3, in this process. Furthermore, analysis of different subassemblies accumulated in mitochondria of cells depleted in specific subunits, allowed us to propose that NDUFA5 and NDUFA3 participate in the assembly of the electron transferring Q module and/or are required for its stabilization during assembly. Since in NDUFA12-depleted cells, assembly of the Q module doesn't seem to be affected, it is more likely that this subunit facilitates interaction of this module with the membrane arm. Recently, it has been reported that NDUFA12 subunit, similarly to NDUFV1 and other constituents of the N-module, is added after the formation of the 830 kDa subcomplex [47]. However, unlike all known N-module mutants (NDUFV1, NDUFS4, NDUFS6 and NDUFA2), accumulating the 830 kDa complex [12,29,30,31,14,11], our study shows that the NDUFA12 depleted cells do not contain this subcomplex. Therefore, the suggested model, including NDUFA12 within N-module during CI assembly, seems to be arguable [38].

Together our data point to the functional importance of CI supernumerary subunits and are consistent with the hypothesis that at least some of them were acquired during evolution to facilitate CI assembly and stabilize its structure.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.03.046.

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