Biochimica et Biophysica Acta 1787 (2009) 502–517

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Review

Pathogenetic mechanisms in hereditary dysfunctions of complex I of the respiratory chain in neurological diseases

Sergio Papa [a,b,*], Vittoria Petruzzella [a,b], Salvatore Scacco [a], Anna Maria Sardanelli [a], Arcangela Iuso [a], Damiano Panelli [b], Rita Vitale [a], Raffaella Trentadue [a], Domenico De Rasmo [a], Nazzareno Capitanio [c], Claudia Piccoli [c], Francesco Papa [d], Michele Scivetti [d], Enrico Bertini [e], Teresa Rizza [e], Giuseppe De Michele [f]

[a] Department of Medical Biochemistry, Biology and Physics, University of Bari, Italy
[b] Institute of Biomembranes and Bioenergetics, Italian Research Council, Bari, Italy
[c] Department of Biomedical Sciences, University of Foggia, Foggia, Italy
[d] Department of Odontostomatology and Surgery, University of Bari, Bari, Italy
[e] Department of Laboratory Medicine, Unit of Molecular Medicine, Bambino Gesù Hospital IRCCS, Rome, Italy
[f] Department of Neurological Sciences, Federico II University, Naples, Italy

ARTICLE INFO

Article history:
Received 12 November 2008
Received in revised form 23 December 2008
Accepted 30 December 2008
Available online 10 January 2009

Keywords:
Complex I
NDUFS4
NDUFS1
ROS balance
mtDNA mutation
Mitochondrial encephalopathy
PINK1
Familiar Parkinsonism
Chronic epilepsy

ABSTRACT

This paper covers genetic and biochemical aspects of mitochondrial bioenergetics dysfunction in hereditary neurological disorders associated with complex I defects. Three types of hereditary complex I dysfunction are dealt with: (i) homozygous mutations in the nuclear genes *NDUFS1* and *NDUFS4* of complex I, associated with mitochondrial encephalopathy; (ii) a recessive hereditary epileptic neurological disorder associated with enhanced proteolytic degradation of complex I; (iii) homoplasmic mutations in the *ND5* and *ND6* mitochondrial genes of the complex, cohexistent with mutation in the nuclear *PINK1* gene in familial Parkinsonism. The genetic and biochemical data examined highlight different mechanisms by which mitochondrial bioenergetics is altered in these hereditary defects of complex I. This knowledge, besides clarifying molecular aspects of the pathogenesis of hereditary diseases, can also provide hints for understanding the involvement of complex I in sporadic neurological disorders and aging, as well as for developing therapeutical strategies.

1. Introduction

Dysfunctions of complex I of the respiratory chain encompass more than 30% of hereditary mitochondrial encephalopathies [1–3]. Complex I defects have also been observed in other neurological disorders [4,5], like sporadic [4,5] and familiar Parkinson disease (PD) [6,7], hereditary spastic paraplegia [8], Friedreich Ataxia [4], as well as in aging [9,10]. Once the genetic basis of hereditary complex I deficiencies has been defined, much more work is needed to understand, as in the case of many other hereditary diseases, the pathways going from the mutated gene to the biochemical, cellular, tissue disorder and ultimately to the clinical course of the disease. Clarification of this issue represents today a major challenge in biomedical research.

Complex I is the largest enzyme of the mammalian oxidative phosphorylation system. With a molecular weight of $\sim 1 \cdot 10^6$ Da it contains a series of redox centers (one FMN, seven FeS-centers and two protein bound semiquinone/quinol couples) which catalyze the stepwise transfer of electrons from NADH to ubiquinone of the pool, coupled to the translocation of $4H^+/2e^-$ from the mitochondrial matrix to the cytosol [11–19] (Fig. 1). Complex I is made up of 45 protein subunits [20], of which seven are encoded by the mitochondrial genome, the others by nuclear genes [17,21]. Fourteen conserved subunits are essential for the redox and proton pumping activity [11,17]. Some of the supernumerary subunits participate in the assembly of the complex [22–25], others appear to be involved in regulatory function [17,25–28]. Complex I is a regulable pacemaker of the mitochondrial respiratory function [25–28], is a major site of cellular oxygen superoxide production [11,25,29–33] and is involved in apoptosis [34–36] and age-related functional decline [9,10].

The functional level of complex I in mammalian cells depends on: (i) coordinated expression of nuclear and mitochondrial genes; (ii) post-translational subunit modification; (iii) mitochondrial import/maturation of nuclear encoded subunits; (iv) subunits interaction and stepwise assembly; (v) proteolytic processing.

* Corresponding author. Department of Medical Biochemistry, Biology and Physics, University of Bari, 70124 Bari, Italy. Tel.: +39 080 5448541; fax: +39 080 5448538.
E-mail address: papabchm@cimedoc.uniba.it (S. Papa).

0005-2728/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2008.12.018

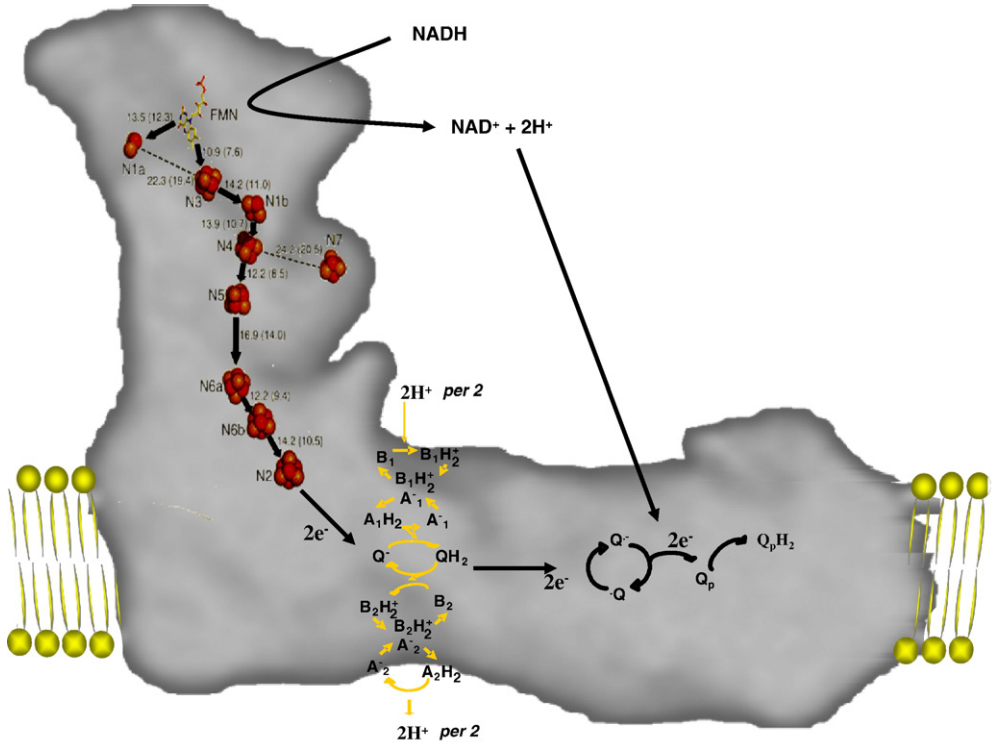


Fig. 1. Shape and electron and proton transfer pathways in complex I. The shape was obtained from high resolution electron microscopy image reconstitution of *N. crassa* complex I [15]. The location of the redox centers [12,16] is obtained from the X-ray crystallographic structure of the hydrophilic moiety of the *T. thermophilus* complex I (see Ref. [16]). In yellow a tentative ubiquinone-gated proton pump mechanism is shown [18], which translocates $4H^+$ from the matrix space to the cytosol *per* two electrons flowing from $NADH_2$ to ubiquinone of the pool. In this model cooperative redox-linked H^+ transfer by the N2-PSST subunit mediates proton consumption from the N space in the reduction of a protein stabilized $UQ^{\bullet -}$ to UQH_2 ($UQ_{NF}^{\bullet -}$ in Ref. [19]). Protons are then released toward the P space upon electron transfer from this UQH_2 to a protein stabilized $UQ/UQ^{\bullet -}$ couple (in black) acting as electron transfer center (Q_{NS} in Ref. [19]) and from this to the ubiquinone of the pool [18,25].

This paper will review aspects of the molecular pathogenetic mechanisms in three types of hereditary dysfunctions of complex I in neurological diseases: (i) homozygous mutations in the nuclear genes *NDUFS1* and *NDUFS4*, encoding for two structural subunits of complex I, both associated with mitochondrial encephalopathy; (ii) a recessive hereditary epileptic neurological disorder associated with enhanced proteolytic degradation of complex I; (iii) homoplasmic mutations in the *ND5* and *ND6* mitochondrial gene of the complex, cohexistent with mutation in the nuclear *PINK1* gene in a familial case of PD.

2. Homozygous mutations in *NDUFS1* and *NDUFS4* genes of complex I in mitochondrial encephalopathy

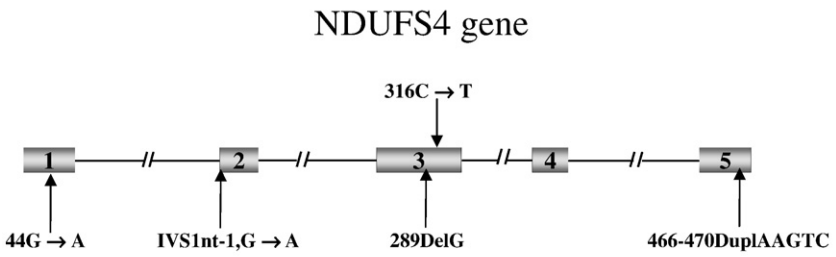
Papa et al. [22,37–39] have studied the impact on mitochondrial functions of mutations in the nuclear *NDUFS4* and *NDUFS1* genes of complex I, in children affected by neurological disease.

The 18 kDa (AQDQ) subunit [20,21], encoded by the *NDUFS4* gene, is essential for the assembly of the complex [22,23]. It is phosphorylated in murine [40,41] and human [22,37] cell cultures "in vivo", by the cAMP-dependent protein kinase A, this phosphorylation being apparently involved in activation of the NADH-ubiquinone oxidoreductase activity of the complex [22,37,40,41] and prevention of accumulation of oxygen free radicals [25,42].

Import in mitochondria of the NDUFS4 precursor protein, with the N-terminal mitochondrial targeting sequence, expressed "in vitro" in a reticulocyte lysate [43,44], or "in vivo" in transfected Hela cells [44], and its cleavage to the mature form, is promoted by phosphorylation of the protein by PKA [44]. In fibroblasts from control subjects and a neurological patient, with mutation in the *NDUFS4* gene resulting in disappearance of the 18 kDa (AQDQ) subunit, the newly imported protein was assembled in complex I [43]. These findings shed light on the mechanism by which PKA mediated phosphorylation of the NDUFS4 subunit can result in promotion in cell cultures "in vivo" of the NADH-ubiquinone oxidoreductase activity of the complex. It is not clear whether the NDUFS4 subunit is assembled in the complex in the phosphorylated or dephosphorylated form. In the inner mitochondrial compartment a phosphatase activity, which dephosphorylates the NDUFS4 protein has, in fact, been detected [45]. These observations could explain negative results of attempts to detect the phosphorylated form of the 18 kDa (AQDQ) protein [46] in complex I in isolated bovine heart mitochondria and the purified complex [47]. For a more detailed discussion of this issue see, however Ref. [25].

Four pathological exonic mutations [38,48,49], one intronic mutation [50], and a deletion of exons 3 and 5 [51] in the *NDUFS4* gene [52] have been found in different neurological patients (Fig. 2). The impact on mitochondrial functions of three of these mutations in the coding sequence of the *NDUFS4* gene has been characterized: a base duplication at position 466–470 in exon 5, which destroyed the RVS phosphorylation site in the carboxy terminus [48], a single base deletion at position 289/290 in exon 3, introducing a premature termination codon (PTC) [49] and a nonsense mutation in the first exon causing premature termination after only 14 aminoacid residues of the protein [38] (Fig. 2). Not only in this last mutation, as expected, but also in the other two the entire 18 kDa (AQDAQ) subunit disappeared from the patient's fibroblasts [22]. All the three NDUFS4 mutations resulted in defective assembly of complex I, with the appearance of a non-functional lower molecular weight subcomplex (Fig. 3, [22]) and complete suppression of the NADH-ubiquinone oxidoreductase activity which did not respond any longer to cAMP activation (Fig. 4, [22]).

The 75 kDa-FeS protein encoded by the *NDUFS1* gene is the largest conserved subunit of complex I, which contains the N1b, N4, N5 Fe–S



Mutations	Disease associated	References
44G → A	Leigh-like syndrome	[38]
IVS1nt-1,G → A	Leigh syndrome	[50]
289DelG	Leigh-like syndrome	[49]
316C → T	Leigh-like syndrome	[49]
466-470DuplAAGTC	Leigh syndrome	[48]
exons 3,5 deletion	Not specified	[51]

Fig. 2. Structure of the *NDUFS4* human gene of complex I with location of mutations found in neurological patients with complex I deficiency. In the table the four exonic mutations, the intronic mutation shown in the structure of the gene (A) and a sixth mutation with deletion of a region including exons 3 and 5, with the associated diseases are listed. For details see text, Ref. [52] and the references in the table.

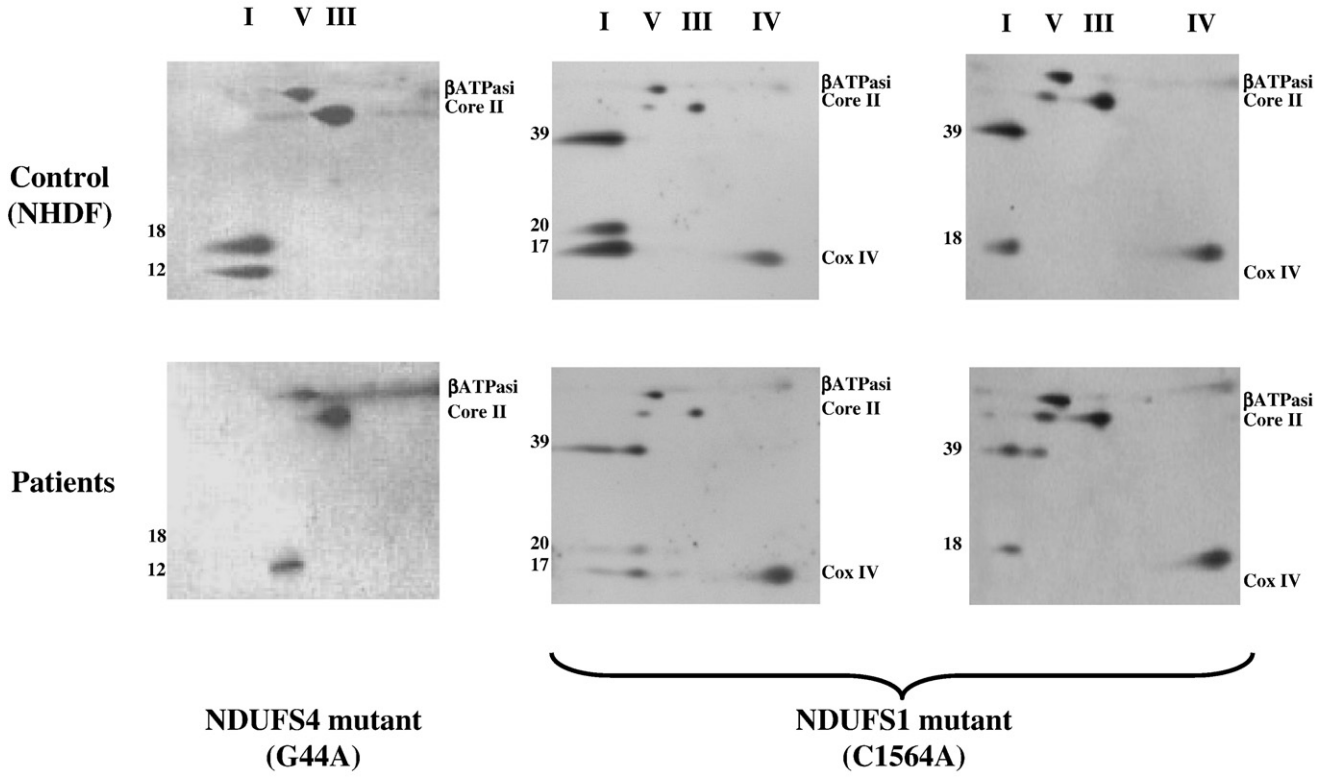


Fig. 3. Two dimensional electrophoretic pattern of respiratory complexes (I, III and IV) and ATP synthase complex (V) in mitoplasts from control (NHDF) and patient's fibroblasts. The complexes were separated by Blue Native electrophoresis and resolved in their subunits in a second dimension SDS-PAGE. Subunits were detected with specific antibodies. For details see text and Ref. [39] from which the figure is reproduced with permission.

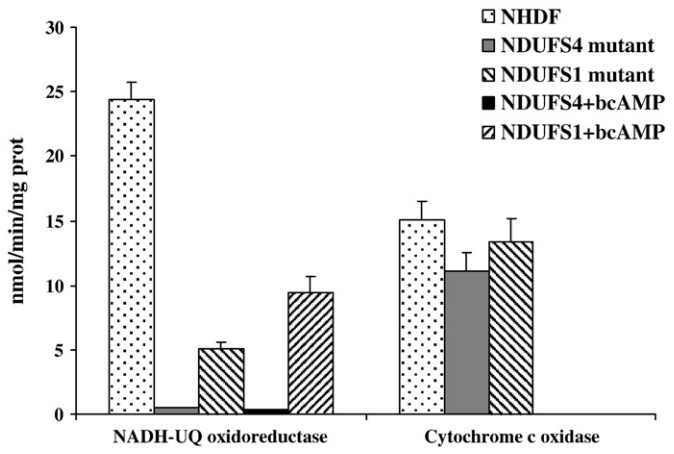


Fig. 4. NADH-ubiquinone oxidoreductase activity of complex I in mitoplasts from control (NHDF) and patient's fibroblasts. Effect of dibutyryl-cAMP. The specific enzymatic activities (V_{max}) were corrected for the rotenone insensitive reaction. Where indicated the fibroblast cultures were exposed to 100 μM dibutyryl-cAMP for 60 min. Data from Refs. [37–39]. For details see Refs. [37–39] and text.

clusters in the electron transfer pathway of the enzyme [12,16] (Figs. 1 and 5). Large scale deletions and point mutations in the *NDUFS1* gene have been found in patients with mitochondrial encephalopathy [53]. Iuso et al. [39] have characterized the functional consequences of a C1564A homozygous mutation in the *NDUFS1* gene (Q522K replacement in the 75 kDa protein) in a child affected by progressive leukodystrophy [3]. Glutamine 522 of the human protein is replaced by asparagine in the corresponding subunit (*NqO3* gene) of the *Thermus thermophilus* complex I. The X-ray crystallographic structure of the bacterial complex I [16] showed that this asparagine is located in subdomain IV of the C-terminal part, which undergoes conformational change to an open configuration upon reduction of the complex by NADH (Fig. 5) [16].

In the fibroblasts of the patient harbouring the C1564A mutation the content of the mitochondrial normally assembled complex decreased significantly as compared to normal cells and a lower molecular mass of the complex appeared (Fig. 3) [39]. The mutation resulted also in a marked, but not complete, suppression of the NADH-ubiquinone oxidoreductase activity (Fig. 4) and depression of the mitochondrial membrane potential [39]. In the fibroblasts with the NDUFS1 mutation, but not in those with the NDUFS4 mutation, there was a large accumulation of H_2O_2 and intramitochondrial $O_2^{\bullet -}$ (Fig. 6).

Complex I is generally considered to represent the major mitochondrial source of $O_2^{\bullet -}$ in mammalian cells [29–33]. The complete suppression of the normal rotenone-sensitive NADH-ubiquinone oxidoreductase, caused by the deletion of the NDUFS4 encoded 18 kDa subunit, is likely to result from inhibition of a redox step that is also involved in the direct reduction of O_2 to $O_2^{\bullet -}$ [30–33]. In the case of the NDUFS1 mutation, the inhibition of the NADH-ubiquinone oxidoreductase can be attributed to altered function of the 75 kDa Fe–S protein encoded by this gene. The Q522K substitution can promote direct oxidation by molecular oxygen of the NDUFS1 Fe–S center(s) or of other redox centers on the substrate side, once they are reduced by NADH.

In the NDUFS1 mutant the depression of the activity of complex I (Fig. 4) and of the membrane potential [39] was counteracted by the addition to the fibroblasts culture of dibutyryl-cAMP, which also prevented the accumulation of oxygen free radicals [39]. Both these rescue effects of cAMP have been generally observed in a variety of human and murine cell cultures, under conditions in which the

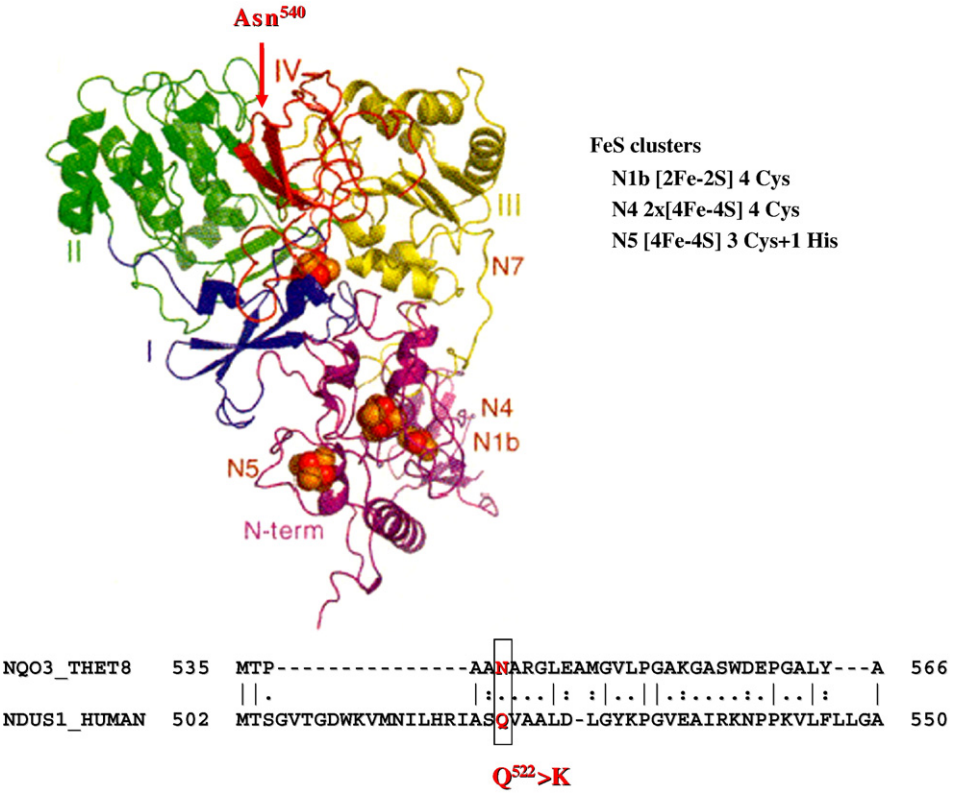


Fig. 5. X-ray crystallographic structure of the NqO3 subunit of *T. thermophilus* complex I (homologous of the human NDUFS1, 75 kDa FeS subunit). Redrawn from Ref. [16]. The FeS cluster associated to the subunit and the aminoacid sequence of subdomain IV of the c-terminal part of the NqO3 and NDUFS1 proteins are presented. The Gln 522, replaced by Lys in the NDUFS1 C1564A mutation, is shown in red. In red the homologous asparagine in the NqO3 corresponding region is also shown.

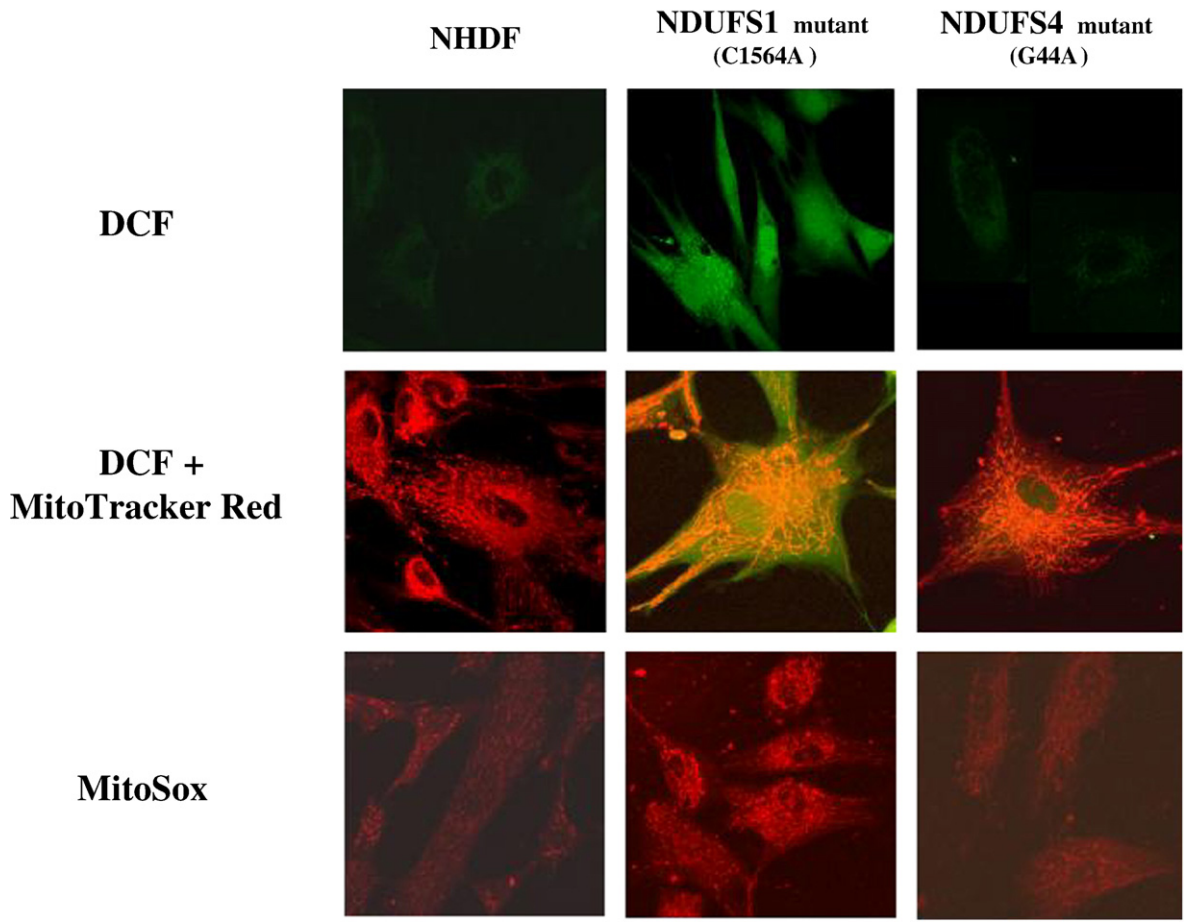


Fig. 6. Laser scanning confocal images of H_2O_2 and $O_2^{\bullet -}$ levels in control (NHDF) and patient's fibroblasts. H_2O_2 was detected by the green fluorescence of DCF; mitotracker red fluorescence detected mitochondrial membrane potential; mitosox red fluorescence detected intra-mitochondrial $O_2^{\bullet -}$ Reproduced with permission from Ref. [39]. For details see Ref. [39] and text.

nucleotide did not exert any effect on components of ROS scavenger systems [25,42, see also 32].

A study of the NDUFS4 transcripts in the fibroblasts from three patients harbouring three different mutations in the gene revealed new aspects of the transcription of this gene (Fig. 7). The five exons of the gene result in a canonical mRNA coding for 175 residue protein (the precursor of the 18 kDa (AQDQ) subunit of complex I) [22]. In the patient with the point deletion at position 289/290 in exon 3 the transcript level was much reduced as compared to control cells [22] (Fig. 7). The introduction of the PTC in the middle of the transcript elicited its degradation by non sense mediated decay (NMD) [22] a mechanism of quality control that ensures fidelity of gene expression [54]. This explains the disappearance of the 18 kDa (AQDQ) protein from the patient's cells [22]. In the case of the 5 bp duplication at position 466/470 in the fifth-exon the transcript level was normal (Fig. 7) [22]. In this case the disappearance of the overall protein could be due to defective translation of the mutated transcript and/or proteolytic degradation of the longer translated product. In the 44G→A non sense mutation in the first exon, disappearance of the mature protein was expected. This mutation which introduced a PTC in the proximity of the canonical AUG start codon, rather than eliciting NMD degradation of the canonical mRNA, upregulated three PTC containing alternative transcripts [55,56] (Fig. 8). The splice variants 1 and 2 (SV1 and SV2) result from insertion between exons 2 and 3 of a cryptoexon which uses two alternative acceptor sites (Fig. 8). The splice variant 3 (SV3) derives from exon 2 skipping [55,56]. These three alternative transcripts are produced also in normal cells but their level is kept low by RNA surveillance mechanisms. SV1 and SV2 are degraded in the cytosol by NMD, the level of SV3 is down-regulated directly in the nucleus [56]. The 44 G→A non sense mutation inactivated in the patient fibroblasts NMD degradation of SV1 and SV2 and nuclear down regulation of SV3 (Fig. 8) [56]. In a neurological patient with a homozygous splice acceptor site mutation in intron 1 (IV Snt-1, G→A) of the *NDUFS4* gene (Fig. 9) only a mRNA transcript, in which exon 2 was skipped, was detected [50]. Amplification of this transcript and sequencing showed that it corresponded exactly to the PTC containing SV3 isoform detected in the patient with G44A nonsense exonic mutation in the *NDUFS4* gene (Fig. 9) [56]. The accumulation of the aberrant alternative transcripts, caused by the exonic or the intronic mutation in the *NDUFS4* gene, can represent another deleterious event contributing to the pathogenetic mechanism of the mutations in neurological patients.

In summary the NDUFS4 mutations, which were associated with earlier onset and severe disease course resulted in: (i) disappearance of the 18 kDa (AQDQ) protein it encodes; (ii) block of the last step in the assembly of the complex; (iii) full suppression of the NADH-ubiquinone oxidoreductase activity; (iv) loss of cAMP promotion of the activity; (v) loss of Pasteur effect with enhanced glycolitic activity and chronic lactic acidosis; (vi) accumulation of unproductive alternative transcripts of the gene [22,50,55,56].

The C1564A NDUFS1 mutation, which was associated with a less severe disease, did not suppress completely the NADH-ubiquinone oxidoreductase activity of the complex. The decrease of the level of mature complex I, could be due to proteolytic degradation of

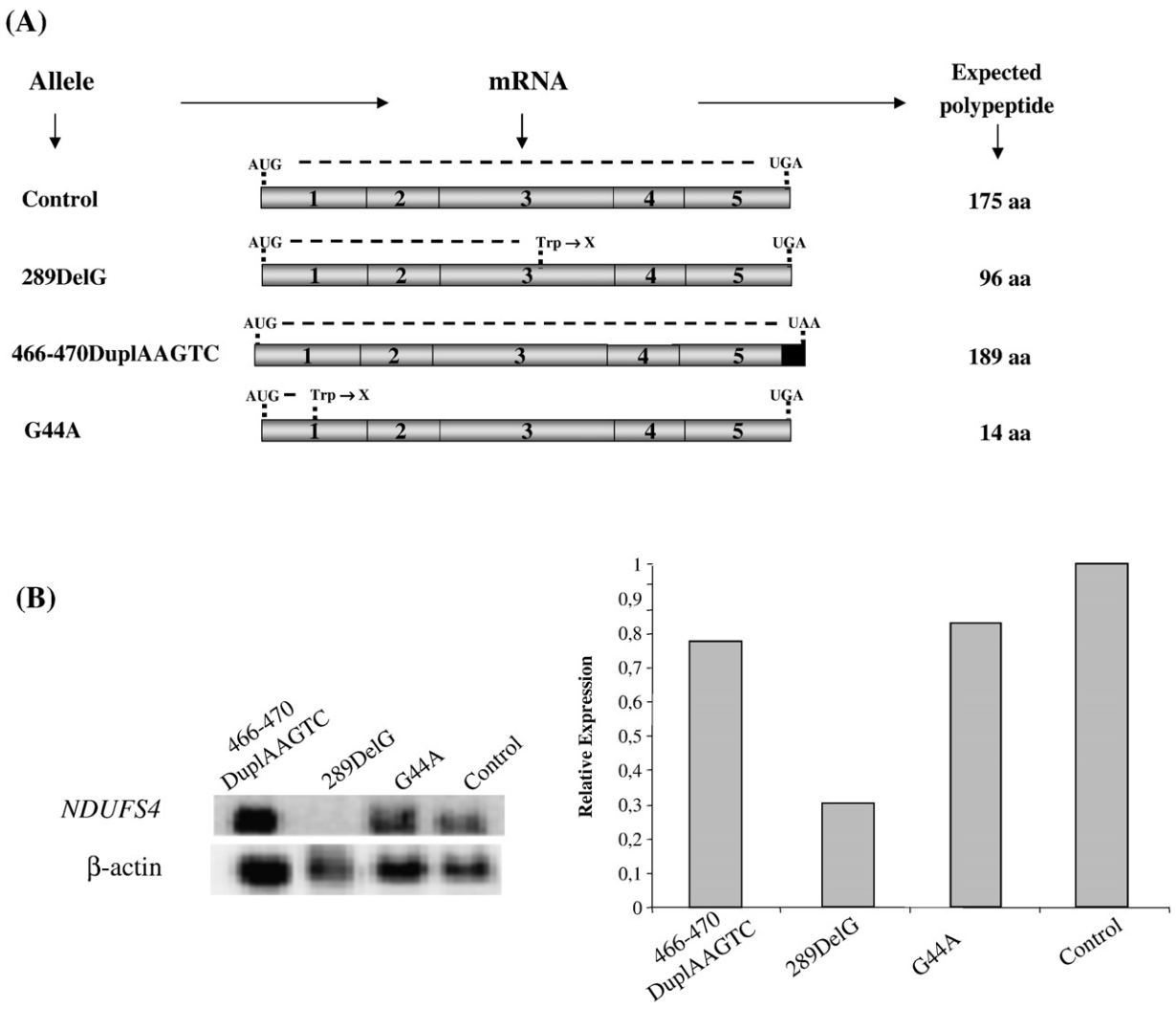


Fig. 7. Structure of normal NDUFS4 mRNA and mRNAs generated by the NDUFS4 pathological alleles and their steady-state levels in fibroblasts cultures. (A) In the G44A mutation as well as in the 289/290G deletion the aminoacids 15 and 96 change respectively from a tryptophan to a stop codon; the AAGTC 466–470 Dupl caused a frame shift with 14 aminoacids elongation of the protein (in black) and disrupted the phosphorylation site (RVS 129–131). (B) Northern blots (on the left) of total RNA from fibroblasts of a healthy child as control and patients with AAGTC 466–470 Dupl, 289DelG, and G44A, hybridized with a radiolabelled *NDUFS4* cDNA region. A β-actin probe estimated the amount of loaded RNA. Real time PCR (on the right) of total RNA from fibroblasts of a control, AAGTC 466–470 Dupl, 289DelG, and G44A patients. The relative expression levels of the *NDUFS4* transcripts with respect to the *GAPDH* transcript, used as internal reference, are presented. The experimental data in (B) are reproduced with permission from Ref. [22]. For details see text.

the complex, promoted by the enhanced production of ROS. The Q522K replacement in the 75 kDa protein could alter the redox function of the FeS centers (N1b, N4, N5) in this subunit and/or other redox components, resulting in inhibition of the normal forward electron transfer activity and enhanced direct reduction of O_2 to $O_2^{\bullet -}$.

3. Proteolytic degradation of complex I in a progressive hereditary epilepsy

Mutational gene analysis and cellular biochemical investigations carried out in our laboratories on two male sibs, affected recessively by inherited progressive cerebral atrophy, lactic acidosis and drug resistant epilepsy, reveal a new type of hereditary pathological dysfunction of complex I. Primary fibroblast cultures of both children exhibited a definite decline of the NADH-ubiquinone oxidoreductase, more severe in one of the two patients (Fig. 10A). This was reflected in a lower mitochondrial content of complex I, as shown by decreased level of the mitochondrial encoded ND6 and the nuclear encoded NDUFA9 and NDUFB6 subunits (Fig. 10B). The activity and content of cytochrome *c* oxidase and complex V (ATP synthase) subunits were, on the other hand, normal. Differently from what observed in the mutations of the *NDUFS1* and *NDUFS4* gene of complex I no lower molecular form of complex I was detectable.

Extensive mutational analysis did not show any pathogenetic mutation in the seven mitochondrial genes and 38 nuclear genes of complex I, except homozygous or heterozygous substitutions already reported in EST (Expressed Sequence Tags) data banks and ANP (single nucleotide polymorphisms) (http://www.ensembl.org).

The addition to the patient's fibroblast culture of a cocktail of protease inhibitors did rescue the level of subunits of complex I and the NADH-ubiquinone oxidoreductase activity (Fig. 10). No effect of the protease inhibitors was exerted on the subunit level and activity of cytochrome *c* oxidase and complex V.

Analysis of the import of the NDUFS4 precursor protein in mitochondria isolated from fibroblasts of the two children shows that, whilst the binding of the precursor at the organelle surface was only partially decreased, the mature form of the imported protein, detected in the mitochondria from control fibroblasts, disappeared in

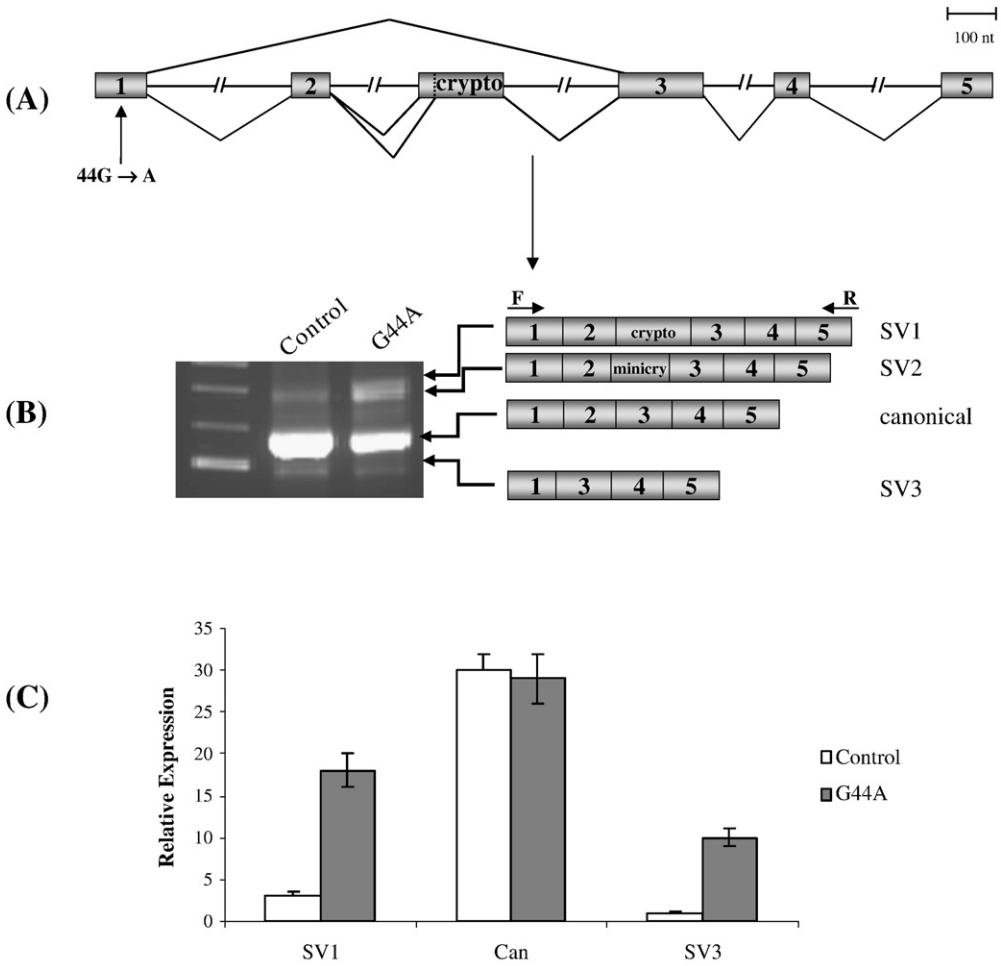


Fig. 8. Impact of the exonic G44A mutation on the expression of the *NDUFS4* gene. (A) The scheme shows the mechanism of production of the alternative splicing products (B) normally produced by the *NDUFS4* gene but up-regulated in the G44A mutation fibroblasts. (B) RT-PCR performed using two primers encompassing all the NDUFS4 ORF. (C) Real-time PCR in total RNA from fibroblasts of a control and G44A patient. The relative expression levels of the *NDUFS4 alternative* transcripts as well as the canonical mRNA with respect to the *GAPDH* transcript, used as internal reference, are presented. Reproduced with permission from Refs. [55,56].

those from both patient's fibroblasts (Fig. 11). The mature imported form of the NDUFS4 protein was again detectable in the patient's mitochondria, when the import assay was carried out in the presence of protease inhibitors (Fig. 11).

The above results indicate that the decreased content of functional complex I in the patient's fibroblasts is due to digestion of the complex by a protease present in the intra-mitochondrial space. It has been reported that calpain 10, a member of a family of 14 cysteine proteases, is localized in mitochondria and its activation by Ca^{2+} results in digestion of complex I subunits, including the mitochondrial encoded ND6 [57]. Preliminary confocal microscopy analysis of calpain activity based on the fluorescence of the cleavage product of the substrate SLLVY-AMC [57], revealed enhanced activity of the enzyme in both patient's fibroblasts, apparently colocalized with mitochondria (results not shown). Further work is in progress in our laboratories to identify the genetic factors involved in the activation of the proteolytic activity.

4. Homoplasmic mutations in the *ND5* and *ND6* mitochondrial genes of complex I in PINK1 familial Parkinsonism

Whilst the cause of sporadic PD remains unclear, several genes have been identified as responsible for hereditary forms of PD [4,58–60]. Mutations in the phosphatase and tensin homolog (PTEN) induced serine/threonine putative kinase-1 (*PINK1*) gene (Fig. 12) represent the second most common cause of autosomal recessive Parkinsonism (ARP) [61,62].

Recent work from different groups provides converging evidence showing that the [8] PINK1 kinase is involved in the degradation of unfolded or oxidized mitochondrial proteins [63–65], autophagy of damaged mitochondria [66–69] and mitochondrial fission/fusion [70]. PINK1 with an N-terminal mitochondrial targeting sequence is essentially localized in mitochondria. It has been reported from time to time that PINK1 is associated with the inner membrane [63,64,71], the intermembrane space [64,65,71] and the outer membrane [72]. The catalytic domain of the outer membrane PINK1 faces the cytosolic space [72] where it phosphorylates the E3 ubiquitin ligase parkin [67,73], product of the *PARK2* gene, whose mutations represent the first most common cause of autosomal recessive Parkinsonism [74]. Parkin phosphorylation promotes its translocation from cytosol to mitochondria [73], where it participates to autophagy of damaged mitochondria [66–69]. PINK1 has also been reported to phosphorylate TRAP1 (HSP75) [64] and to regulate, through an indirect phosphorylation mechanism, the protease HtrA2 [65], both these proteins being involved in quality control of oxidized mitochondrial proteins. Since TRAP1 and HtrA2 reside in the intramitochondrial space it is possible that PINK1 can partition between the outer and the inner mitochondrial location in response to the prevailing cellular conditions [see also 75].

Different mutations in the *PINK-1* gene have been detected in ARP and PD cases with an early onset [61,76–78] (Fig. 12). Even if the

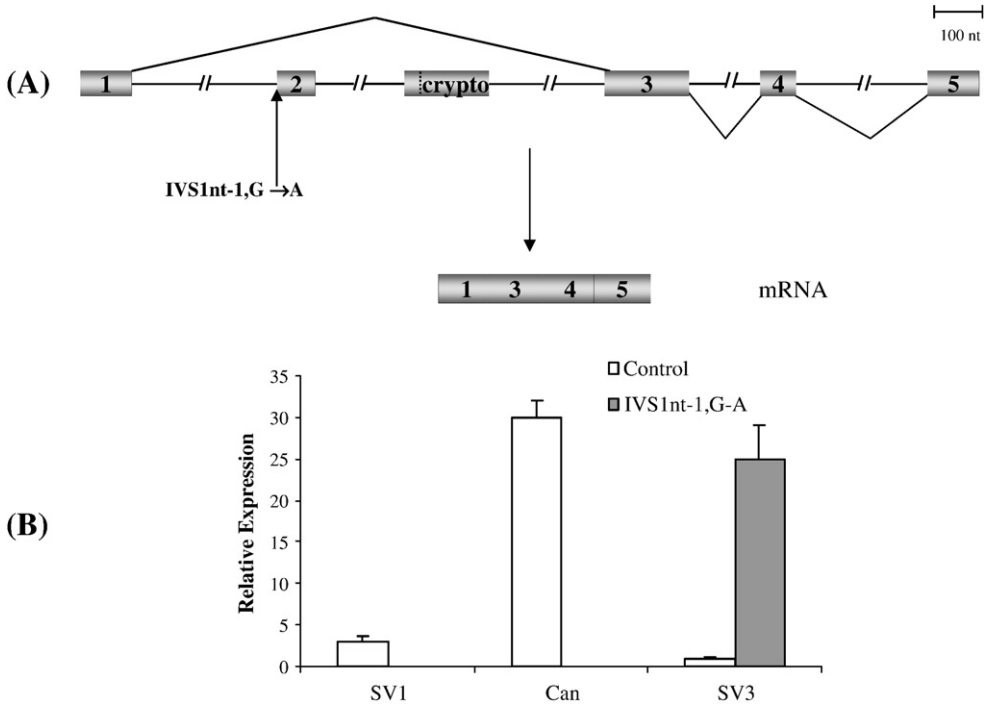


Fig. 9. Impact of the intronic IVS1nt-1,G-A mutation on the expression of the *NDUFS4* gene. (A) The scheme shows the only splicing product generated by the IVS1nt-1,G-A mutated *NDUFS4* gene. (B) Real-time PCR of total RNA from fibroblasts of a control and the IVS1nt-1,G-A patient. The relative expression level of the *NDUFS4* transcripts with respect to the *GAPDH* transcript, used as internal reference, is presented. Reproduced with permission from Ref. [56].

heterozygous state of subjects carrying PINK-1 mutations does not generally evolve towards the disease, in some cases it does albeit with a later onset [78,79]. This would suggest that dysfunction of PINK1 may promote the disease in combination with other acquired or inherited defect(s). Mutations in mitochondrial DNA have, on the other hand, been reported in PD [67,80]. Our group has characterized the mitochondrial genotype and function in a female patient carrying a homozygous W437X PINK-1 mutation [6,7], resulting in a C-terminus truncated kinase with depressed kinase activity (Fig. 12) [81]. The same mutation was present in the heterozygous state in both the patient's parents [7] (see Fig. 12C). The fibroblasts of the homozygous W437X patient showed a significant depression of the oligomycin sensitive, endogenous respiratory activity both in the coupled and CCCP-uncoupled state, accounting in the last case for 50% depression as compared to control value (Fig. 13A). The respiratory control ratio was also lower. Direct enzymatic assays showed in the patient's fibroblasts normal specific activities of complex I and III of the respiratory chain (Fig. 13B). The specific activity of complex IV was also normal when assayed with added cytochrome *c* (a), but depressed when assayed with ascorbate plus TMPD (b), a condition that depends on the endogenous mitochondrial content of cytochrome *c* (Fig. 13B). The cellular level of ATP was higher in patient's fibroblasts than in controls when cells were grown in a glucose medium, but significantly lower in a galactose medium (Fig. 13C), a condition in which ATP is essentially produced by mitochondrial oxidative phosphorylation [82].

Two dimensional blue-native/SDS gel-electrophoresis combined with immunoblotting detection of constituent subunits of oxidative phosphorylation complexes showed no significant change in the content and subunit pattern of complexes I, III, IV and V (F_0F_1 ATP-synthase) in the patient's fibroblasts as compared to control [6,7].

Analysis with a specific fluorescent antibody showed that the content of cytochrome *c*, whose cellular distribution in control fibroblasts mimicked the mitochondrial reticular network, was markedly reduced in the patient's fibroblasts (Fig. 14A). Similar analysis with specific fluorescent antibodies showed that in the patient's fibroblasts there was no decrease in the level of the mitochondrial β-subunit of the ATP synthase and subunit IV of complex IV (Fig. 14A) [6].

Confocal microscopy analysis with fluorescent probes revealed in patient's fibroblasts significant accumulation of H_2O_2 with a reticular distribution resembling the mitochondrial network (Fig. 14B). The MitoSox probe, which accumulates in mitochondria and reacts specifically with $O_2^{\bullet -}$, revealed accumulation of this oxygen reactive species in patient's mitochondria [7]. The accumulation of H_2O_2 in patient's fibroblasts was not prevented by apocynin, which specifically inhibits the plasma membrane NADPH oxidase [83] neither by dibutyryl-cAMP. On the contrary DPI, which binds irreversibly to the prosthetic flavin moiety of redox enzymes, including complex I [84], prevented completely the accumulation of H_2O_2 in the patient's fibroblasts (Fig. 14B). The accumulation of ROS in patient's fibroblasts was not due to a deficiency of the ROS scavenger capacity [6]. The depressed production of ATP by mitochondrial oxidative phosphorylation and the concomitant ROS accumulation, detected in the patient's fibroblasts could both result from the combined impact of complex I dysfunction, caused by a ND6 mutation [7] (see below), and the decreased mitochondrial content of cytochrome *c* [6]. The PINK-1 mutation can be responsible for this decrease of mitochondrial cytochrome *c* content. Defective phosphorylation by the mutated PINK1 kinase activity of TRAP1 has been reported to abrogate a protective effect exerted by this chaperon protein in preventing cytochrome *c* release from mitochondria [64].

Quantitative PCR showed that normal and patient's fibroblasts had a comparable content of mtDNA amounting to around 1000 mtDNA copy number/cell [7]. Sequence analysis of mtDNA of the homozygous patient's fibroblasts showed a number of single nucleotide changes (SNCs) disseminated throughout the entire mtDNA (Fig. 15) [7]. All but two were already reported as known polymorphisms (http://www.mitomap.org). These two SNCs were m.A12397G and m.T14319.C in the *ND5* and *ND6* genes, respectively. Notably, both were homoplasmic [7] and confirmed in DNA extracted from the patient's blood. Both the ND5 and ND6 mutations were also found in the

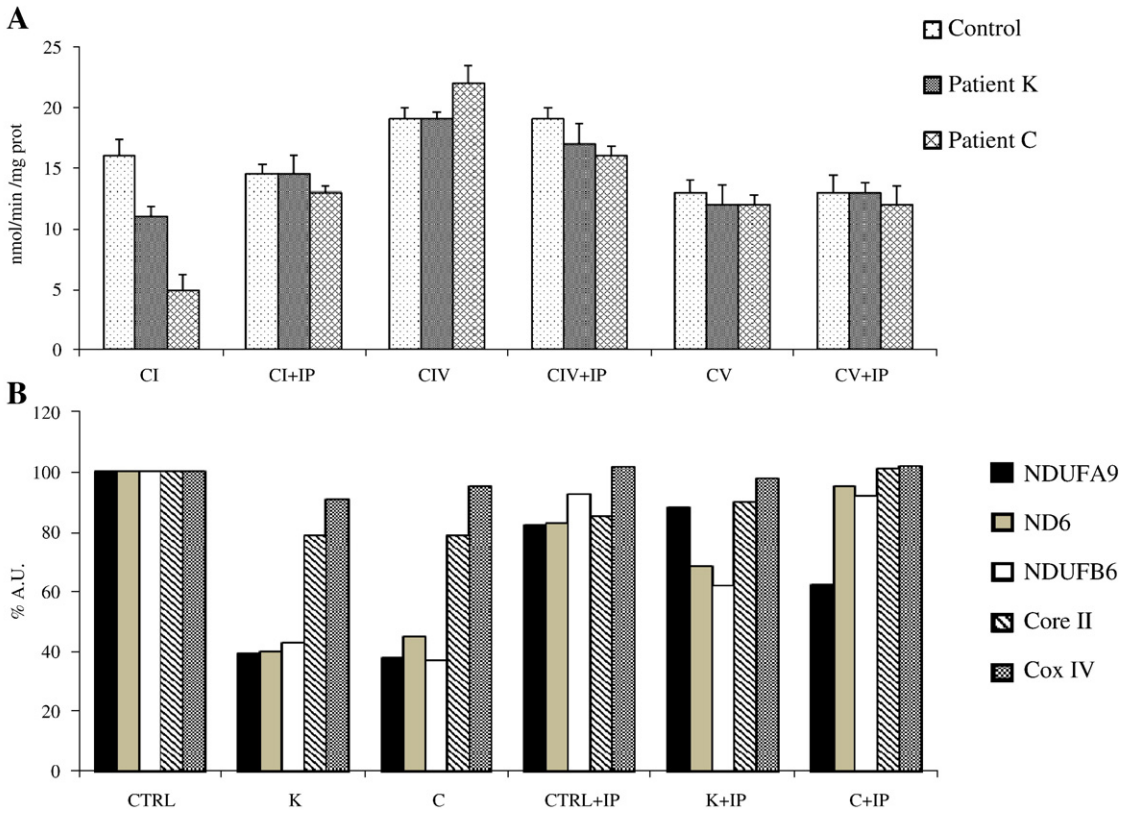


Fig. 10. Enzymatic activities of complexes I, IV and V and subunit levels of complexes I, III and IV in mitoplasts from control and progressive hereditary epilepsy patient's (K and C) fibroblasts, before and after treatment with protease inhibitors (IP). Where indicated fibroblast cultures were treated for 48 h with the protease inhibitors cocktail (SIGMA) at 1:400 dilution (dissolved in DMSO and containing aprotinin, bestatin, leupeptin, pepstatin A and E-64 which is, in particular, specific for calpains). Panel A: Enzymatic activities, means ± standard error of 3 or more determinations. NADH-UQ oxidoreductase activity was measured as described in [22]; cytochrome *c* oxidase activity was determined as in [39] and ATP hydrolase activity as in [7]. Panel B: Immunoblot and relative densitometric units of complexes I, III and IV subunits. Two-dimensional gel analysis (Blue Native PAGE/SDS-PAGE) of the mitoplasts and immunoblotting were carried out as in Ref. [39]. Monoclonal antibodies against subunits of complex I (NDUFA9, ND6, NDUFB6), the core II subunit of complex III and the subunit IV of complex IV were from Molecular Probes (Invitrogen). Detection by chemiluminescence reaction was carried out by using the Western Lightning Chemiluminescence Reagent Plus, followed by exposure to Hyperfilm (Amersham Pharmacia). Quantification was made by densitometric analyses of bands intensity (VersaDoc Imaging System model 1000-Bio-Rad).

homoplasmic state in the mtDNA of the patient's mother blood. Conversely, the same analysis carried out on the patient's father blood resulted in a normal ND5 and ND6 genotype [7]. The mother presented a late onset PD, the father at the age of 70 was still exempt from the disease (see Fig. 12C). Collectively these results demonstrate that the homoplasmic mtDNA mutations found in the patient cohexisted with the PINK1 mutation at birth and were inherited by the mother, which carried the same homoplasmic mtDNA mutations.

ND5 and ND6 are two of the seven mtDNA encoded, membrane embedded, subunits of complex I. The mt A12397G missense mutation changes a threonine in alanine at position 21 of the ND5 subunit (in a hydrophilic segment likely exposed to the inter-membrane mitochondrial space) [7]. Various ND6 mutations leading to aminoacid changes have been found, associated with mitochondrial diseases [85,86] and tumour cell metastasis [87]. Some of these mutations result in changes in the kinetics parameters of complex I [85,86] and/or production of oxygen free radicals [87,88] (see Fig. 16). Site directed mutagenesis in the NuoJ subunit (counterpart of the mammalian ND6) of *E. coli* complex I resulted in suppression of the proton pumping activity of the complex [89]. Similar site-directed mutagenesis indicated that the NuoJ subunit also delineates the binding site of ubiquinone [90]. In both cases [89,90] the assembly of complex I and its specific oxidoreductase activity were unaffected by the mutations as, in fact, found in the mitochondria from the patient of our study. The mt T14319C mutation changes an asparagine in aspartic acid at position 119 of the ND6 subunit. This mutation is located in the fifth of six predicted transmembrane helices (TMH) [7]. A comparison of the hydropathy plot between the ND6 wild-type and the N119D mutant resulted for the latter in a pronounced destabilizing effect of the TMH-V [7]. Enzyme kinetic analysis of the NADH-ubiquinone oxidoreductase activity of complex I in normal and patient's fibroblasts showed an almost fivefold decrease of the apparent Km for both ubiquinone and NADH in the patient's sample [7]. Conversely, the estimated maximal rate of complex I was practically unchanged when ubiquinone was used as limiting substrate, whereas a 26% inhibition of the V_{max} was observed in patient's fibroblasts titrated with NADH [7].

The changes in the Km of the substrates in the patient's NADH: ubiquinone oxidoreductase activity would argue in favour of alteration in the catalytic feature of the enzyme. This, without compromising the overall electron transfer in the mitochondrial respiratory chain, may be, nevertheless, responsible for the enhanced production of ROS [see also Refs. 87,88]. The higher affinity for ubiquinone of the patient's complex I could predispose to a longer occupancy of the reduced quinone or of the semiquinone radical species at the catalytic site(s), with altered reduction potential of the upstream redox centers.

It can be recalled here that exposure of rats to complex I inhibitors like rotenone and 1-methyl-4-phenylpyridinium (MPP+) causes oxidative stress and reproduces features of PD [91,92]. It has, also, been reported that the anti-apoptotic proteolytic activity of HtrA2, downregulated in PINK1-mutated PD patients [65], is enhanced [93] by interaction with the protein product of *GRIM19* gene (cell death regulatory gene induced by interferon and retinoic acid) [34]. The protein product of GRIM-19 has been identified as a subunit of

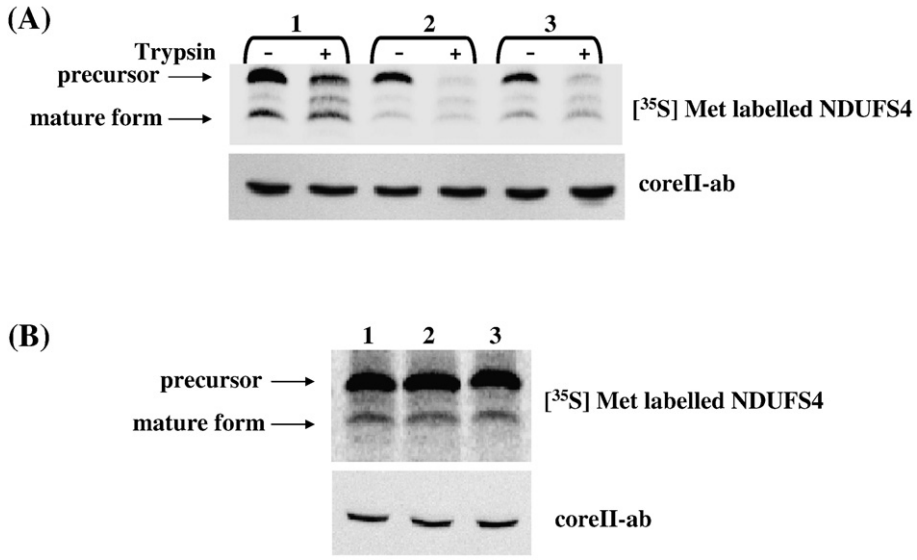
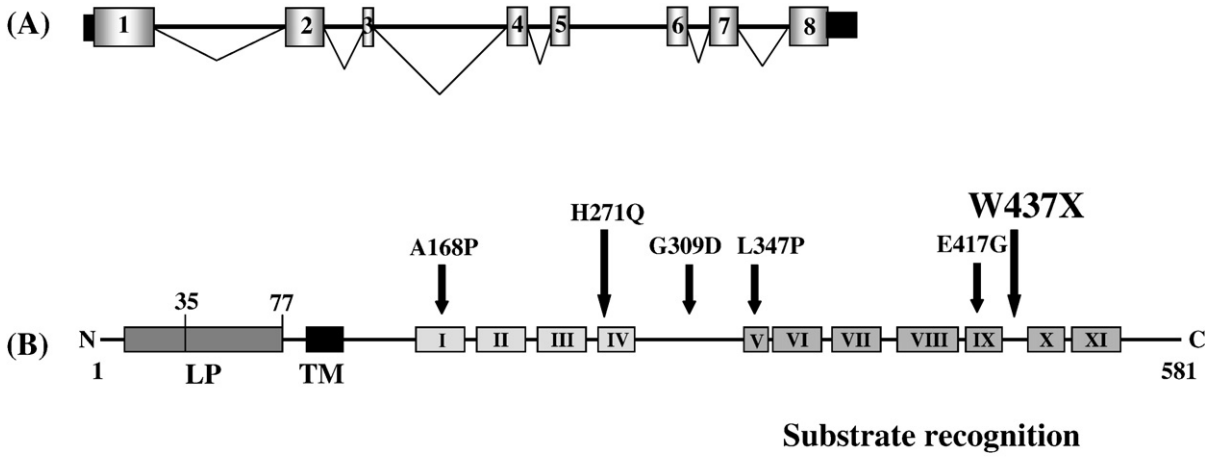
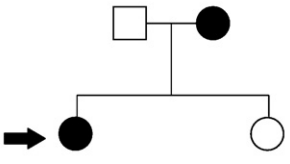


Fig. 11. Import of [^{35}S]Met-labelled NDUFS4 protein in mitochondria isolated from human cell cultures. In vitro transcription and translation of radiolabelled [^{35}S] methionine NDUFS4 precursor protein were performed in Rabbit Reticulocyte Lysate System (RRL). Mitochondria were isolated from control and patients fibroblasts. Cells were collected by centrifugation at 500 *g* for 3′ and homogenized in ice-cold homogenization buffer (300 mM sucrose, 1 mM EGTA, and 20 mM MOPS, pH 7.4). Nuclei and cellular debris were pelleted by centrifugation at 600 *g* for 15 min. The supernatant was centrifuged at 10,000 *g* for 25 min, resulting in a crude mitochondrial pellet. Mitochondria were immediately employed for import experiments. Panel A, RRL translation product were incubated with freshly isolated mitochondria, from control (lanes 1) or from fibroblasts from patient K (lanes 2) or patient C (lanes 3), in BSA buffer (20 mM HEPES-KOH pH 7.4, 250 mM sucrose, 80 mM potassium acetate, 5 mM magnesium acetate, BSA 3%) plus 10 mM sodium succinate and 2 mM ATP at for 90 min. at 37 °C. Mitochondria were spun down from the import mixture before or after trypsin treatment (0.5 μg per 50 μg mitochondrial proteins, 35 min on ice). The solubilized pellets were analyzed by SDS-PAGE and autoradiography. Panel B, RRL translation product were incubated with freshly isolated mitochondria, from control fibroblasts (lane 1), fibroblasts from patient K (lane 2) or from patient C (lane 3), as described above, in the presence of protease inhibitors cocktail (dilution 1:600). Aliquots of the all samples were subjected to western blotting with antibody against the core II subunit of bc1 complex for other experimental details see Ref. [44].





(C)

	PD ONSET (Y)	G1311A, W437X PINK1 MUTATION	ND5 A12397G	ND6 T14319C
PATIENT	22	HOMOZYGOTE	homopl.	homopl.
MOTHER	53	HETEROZYGOTE	homopl.	homopl.
FATHER	—	HETEROZYGOTE	absent	absent

Fig. 12. Structure of the gene (A) coding for the PTEN induced kinase 1 (PINK1) protein (B) localized in the inner mitochondrial membrane. In B, the domains of the PINK1 protein (from ExPASy Proteomics Server; http://www.expasy.org/) and the mutations found in patients with familiar Parkinson are shown. (C) PINK1 G1311A (W437X) mutation in the alleles and ND5 and ND6 mutations in the patient studied and her parents. Onset age of the clinical manifestations of the disease is also shown.

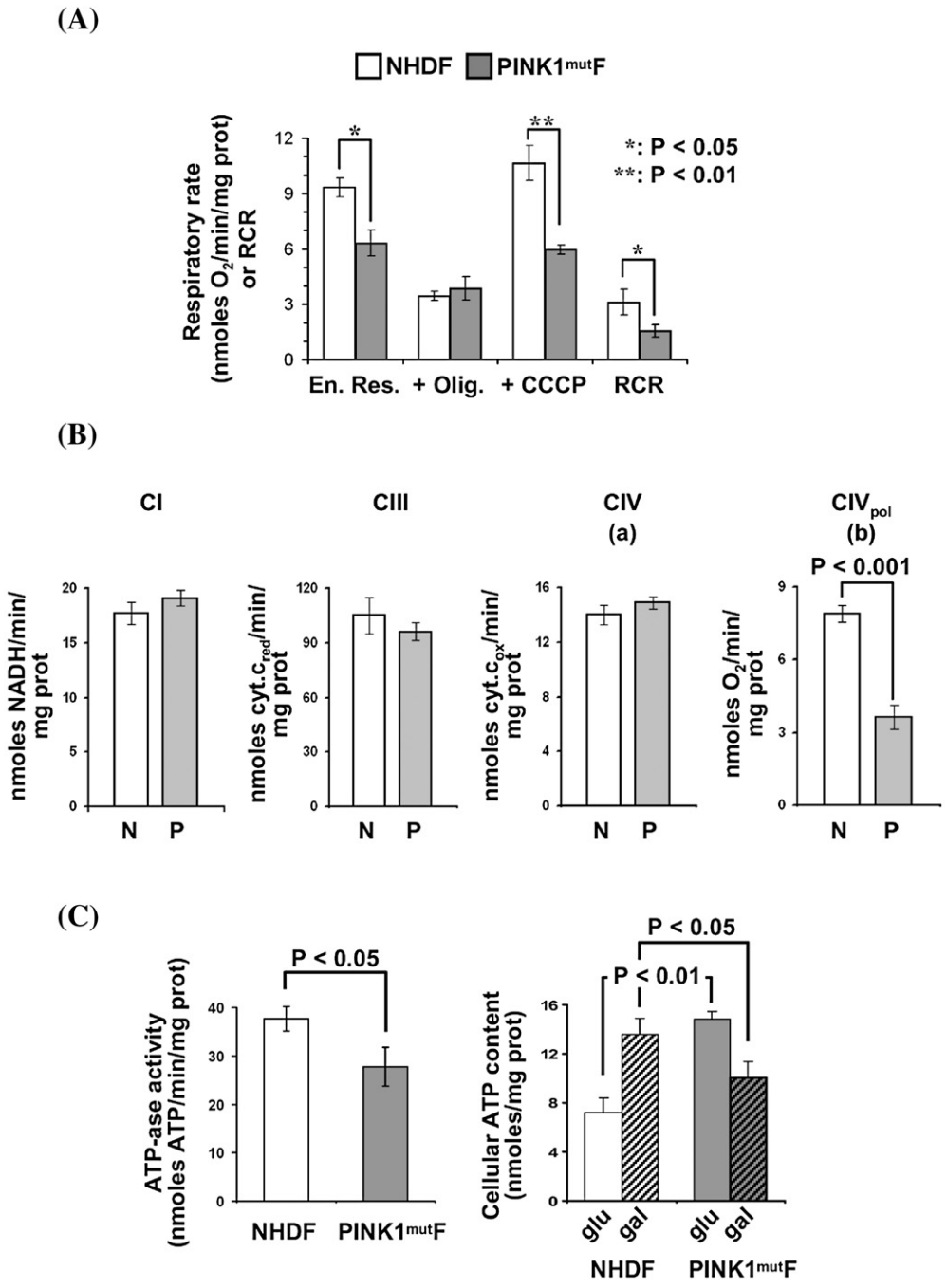


Fig. 13. Functional pattern of the oxidative phosphorylation system in control fibroblasts (NHDF) and fibroblasts from the Parkinson patients with the homozygous G1311A mutation in PINK1. (A) Endogenous respiration rate in the absence or presence of oligomycin (state 4) or of the uncoupler CCCP, RCR, respiratory control ratio. (B) Specific enzymatic activity of complexes I, III and IV (a, with added cytochrome *c*; b, supported by ascorbate plus TMPD). (C) ATPase activity of complex V and ATP content in fibroblast cultures grown in glucose or galactose medium. Reproduced with permission from Ref. [6]. For details see Ref. [6] and text.

complex I [35], is constitutively phosphorylated [36] and is essential for the assembly/function of the complex [94]. This intriguing PINK1/HtrA2/complex I interaction seems to deserve further study. It has, for example, been found that all-trans retinoic acid increases, by a post-transcriptional mechanism, the mitochondrial level of the protein product of GRIM-19 in complex I [95]. It would be of interest to investigate whether this effect of retinoic acid, which resulted, however, in a marked inhibition of the NADH-ubiquinone oxidoreductase activity [95], is associated with the reported stimulation by retinoic acid of protein phosphatase activity [96,97].

What presented provides an in sight into the possible combined impact of PINK1 and mtDNA mutations in the development of Parkinsonism, as exemplified by the familial case examined (see Fig. 12). In addition to the germinal ones present at birth, mutations in mitochondrial genes of complex I, i, can accumulate in the life span, in particular with aging. This can result *per se* in general decline of the functional capacity of oxidative phosphorylation complexes [9,10,98]. Complex I due to its gene and protein complexity appears to be an hotspot for gene mutations and protein damages.

The decline in the mitochondrial bioenergetics function, particularly critical in neurons due to their high ATP demand [17], is normally counteracted by defence mechanisms which encompass ROS scavenger systems [33,42,99], quality control of DNA [100], RNA transcripts [54,56] and oxidized proteins [63–65], autophagy of damaged

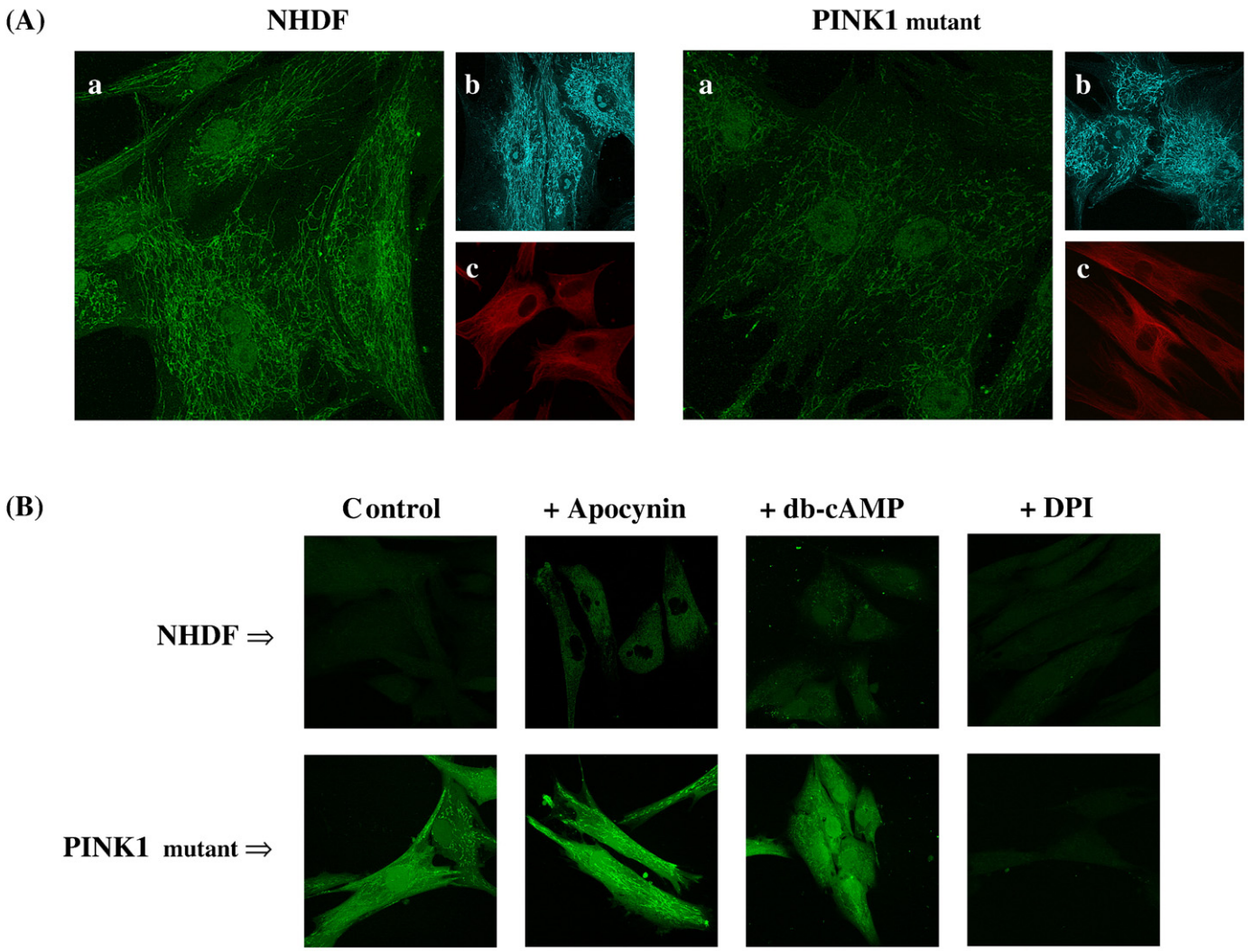


Fig. 14. Confocal microscopy analysis of cytochrome *c* content and H_2O_2 level in control (NHDF) and PINK1 mutant fibroblasts. A): (a) Immunocytochemical detection of cytochrome *c* in normal and patient's fibroblasts was performed by binding of a secondary FITC-conjugated Ab to a primary mAb for cytochrome *c*. (b) Immunocytochemistry for detection of the β-subunit of complex V and of subunit IV of cytochrome *c* oxidase (c) are also shown; in the latter a rhodamine-conjugated secondary Ab was used. B): H_2O_2 generation and localization in control (NDHF) and patient's PINK1 mutated fibroblasts. Fluorescence of the H_2O_2-probe DCF was visualised in live fibroblasts by laser scanning confocal microscopy. Reproduced with permission from Ref. [6]. See text and Ref. [6] for details.

organelles [66–68]. The PINK1 kinase, in combination with the products of other genes like PARK2, DJ-1 [67,75,101], whose mutations are associated with familial Parkinsonism, plays an essential role in the quality control of altered mitochondrial systems.

In the family examined here the homozygous ND5 and ND6 mutations were both present in the patient's mother, heterozygous for the PINK1 mutation. She presented a Parkinson syndrome in her fifties. Thus the mtDNA mutation predisposed the PINK1 heterozygous subject to a relatively late development of the disease (Fig. 12). In the patient, the homozygous PINK1 mutation, impairing quality control processes of damaged mitochondria, anticipated the decay of mitochondrial functions associated with the cohexistent homoplasmic mutations in the *ND5* and *ND6* genes, with an early PD onset (age 22 years). The PINK1 heterozygous mutation, in the absence of ND5 and ND6 mutations, would not result in Parkinsonism, as the case of the father still exempt from the disease in his seventies.

5. Conclusions

The genetic and biochemical observations examined in this paper highlight different mechanisms by which genetic defects of complex I alter the mitochondrial bioenergetics function in hereditary neurological diseases. Mutations in the *NDFUFS4* and *NDUFS1* nuclear genes of the complex are respectively associated with encephalopathy disorders with different clinical features [3]. The NDUFS4 mutations (18 kDa AQDQ subunit), which are most unfavourable, result in disappearance of the subunit encoded by the gene, block of the last step of the assembly of the complex, complete loss of the enzymatic activity and its activation by cAMP, accumulation, for some of the mutations, of unproductive alternative transcripts, severe chronic lactic acidosis. These alterations underscore the fatal outcome of the disease, whose conceivable treatment might rest on wild-type gene transfection.

The NDUFS1 mutation (75 kDa FeS subunit), which is associated with a less severe encephalopathy, results in marked but incomplete depression of the content and enzymatic activity of complex I and enhancement of ROS production, both these effects being reversed by cAMP. The mature complex is converted to a small form, apparently by proteolytic degradation promoted by the same ROS produced as a consequence of the mutated 75 kDa subunit. In this case treatment with β-adrenergic agonists and antioxidants could exert a beneficial effect on the clinical course of the disease.

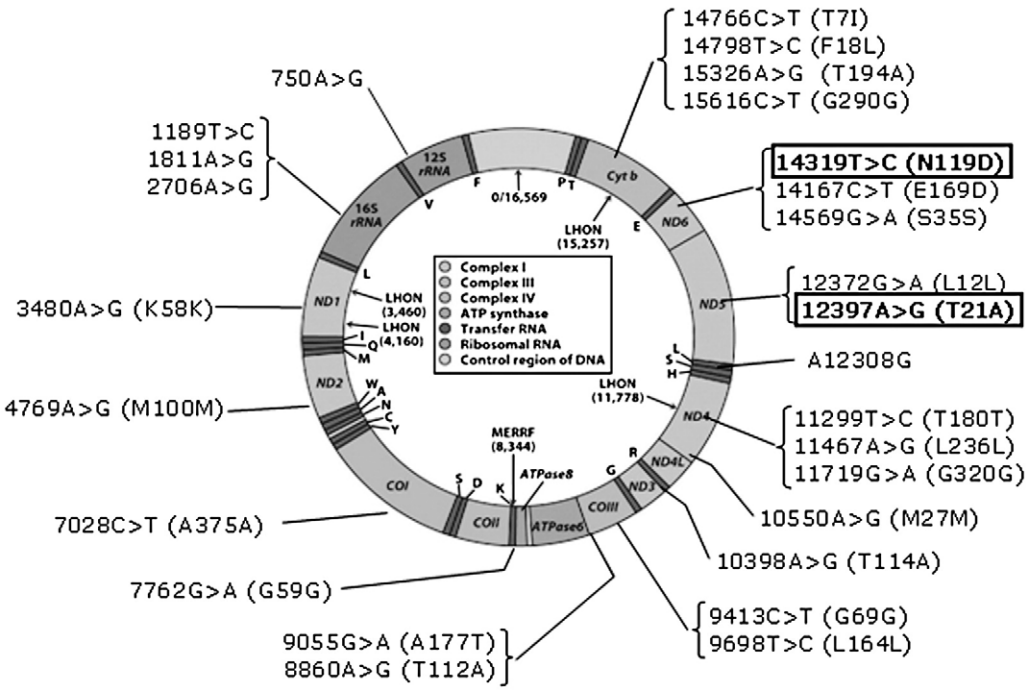


Fig. 15. Results of the mutational analysis of the entire mtDNA of the Parkinson patient with homozygous G1311A mutation in the *PINK1* gene. Reproduced with permission from Ref. [7]. See text and Ref. [7] for details.

In the case of familiar chronic epilepsy and progressive encephalopathy, the deficit of complex I appeared to be associated with a genetically determined enhanced proteolytic degradation of the complex by a mitochondrial protease, possibly of the calpain family. The genetic determinants and the biochemical mechanisms leading to this enhanced proteolytic activity remain to be elucidated. It might be recalled, here, that in spastic paraplegia, loss of paraplegin, a putative mitochondrial AAA protease, results, on the other hand, in defective assembly of complex I and increased sensitivity to oxidative stress [8,102]. Thus mitochondrial proteases can play a dual role in the

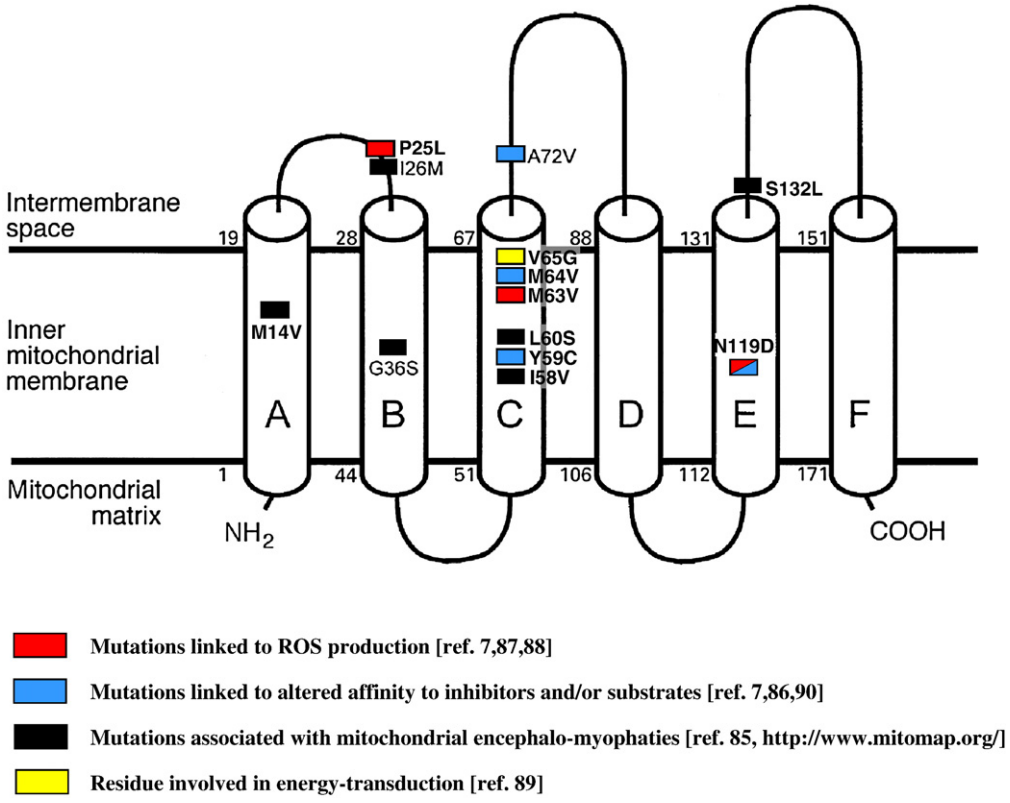


Fig. 16. Localization of reported mutations in the ND6 protein secondary structure. The secondary structure and membrane topology for the human ND6 protein was predicted by the hydropathy plot. The model predicts six putative hydrophobic transmembrane helices, connected each other by five loops (two external and three internal loops). Pathogenic changes in aminoacid residues are reported with the respective references.

control of the functional level of complex I. Those like paraplegin are essential for subunit maturation and assembly of the complex; their mutational inactivation impairs formation of the mature complex [102]. The second group of proteases, which appear to include member(s) of the calpain family, are involved in the proteolytic degradation of subunits of the complex [57]. Genetic factors leading to anomalous enhancement of this proteolysis results in a pathological decline of the functional content of complex I.

Genetic and functional analysis of mitochondria in the case of the familiar PD associated with the W437X PINK1 mutation revealed that co-segregation of mutations in the *ND5* and *ND6* mitochondrial genes of complex I had an impact in determining the development and the clinical course of the disease. This finding underscores the importance of mutational analysis of mitochondrial DNA in patients with hetero- or homozygous familial forms of Parkinsonism as well as in sporadic PD. Identification of mtDNA mutations in PD could contribute to develop therapeutical measures for preventing the progress of this disease.

The features of the hereditary complex I dysfunctions described, besides clarifying the molecular basis of the pathogenetic mechanism of the associated neurological disorders, can shed light on the involvement of complex I dysfunction in sporadic neurological diseases, cancer and aging.

Acknowledgements

This work was supported by: National Project on “Molecular Mechanisms, Physiology and Pathology of Membrane Bioenergetics System”, 2005-Ministero dell' Istruzione, dell' Università e della Ricerca (MIUR), Italy; Research Grant, University of Bari and Research Grant Foundation Cassa di Risparmio di Puglia.

References

[1] J.L. Loeffen, J.A. Smeitink, J.M. Trijbels, A.J. Janssen, R.H. Triepels, R.C. Sengers, L.P. van den Heuvel, Isolated complex I deficiency in children: clinical, biochemical and genetic aspects, Hum. Mutat. 15 (2000) 123–134.
[2] V. Petruzzella, S. Papa, Mutations in human nuclear genes encoding for subunits of mitochondrial respiratory complex I: the *NDUFS4* gene, Gene 286 (2002) 149–154.
[3] M. Bugiani, F. Invernizzi, S. Alberio, E. Briem, E. Lamantea, F. Carrara, I. Moroni, L. Farina, M. Spada, M.A. Donati, G. Uziel, M. Zeviani, Clinical and molecular findings in children with complex I deficiency, Biochim. Biophys. Acta 1659 (2004) 136–147.
[4] M. Orth, A.H.V. Schapira, Mitochondria and degenerative disorders, Am. J. Med. Genet. 106 (2001) 27–36.
[5] D.J. Pagliarini, S.E. Calvo, B. Chang, S.A. Sheth, S.B. Vafai, S.E. Ong, G.A. Walford, C. Sugiana, A. Boneh, W.K. Chen, D.E. Hill, M. Vidal, J.G. Evans, D.R. Thorburn, S.A. Carr, V.K. Mootha, A mitochondrial protein compendium elucidates complex I disease biology, Cell 134 (2008) 112–123.
[6] C. Piccoli, A.M. Sardanelli, R. Scrima, M. Ripoli, G. Quarato, A. D'Aprile, F. Bellomo, S. Scacco, G. De Michele, A. Filla, A. Iuso, D. Boffoli, N. Capitanio, S. Papa, Mitochondrial respiratory dysfunction in familiar Parkinsonism associated with PINK1 mutation, Neurochem. Res. 33 (2008) 2565–2574.
[7] C. Piccoli, M. Ripoli, G. Quarato, R. Scrima, A. D'Aprile, D. Boffoli, M. Margaglione, C. Criscuolo, G. De Michele, A. Sardanelli, S. Papa, N. Capitanio, Coexistence of mutations in PINK1 and mitochondrial DNA in early onset parkinsonism, J. Med. Genet. 45 (2008) 596–602.
[8] L. Atorino, L. Silvestri, M. Koppen, L. Cassina, A. Ballabio, R. Marconi, T. Langer, G. Casari, Loss of m-AAA protease in mitochondria causes complex I deficiency and increased sensitivity to oxidative stress in hereditary spastic paraplegia, J. Cell Biol. 163 (2003) 777–787.
[9] S. Papa, Mitochondrial oxidative phosphorylation changes in the life span. Molecular aspects and physiopathological implications, Biochim. Biophys. Acta 1276 (1996) 87–105.
[10] B. Ventura, M.L. Genova, C. Bovina, G. Formiggini, G. Lenaz, Control of oxidative phosphorylation by Complex I in rat liver mitochondria: implications for aging, Biochim. Biophys. Acta 1553 (2002) 249–260.
[11] J.E. Walker, The NADH: ubiquinone oxidoreductase (complex I) of respiratory chains, Q. Rev. Biophys. 25 (1992) 232–253.
[12] T. Ohnishi, Iron–sulfur clusters/semiquinones in complex I, Biochim. Biophys. Acta 1364 (1998) 186–206.
[13] T. Yagi, A. Matsuno-Yagi, The proton-translocating NADH-quinone oxidoreductase in the respiratory chain: the secret unlocked, Biochemistry 42 (2003) 2266–2274.
[14] U. Brandt, Energy converting NADH:quinone oxidoreductase (complex I), Annu. Rev. Biochem. 75 (2006) 69–92.
[15] T. Friedrich, B. Böttcher, The gross structure of the respiratory complex I: a Lego System, Review. Biochim. Biophys. Acta 1657 (2004) 71–83.
[16] L.A. Sazanov, P. Hinchliffe, Structure of the hydrophilic domain of respiratory complex I from *Thermus thermophilus*, Science 311 (2006) 1430–1436.
[17] S. Papa, V. Petruzzella, S. Scacco, Structure, redox-coupled protonmotive activity and pathological disorders of respiratory chain complexes, in: G. Gibson, G.A. Dienel (Eds.), 3rd Edition, Handbook of Neurochemistry and Molecular Neurobiology, Vol. 5, Springer-Verlag, Berlin Heidelberg, 2007, pp. 94–118.
[18] S. Papa, N. Capitanio, G. Villani, Proton pumps of respiratory chain enzymes, in: S. Papa, F. Guerrieri, J.M. Tager (Eds.), Frontiers of Cellular Bioenenergetics: Molecular Biology, Biochemistry and Physiopathology, Plenum Press, London, 1999, pp. 49–88.
[19] T. Ohnishi, J.C. Salerno, Conformation-driven and semiquinone-gated proton-pump mechanism in the NADH-ubiquinone oxidoreductase (complex I), FEBS Lett. 579 (2005) 4555–4561.
[20] J. Carroll, I.M. Fearnley, J.M. Skehel, R.J. Shannon, J. Hirst, J.E. Walker, Bovine complex I is a complex of 45 different subunits, J. Biol. Chem. 281 (2006) 32724–32727.
[21] J. Hirst, J. Carroll, I.M. Fearnley, R.J. Shannon, J.E. Walker, The nuclear encoded subunits of complex I from bovine heart mitochondria, Biochim. Biophys. Acta 1604 (2003) 135–150.
[22] S. Scacco, V. Petruzzella, S. Budde, R. Vergari, R. Tamborra, D. Panelli, L.P. van den Heuvel, J.A. Smeitink, S. Papa, Pathological mutations of the human *NDUFS4* gene of the 18-kDa (AQDQ) subunit of complex I affect the expression of the protein and the assembly and function of the complex, J. Biol. Chem. 278 (2003) 44161–44167.
[23] H. Antonicka, I. Ogilvie, T. Taivassalo, R.P. Anitori, R.G. Haller, J. Vissing, N.G. Kennaway, E.A. Shoubridge, Identification and characterization of a common set of complex I assembly intermediates in mitochondria from patients with complex I deficiency, J. Biol. Chem. 278 (2003) 43081–43088.
[24] I.E. Scheffler, N. Yadava, P. Potluri, Molecular genetics of complex I-deficient Chinese hamster cell lines, Biochim. Biophys. Acta 1659 (2004) 160–171.
[25] S. Papa, D. De Rasmo, S. Scacco, A. Signorile, Z. Technikova-Dobrova, G. Palmisano, A.M. Sardanelli, F. Papa, D. Panelli, R. Scaringi, A. Santeramo, Mammalian complex I: a regulable and vulnerable pacemaker in mitochondrial respiratory function, Biochim. Biophys. Acta 1777 (2008) 719–728.
[26] M. Hüttemann, I. Lee, L. Samavati, H. Yu, J.W. Doan, Regulation of mitochondrial oxidative phosphorylation through cell signalling, Biochim. Biophys. Acta 1773 (2007) 1701–1720.
[27] C. Remacle, M.R. Barbieri, P. Cardol, P.P. Hamel, Eukaryotic complex I: functional diversity and experimental systems to unravel the assembly process, Mol. Genet. Genomics 280 (2008) 93–110.
[28] N. Yadava, P. Potluri, I.E. Scheffler, Investigations of the potential effects of phosphorylation of the MWFE and ESSS subunits on complex I activity and assembly, Int. J. Biochem. Cell. Biol. 40 (2008) 447–460.
[29] E. Cadenas, K.J. Davies, Mitochondrial free radical generation, oxidative stress, and aging, Free Radic. Biol. Med. 29 (2000) 222–230.
[30] V.G. Grivennikova, A.D. Vinogradov, Generation of superoxide by the mitochondrial Complex I, Biochim. Biophys. Acta 1757 (2006) 553–561.
[31] R. Fato, C. Bergamini, S. Leoni, P. Strocchi, G. Lenaz, Generation of reactive oxygen species by mitochondrial complex I: implications in neurodegeneration, Neurochem. Res. 33 (2008) 2487–2501.
[32] S. Verkaart, W.J. Koopman, J. Cheek, S.E. van Emst-de Vries, L.W. van den Heuvel, J.A. Smeitink, P.H. Willems, Mitochondrial and cytosolic thiol redox state are not detectably altered in isolated human NADH:ubiquinone oxidoreductase deficiency, Biochim. Biophys. Acta 1772 (2007) 1041–1051.
[33] M.P. Murphy, How mitochondria produce reactive oxygen species, Biochem. J. 417 (2009) 1–13.
[34] J.E. Angell, D.J. Lindner, P.S. Shapiro, E.R. Hofmann, D.V. Kalvakolanu, Identification of GRIM-19, a novel cell death-regulatory gene induced by the interferon- and retinoic acid combination, using a genetic approach, J. Biol. Chem. 275 (2000) 33416–33426.
[35] I.M. Fearnley, J. Carroll, R.J. Shannon, M.J. Runswick, J.E. Walker, J. Hirst, GRIM-19, a cell death regulatory gene product, is a subunit of bovine mitochondrial NADH: ubiquinone oxidoreductase (complex I), J. Biol. Chem. 276 (2001) 38345–38348.
[36] G. Palmisano, A.M. Sardanelli, A. Signorile, S. Papa, M.R. Larsen, The phosphorylation pattern of bovine heart complex I subunits, Proteomics 7 (2007) 1575–1583.
[37] S. Papa, S. Scacco, A.M. Sardanelli, R. Vergari, F. Papa, S. Budde, L. van den Heuvel, J. Smeitink, Mutation in the NDUFS4 gene of complex I abolishes cAMP-dependent activation of the complex in a child with fatal neurological syndrome, FEBS Lett. 489 (2001) 259–262.
[38] V. Petruzzella, R. Vergari, I. Puzziferri, D. Boffoli, E. Lamantea, M. Zeviani, S. Papa, A nonsense mutation in the NDUFS4 gene encoding the 18 kDa (AQDQ) subunit of complex I abolishes assembly and activity of the complex in a patient with Leigh-like syndrome, Hum. Mol. Genet. 10 (2001) 529–535.
[39] A. Iuso, S. Scacco, C. Piccoli, F. Bellomo, V. Petruzzella, R. Trentadue, M. Minuto, M. Ripoli, N. Capitanio, M. Zeviani, S. Papa, Dysfunctions of cellular oxidative metabolism in patients with mutations in the *NDUFS1* and *NDUFS4* genes of complex I, J. Biol. Chem. 281 (2006) 10374–10380.
[40] S. Scacco, R. Vergari, R.C. Scarpulla, Z. Technikova-Dobrova, A.M. Sardanelli, R. Lambo, V. Lorusso, S. Papa, cAMP-dependent phosphorylation of the nuclear encoded 18 kDa (IP) subunit of respiratory complex I and activation of the complex in serum-starved mouse fibroblast cultures, J. Biol. Chem. 275 (2000) 17578–17582.

[41] Z. Technikova-Dobrova, A.M. Sardanelli, F. Speranza, S. Scacco, A. Signorile, V. Lorusso, S. Papa, Cyclic adenosine monophosphate-dependent phosphorylation of mammalian mitochondrial proteins: enzyme and substrate characterization and functional role, Biochemistry 40 (2001) 13941–13947.
[42] C. Piccoli, S. Scacco, F. Bellomo, A. Signorile, A. Iuso, D. Boffoli, R. Scrima, N. Capitanio, S. Papa, cAMP controls oxygen metabolism in mammalian cells, FEBS Lett. 580 (2006) 4539–4543.
[43] M. Lazarou, M. McKenzie, A. Ohtake, D.R. Thorburn, M.T. Ryan, Analysis of the assembly profiles for mitochondrial- and nuclear-DNA-encoded subunits into complex I, Mol. Cell Biol. 27 (2007) 4228–4237.
[44] D. De Rasmo, D. Panelli, A.M. Sardanelli, S. Papa, cAMP-dependent protein kinase regulates the mitochondrial import of the nuclear encoded NDUFS4 subunit of complex I, Cell Signal. 20 (2008) 989–997.
[45] A. Signorile, A.M. Sardanelli, R. Nuzzi, S. Papa, Serine (threonine) phosphatase(s) acting on cAMP-dependent phosphoproteins in mammalian mitochondria, FEBS Lett. 512 (2002) 91–94.
[46] W.A. Tao, B. Wollscheid, R. O'Brien, J.K. Eng, X.J. Li, B. Bodenmiller, J.D. Watts, L. Hood, R. Aebersold, Quantitative phosphoproteome analysis using a dendrimer conjugation chemistry and tandem mass spectrometry, Nat. Methods 2 (2005) 591–598.
[47] R. Chen, I.M. Fearnley, S.Y. Peak-Chew, J.E. Walker, The phosphorylation of subunits of complex I from bovine heart mitochondria, J. Biol. Chem. 279 (2004) 26036–26045.
[48] L. van den Heuvel, W. Ruitenbeek, R. Smeets, Z. Gelman-Kohan, O. Elpeleg, J. Loeffen, F. Trijbels, E. Mariman, D. de Bruijn, J. Smeitink, Demonstration of a new pathogenic mutation in human complex I deficiency: a 5-bp duplication in the nuclear gene encoding the 18-kD (AQDQ) subunit, Am. J. Hum. Genet. 62 (1998) 262–268.
[49] S.M. Budde, L.P. van den Heuvel, A.J. Janssen, R.J. Smeets, C.A. Buskens, L. DeMeirleir, R. Van Coster, M. Baethmann, T. Voit, J.M. Trijbels, J.A. Smeitink, Combined enzymatic complex I and III deficiency associated with mutations in the nuclear encoded *NDUFS4* gene, Biochem. Biophys. Res. Commun. 18 (2000) 63–68.
[50] P. Benit, J. Steffann, S. Lebon, D. Chretien, N. Kadhom, P. de Lonlay, A. Goldenberg, Y. Dumez, M. Dommergues, P. Rustin, A. Munnich, A. Rotig, Genotyping microsatellite DNA markers at putative disease loci in inbred/multiplex families with respiratory chain complex I deficiency allows rapid identification of a novel nonsense mutation (IVS1nt-1) in the *NDUFS4* gene in Leigh syndrome, Hum. Genet. 112 (2003) 563–566.
[51] A. Rötig, S. Lebon, E. Zinovieva, J. Mollet, E. Sarzi, J.P. Bonnefont, A. Munnich, Molecular diagnostics of mitochondrial disorders, Biochim. Biophys. Acta 1659 (2004) 129–135.
[52] T. Emahazion, A.J. Brookes, Mapping of the *NDUFA2*, *NDUFA6*, *NDUFA7*, *NDUFB8*, and *NDUFS8* electron transport chain genes by intron based radiation hybrid mapping, Cytogenet. Cell Genet. 82 (1998) 114–118.
[53] P. Bénit, D. Chretien, N. Kadhom, P. de Lonlay-Debeney, V. Cormier-Daire, A. Cabral, S. Peudenier, P. Rustin, A. Munnich, A. Rötig, Large-scale deletion and point mutations of the nuclear *NDUFV1* and *NDUFS1* genes in mitochondrial complex I deficiency, Am. J. Hum. Genet. 68 (2001) 1344–1352.
[54] O. Isken, L.E. Maquat, Quality control of eukaryotic mRNA: safeguarding cells from abnormal mRNA function, Genes Dev. 21 (2007) 1833–1856.
[55] V. Petruzzella, D. Panelli, A. Torraco, A. Stella, S. Papa, Mutations in the *NDUFS4* gene of mitochondrial complex I alter stability of the splice variants, FEBS Lett. 579 (2005) 3770–3776.
[56] D. Panelli, V. Petruzzella, R. Vitale, D. De Rasmo, A. Munnich, A. Rötig, S. Papa, The regulation of PTC containing transcripts of the human *NDUFS4* gene of complex I of respiratory chain and the impact of pathological mutations, Biochimie 90 (2008) 1452–1460.
[57] D.D. Arrington, T.R. Van Vleet, R.G. Schnellmann, Calpain 10: a mitochondrial calpain and its role in calcium-induced mitochondrial dysfunction, Am. J. Physiol. Cell Physiol. 291 (2006) C1159–C1171.
[58] M.J. Farrer, Genetics of Parkinson disease: paradigm shifts and future prospectsì, Nat. Rev. Genet. 7 (2006) 306–318.
[59] M.C.J. Dekker, V. Bonifati, C.M. van Duijn, Parkinson's disease: piecing together a genetic jigsaw, Brain 126 (2003) 1722–1733.
[60] T. Gasser, Genetics of Parkinson's disease, Curr. Opin. Neurol. 18 (2005) 363–369.
[61] E.M. Valente, P.M. Abou-Sleiman, V. Caputo, M.M. Muqit, K. Harvey, S. Gispert, Z. Ali, D. Del Turco, A.R. Bentivoglio, D.G. Healy, A. Albanese, R. Nussbaum, R. González-Maldonado, T. Deller, S. Salvi, P. Cortelli, W.P. Gilks, D.S. Latchman, R.J. Harvey, B. Dallapiccola, G. Auburger, N.W. Wood, Hereditary early-onset Parkinson's disease caused by mutations in PINK1, Science 304 (2004) 1158–1160.
[62] E.M. Valente, S. Salvi, T. Ialongo, R. Marongiu, A.E. Elia, V. Caputo, L. Romito, A. Albanese, B. Dallapiccola, A.R. Bentivoglio, PINK1 mutations are associated with sporadic early-onset parkinsonism, Ann. Neurol. 56 (2004) 336–441.
[63] S. Gandhi, M.M. Muqit, L. Stanyer, D.G. Healy, P.M. Abou-Sleiman, I. Hargreaves, S. Heales, M. Ganguly, L. Parsons, A.J. Lees, D.S. Latchman, J.L. Holton, N.W. Wood, T. Revesz, PINK1 protein in normal human brain and Parkinson's disease, Brain 129 (2006) 1720–1731.
[64] J.W. Pridgeon, J.A. Olzmann, L.S. Chin, L. Li, PINK1 protects against oxidative stress byphosphorylating mitochondrial chaperone TRAP1, PLoS Biol. 5 (2007) e172.
[65] H. Plun-Favreau, K. Klupsch, N. Moisoi, S. Gandhi, S. Kjaer, D. Frith, K. Harvey, E. Deas, R.J. Harvey, N. McDonald, N.W. Wood, L.M. Martins, J. Downward, The mitochondrial protease HtrA2 is regulated by Parkinson's disease-associated kinase PINK1, Nat. Cell Biol. 9 (2007) 1243–1252.
[66] I.E. Clark, M.W. Dodson, C. Jiang, J.H. Cao, J.R. Huh, J.H. Seol, S.J. Yoo, B.A. Hay, M. Guo, Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin, Nature 441 (2006) 1163–1166.
[67] A.H. Schapira, Mitochondria in the aetiology and pathogenesis of Parkinson's disease, Lancet Neurol. 7 (2008) 97–109.
[68] D. Narendra, A. Tanaka, D.F. Suen, R.J. Youle, Parkin is recruited selectively to impaired mitochondria and promotes their autophagy, J. Cell Biol. 183 (2008) 795–803.
[69] H.M. McBride, Parkin mitochondria in the autophagosome, J. Cell Biol. 183 (2008) 757–759.
[70] Y. Yang, Y. Ouyang, L. Yang, M.F. Beal, A. McQuibban, H. Vogel, B. Lu, Pink1 regulates mitochondrial dynamics through interaction with the fission/fusion machinery, PNAS 105 (2008) 7070–7073.
[71] L. Silvestri, V. Caputo, E. Bellacchio, L. Atorino, B. Dallapiccola, E.M. Valente, G. Casari, Mitochondrial import and enzymatic activity of PINK1 mutants associated to recessive parkinsonism, Hum. Mol. Genet. 14 (2005) 3477–3492.
[72] C. Zhou, Y. Huang, Y. Shao, J. May, D. Prou, C. Perier, W. Dauer, E.A. Schon, S. Przedborski, The kinase domain of mitochondrial PINK1 faces the cytoplasm, PNAS 105 (2008) 2022–12027.
[73] Y. Kim, J. Park, S. Kim, S. Song, S.K. Kwon, S.H. Lee, T. Kitada, J.M. Kim, J. Chung, PINK1 controls mitochondrial localization of Parkin through direct phosphorylation, Biochim. Biophys. Res. Comm. 377 (2008) 975–980.
[74] T. Kitada, S. Asakawa, N. Hattori, H. Matsumine, Y. Yamamura, S. Minoshima, M. Yokochi, Y. Mizuno, N. Shimizu, Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism, Nature 392 (1998) 605–608.
[75] R.D. Mills, C.H. Sim, S.S. Mok, T.D. Mulhern, J.G. Culvenor, H.C. Cheng, Biochemical aspects of the neuroprotective mechanism of PTEN-induced kinase-1 (PINK1), J. Neurochem. 105 (2008) 18–33.
[76] V. Bonifati, C.F. Rohé, G.J. Breedveld, E. Fabrizio, M. De Mari, C. Tassorelli, A. Tavella, R. Marconi, D.J. Nicholl, H.F. Chien, E. Fincati, G. Abbruzzese, P. Marini, A. De Gaetano, M.W. Horstink, J.A. Maat-Kievit, C. Sampaio, A. Antonini, F. Stocchi, P. Montagna, V. Toni, M. Guidi, A. Dalla Libera, M. Tinazzi, F. De Pandis, G. Fabbrini, S. Goldwurm, A. de Klein, E. Barbosa, L. Lopiano, E. Martignoni, P. Lamberti, N. Vanacore, G. Meco, B.A. Oostra, Italian Parkinson Genetics Network. Early-onset parkinsonism associated with PINK1 mutations: frequency, genotypes, and phenotypes, Neurology 65 (2005) 87–95.
[77] C. Criscuolo, G. Volpe, A. De Rosa, A. Varrone, R. Marongiu, P. Mancini, E. Salvatore, B. Dallapiccola, A. Filla, E.M. Valente, G. De Michele, PINK1 homozygous W437X mutation in a patient with apparent dominant transmission of parkinsonism, Mov. Disord. 21 (2006) 1265–1267.
[78] C. Zadikoff, E. Rogaeva, A. Djarmati, C. Sato, S. Salehi-Rad, P. St George-Hyslop, C. Klein, A.E. Lang, Homozygous and heterozygous PINK1 mutations: considerations for diagnosis and care of Parkinson's disease patients, Mov. Disord. 21 (2006) 875–879.
[79] P.M. Abou-Sleiman, M.M. Muqit, N.Q. McDonald, Y.X. Yang, S. Gandhi, D.G. Healy, K. Harvey, R.J. Harvey, E. Deas, K. Bhatia, N. Quinn, A. Lees, D.S. Latchman, N.W. Wood, A heterozygous effect for PINK1 mutations in Parkinson's disease? Ann. Neurol. 60 (2006) 414–419.
[80] J. Fukae, Y. Mizuno, N. Hattori, Mitochondrial dysfunction in Parkinson's disease, Mitochondrion 7 (2007) 58–62.
[81] C.H. Sim, D.S. Lio, S.S. Mok, C.L. Masters, A.F. Hill, J.G. Culvenor, H.C. Cheng, C-terminal truncation and Parkinson's diseaseassociated mutations down-regulate the protein serine/threonine kinase activity of PTEN-induced kinase-1, Hum. Mol. Genet. 15 (2006) 3251–3262.
[82] R. Rossignol, R. Gilkerson, R. Aggeler, K. Yamagata, S.J. Remington, R.A. Capaldi, Energy substrate modulates mitochondrial structure and oxidative capacity in cancer cells, Cancer Res. 64 (2004) 985–993.
[83] D.K. Johnson, K.J. Schillinger, D.M. Kwait, C.V. Hughes, E.J. McNamara, F. Ishmael, R.W. O'Donnell, M.M. Chang, M.G. Hogg, J.S. Dordick, L. Santhanam, L.M. Ziegler, J. A. Holland, Inhibition of NADPH oxidase activation in endothelial cells by ortho-methoxy-substituted catechols, Endothelium 9 (2002) 191–203.
[84] A. Majander, M. Finel, M. Wikstrom, Diphenyleneiodonium inhibits reduction of iron–sulfur clusters in the mitochondrial NADH-ubiquinone oxidoreductase (Complex I), J. Biol. Chem. 269 (1994) 21037–21042.
[85] P.F. Chinnery, D.T. Brown, R.M. Andrews, R. Singh-Kler, P. Riordan-Eva, J. Lindley, D.A. Applegarth, D.M. Turnbull, N. Howell, The mitochondrial *ND6* gene is a hot spot for mutations that cause Leber's hereditary optic neuropathy, Brain 124 (2001) 209–218.
[86] V. Carelli, A. Ghelli, L. Bucchi, P. Montagna, A. De Negri, V. Leuzzi, C. Carducci, G. Lenaz, E. Lugaresi, M. Degli Esposti, Biochemical features of mtDNA 14484 (ND6/M64V) point mutation associated with Leber's hereditary optic neuropathy, Ann. Neurol. 45 (1999) 320–328.
[87] K. Ishikawa, K. Takenaga, M. Akimoto, N. Koshikawa, A. Yamaguchi, H. Imanishi, K. Nakada, Y. Honma, J. Hayashi, ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis, Science 320 (2008) 661–664.
[88] R. Gonzalo, E. Garcia-Arumi, D. Llige, R. Marti, A. Solano, J. Montoya, J. Arenas, A.L. Andreu, Free radicals-mediated damage in transmitochondrial cells harboring the T14487C mutation in the *ND6* gene of mtDNA, FEBS Lett. 579 (2005) 6909–6913.
[89] M.C. Kao, S. Di Bernardo, E. Nakamaru-Ogiso, H. Miyoshi, A. Matsuno-Yagi, T. Yagi, Characterization of the membrane domain subunit NuoJ (ND6) of the NADH-quinone oxidoreductase from Escherichia coli by chromosomal DNA manipulation, Biochemistry 44 (2005) 3562–3571.
[90] J. Pätsi, M. Kervinen, M. Finel, I.E. Hassinen, Leber hereditary optic neuropathy mutations in the ND6 subunit of mitochondrial complex I affect ubiquinone reduction kinetics in a bacterial model of the enzyme, Biochem. J. 409 (2008) 129–137.
[91] R. Betarbet, T.B. Sherer, G. MacKenzie, M. Garcia-Osuna, A.V. Panov, J.T.

Greenamyre, Chronic systemic pesticide exposure reproduces features of Parkinson's disease, Nat. Neurosci. 3 (2000) 1301–1306.
[92] R.E. Heikkila, A. Hess, R.C. Duvoisin, Dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine in mice, Science 224 (1984) 1451–1453.
[93] X. Ma, S. Kalakonda, S.M. Srinivasula, S.P. Reddy, L.C. Platanias, D.V. Kalvakolanu, GRIM-19 associates with the serine protease HtrA2 for promoting cell death, Oncogene 26 (2007) 4842–4849.
[94] G. Huang, H. Lu, A. Hao, D.C. Ng, S. Ponniah, K. Guo, C. Lufei, Q. Zeng, X. Cao, GRIM-19, a cell death regulatory protein, is essential for assembly and function of mitochondrial complex I, Mol. Cell Biol. 24 (2004) 8447–8456.
[95] F. Papa, M. Delia, R. Trentadue, D. Panelli, F. Bellomo, R. Serpico, M. Petruzzi, M. De Benedittis, S. Scacco, Differential effects of all-trans retinoic acid on the growth of human keratinocytes and mouth carcinoma epidermoid cultures. Involvement of GRIM-19 and complex I of the respiratory chain, Int. J. Immunopathol. Pharmacol. 20 (2007) 719–729.
[96] S. Vuocolo, E. Purev, D. Zhang, J. Bartek, K. Hansen, D.R. Soprano, K.J. Soprano, Protein phosphatase 2A associates with Rb2/p130 and mediates retinoic acid-induced growth suppression of ovarian carcinoma cells, J. Biol. Chem. 278 (2003) 41881–41889.
[97] B. Xiao, J. Guo, Y. Lou, D. Meng, W. Zhao, L. Zhang, C. Yan, D. Wang, Inhibition of growth and increase of alkaline phosphatase activity in cultured human oral cancer cells by all-trans retinoic acid, Int. J. Oral. Maxillofac. Surg. 35 (2006) 643–648.
[98] M.N. Gadaleta, B. Kadenbach, A.M.S. Lezza, A. Reith, P. Cantatore, D. Boffoli, S. Papa, Age-linked changes in the genotype and phenotype of mitochondria, in: S. Papa, F. Guerrieri, J.M. Tager (Eds.), Frontiers of Cellular Bioenergetics: Molecular Biology, Biochemistry and Physiopathology, Plenum Press, London, 1999, pp. 693–727.
[99] M. Valko, D. Leibfritz, J. Moncol, M.T.D. Cronin, M. Mazur, J. Telser, Free radicals and antioxidants in normal physiological functions and human disease, Int. J. Biochem. Cell Biol. 39 (2007) 44–84.
[100] M. Falkenberg, N.G. Larsson, C.M. Gustafsson, DNA replication and transcription in mammalian mitochondria, Annu. Rev. Biochem. 76 (2007) 679–699.
[101] J.C. Fitzgerald, H. Plun-Favreau, Emerging pathways in genetic Parkinson's disease: autosomal-recessive genes in Parkinson's disease — a common pathway? FEBS J. 275 (2008) 5758–5766.
[102] G. Casari, E. Rugarli, Molecular basis of inherited spastic paraplegias, Curr. Opin. Genet. Dev. 11 (2001) 336–342.