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Mitochondriální poruchy ATP syntázy jaderného původu

Mitochondrial ATP synthase deficiencies of a nuclear genetic origin

Dizertační práce

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ABSTRAKT

ATP syntáza je klíčový enzym buněčného metabolismu a defekty ATP syntázy patří k nejzávažnějším mitochondriálním onemocněním postihujícím dětskou populaci. Cílem této práce bylo identifikovat genetické defekty a popsat patogenní mechanismy narušení biosyntézy ATP syntázy, které vedou k izolované deficienci tohoto enzymu a projevují se jako mitochondriální encefalomyopatie s nástupem v novorozeneckém věku. Studie skupiny 25 pacientů vedla k identifikaci dvou jaderných genů zodpovědných za deficienci ATP syntázy.

První postižený gen byl *TMEM70* kódující neznámý mitochondriální protein. Tento protein byl popsán jako nový asemblační faktor ATP syntázy, první specifický pro vyšší eukaryota. Jeho velikost je 21 kDa, nachází se ve vnitřní mitochondriální membráně a není přítomný v tkáních pacientů. Mutace v *TMEM70* genu byla nalezena u 23 pacientů a ukázala se být nejčastější příčinou deficience ATP syntázy. Studie na buněčných kulturách ukázaly, že defekt enzymu vede ke kompenzačně-adaptačnímu zvýšení komplexů IV a III respiračního řetězce způsobenému posttranskripční regulací jejich biosyntézy.

Druhým postiženým genem byl *ATP5E*, který kóduje malou strukturní epsilon podjednotku ATP syntázy. Záměna konzervovaného Tyr12 za Cys způsobuje významný pokles obsahu ATP syntázy, ale zároveň akumulaci hydrofobní podjednotky c. Tento fenotyp byl také vyvolán snížením exprese *ATP5E* genu pomocí RNA interference v HEK293 buněčné linii a ukazuje na regulační roli podjednotky epsilon v biogenezi enzymu. Podjednotka epsilon pravděpodobně ovlivňuje asemblaci a stabilitu katalytické F_1 části enzymu a inkorporaci hydrofobních podjednotek c do F_1-c oligomeru. Mutace v *ATP5E* genu byla nalezena jen u jednoho pacienta a představuje první mutaci v jaderném strukturním genu ATP syntázy.

Tato dizertační práce byla vypracována na oddělení Bioenergetiky ve Fyziologickém ústavu Akademie věd České republiky, v.v.i. ve spolupráci s Klinikou dětského a dorostového lékařství a Ústavem dědičných metabolických poruch 1. lékařské fakulty Univerzity Karlovy.

Klíčová slova: mitochondrie, oxidativní fosforylace, ATP syntáza, mitochondriální onemocnění, mitochondriální biogeneze, asemblační faktor TMEM70.

ABSTRACT

ATP synthase represents the key enzyme of cellular energy provision and ATP synthase disorders belong to the most deleterious mitochondrial diseases affecting pediatric population. The aim of this thesis was to identify nuclear genetic defects and describe the pathogenic mechanism of altered biosynthesis of ATP synthase that leads to isolated deficiency of this enzyme manifesting as an early onset mitochondrial encephalo-cardiomyopathy. Studies in the group of 25 patients enabled identification of two new disease-causing nuclear genes responsible for ATP synthase deficiency.

The first affected gene was *TMEM70* that encodes an unknown mitochondrial protein. This protein was identified as a novel assembly factor of ATP synthase, first one specific for higher eukaryotes. TMEM70 protein of 21 kDa is located in mitochondrial inner membrane and it is absent in patient tissues. *TMEM70* mutation was found in 23 patients and turned to be the most frequent cause of ATP synthase deficiency. Cell culture studies also revealed that enzyme defect leads to compensatory-adaptive upregulation of respiratory chain complexes III and IV due to posttranscriptional events.

The second affected gene was *ATP5E* that encodes small structural epsilon subunit of ATP synthase. Replacement of conserved Tyr12 with Cys caused pronounced decrease of ATP synthase content and accumulation of hydrophobic subunit c. This phenotype was also induced by *ATP5E* RNAi knockdown in HEK293 cell line and indicated regulatory role of epsilon subunit in enzyme biogenesis that points to assembly and stability of F_1 catalytic part and incorporation of hydrophobic c subunits into F_1-c oligomer. *ATP5E* mutation was found only in one patient and represents the first mutation in nuclear structural gene of ATP synthase.

This thesis has been worked out in the Department of Bioenergetics, Institute of Physiology, Academy of Sciences of the Czech Republic, within collaboration with the Department of Pediatrics and Adolescent Medicine and the Institute of Inherited Metabolic Diseases, 1st Faculty of Medicine, Prague.

Key words: mitochondria, oxidative phosphorylation, ATP synthase, mitochondrial disorders, mitochondrial biogenesis, assembly factor TMEM70.

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ABBREVIATIONS

ANT - adenine nucleotide translocator

BAT - brown adipose tissue

COX - cytochrome *c* oxidase

CoQ - coenzyme Q

DGUOK - deoxyguanosine kinase

dNTP - deoxy nucleoside triphosphate

FMN - flavin mononucleotide

GRACILE - growth retardation, aminoaciduria, cholestasis, iron overload, lacidosis and early death

KSS - Kearns-Sayre syndrome

LHON - Leber's hereditary optic neuropathy

LRPPRC - leucine rich pentatricopeptide repeat cassette

LS - Leigh syndrome

MELAS - mitochondrial encephalomyopathy, lactic acidosis and stroke like episodes

MERRF - myoclonic epilepsy with ragged red fibers (RRF)

MIA - mitochondrial intermembrane space machinery

MILS - maternally inherited Leigh syndrome

MNGIE - mitochondrial neurogastrointestinal encephalomyopathy

MPV17 - mitochondrial inner membrane protein with unknown function

mtDNA - mitochondrial DNA

MTERF - mitochondrial transcriptional termination factor

NARP - neuropathy, ataxia, retinitis pigmentosa

NRF-1/2 - nuclear respiratory factors 1 or 2

OPA1 - nuclear encoded mitochondrial protein with similarity to dynamin-related GTPases; mutations in this protein are associated with optic atrophy type 1

OSCP - oligomycin sensitivity conferral protein

OXPHOS - oxidative phosphorylation system

PAM - presequence translocase-associated motor

PEO - progressive external ophtalmoplegia

PGC-1 - peroxisome proliferator-activated receptor coactivator 1

POLG - polymerase gamma

ROS - reactive oxygen species

RRM2B - p-53-inducible ribonucleotide reductase

SAM - sorting and assembly machinery

mtSSB - mitochondrial single stranded binding protein

SUCLA2 - succinyl CoA ligase

SUCLG1 - succinyl CoA ligase GDP-binding protein subunit alfa

TACO1 - translation activator of Cox1

mTERF - mitochondrial transcription termination factor

TFAM - mitochondrial transcription factor A

TFB1M/TFB2M - mitochondrial transcription factor B1 or B2

TIM - translocases of the inner membrane

TK2 - mitochondrial thymidine kinase

TOM - translocases of the outer mitochondrial membrane

TYMP - thymidine phosphorylase

TWINKLE - mtDNA helicase

WPW - Wolff-Parkinson-White syndrome

1. INTRODUCTION

1.1 Mitochondria

1.1.1 Mitochondrial structure and function

Mitochondria were first described by Altman in the second half of the 19th century [1]. Mitochondria are highly dynamic, semi-autonomous and double membrane organelles that descended from α-proteobacterial endosymbiont [2] and are present in the cytosol of almost all eukaryotic cells. Mitochondria are involved in many important metabolic tasks, such as the regulation of calcium homeostasis and the control of programmed cell death. Most importantly, this is the place were ATP is generated by the oxidative phosphorylation system (OXPHOS).

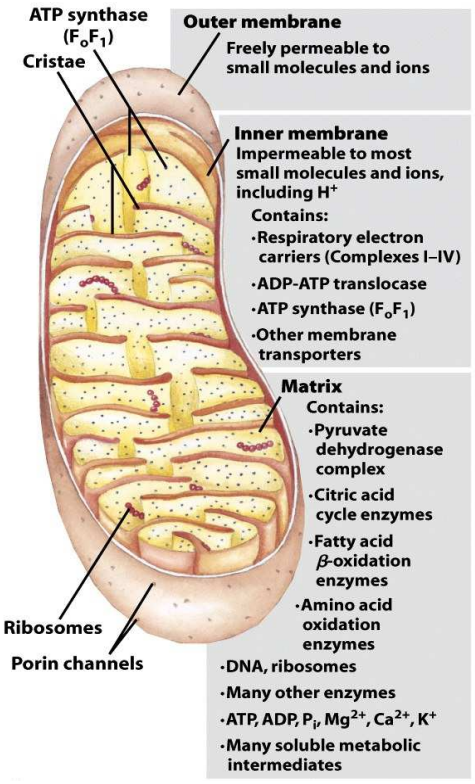

Figure 1 - The mitochondrion
The picture shows an overview of mitochondrial structure and function. Adapted from [3].

Structurally, mitochondria consist of an inner membrane that is surrounded by an outer membrane. The space inside the inner membrane is called the matrix and the space between the two membranes is called the mitochondria intermembrane space. The outer membrane is

permeable for small molecules (to approximately 10 kDa) and ions via integral membrane proteins called porins [3]. In contrast, the inner membrane is almost impermeable; passing through is only possible by special transporters. It involves many invaginations, called cristae; their number varies with the energy demand of particular types of cells. This is because the respiratory machinery is placed here, and thus respiratory rate depends on membrane surface area. The matrix is a gel like substance, which is composed of more than 50% water, inorganic ions and nucleotides. It contains proteins involved in the Krebs cycle, pyruvate oxidation, and in the metabolism of amino acids, fatty acids and steroids. Moreover, the mitochondrial genetic system and protein synthesis machinery are also localized in the matrix [4].

1.1.2 Mitochondrial biogenesis and genetics

Biogenesis of mitochondria is uniquely complex process reflecting dual genomic origin of mitochondrial energetic OXPHOS system. For its proper biogenesis several points have to be fulfilled: (i) nuclear DNA encoded proteins have to be translated in cytoplasm and imported into the organelle in an unfolded state by mitochondrial protein import machinery; (ii) mitochondrial DNA has to be maintained, replicated, transcribed and translated; and (iii) many essential chaperones, proteases and assembly factors have to be present to facilitate proper incorporation of OXPHOS proteins into functional complexes in the inner mitochondrial membrane.

Mitochondrial proteome

One of the most challenging tasks of today's mitochondrial research is to describe and characterize the unknown proteins that are involved in biogenesis of the OXPHOS system. Approximately 1100 mitochondrial proteins have been identified by large-scale proteomics, microscopy and computation. At present, the most complete catalogue of mitochondrial proteins is known as MitoCarta (http://www.broadinstitute.org/pubs/MitoCarta/index.html), authors estimate that it covers approximately 85% of mitochondrial proteome [5]. Importantly, ~300 of them have no known function and the functions of another ~300 are based only on domain annotations and sequence similarity. The knowledge of all mitochondrial proteins is important for identification of new disease-causing genes as mitochondrial disorders belong to the most frequent and severe inherited metabolic diseases. On the other hand, newly described mitochondrial diseases represent important experimental

models for uncovering the mechanisms of biogenesis and function of mammalian OXPHOS system. Some good examples are mitochondrial diseases caused by isolated defects in ATP synthase.

Mitochondrial protein import

Mitochondria are under the control of the mitochondrial and nuclear genomes. However, most of the mitochondrial proteins are products of nuclear genes that encode the structural and catalytic components directly involved in energy metabolism, as well as the proteins of mitochondrial replication, transcription and translation. They are synthesized on free cytosolic ribosomes, often as larger precursors, which are imported into the organelle and directed to different mitochondrial compartments. The translated proteins require cytosolic chaperones HSP70 or HSP90 to keep them in an unfolded state and prevent their aggregations during the import process [6]. Then they are translocated to their final destination with the help of many proteins that form special pores in both mitochondrial membranes organized into TOM (translocases of the outer mitochondrial membrane) and TIM (translocases of the inner membrane) complexes (Fig. 2). The precursor proteins carry additional information that they need to be imported into mitochondria. In most cases, this informative role is fulfilled by a N-terminal cleavable presequence; other proteins carry various integral, cryptic targeting signals [7]. Preproteins enter into mitochondria through general entry gate pores formed by TOM complex and then the precursor protein can follow one of the four major pathways [8].

Firstly, the matrix proteins and some inner membrane proteins are transferred by the TIM23 complex. This inner membrane translocase is specific for proteins with an N-terminal presequence and is associated with mitochondrial molecular chaperone mtHsp70, which forms the core of molecular motor PAM (presequence translocase-associated motor). PAM then drives and completes the transport of the proteins to the matrix. However, some preproteins contain an additional, hydrophobic sorting signal, which stops the translocation from inner membrane and they are released laterally into the inner membrane lipid phase [9]. Secondly, some inner membrane proteins are imported through a carrier translocase called the TIM 22 complex. These proteins, usually the metabolite carriers such as ADP/ATP carrier, phosphate carrier or uncoupling proteins have a noncleaved noncontiguous targeting signal. It means that about 10 amino acid residues of the targeting signal are distributed across the length of the protein. The noncontiguous targeting signals probably help these proteins to cross the mitochondrial outer membrane in loop formation [10, 11].

Thirdly, recently identified redox regulated import pathways facilitate protein import into the intermembrane space. The central component of the mitochondrial intermembrane space machinery (MIA) is MIA40 [12]. Lastly, proteins of the outer membrane, like porins, are integrated with the help of sorting and assembly machinery (SAM). For more information about protein import machinery see [8].

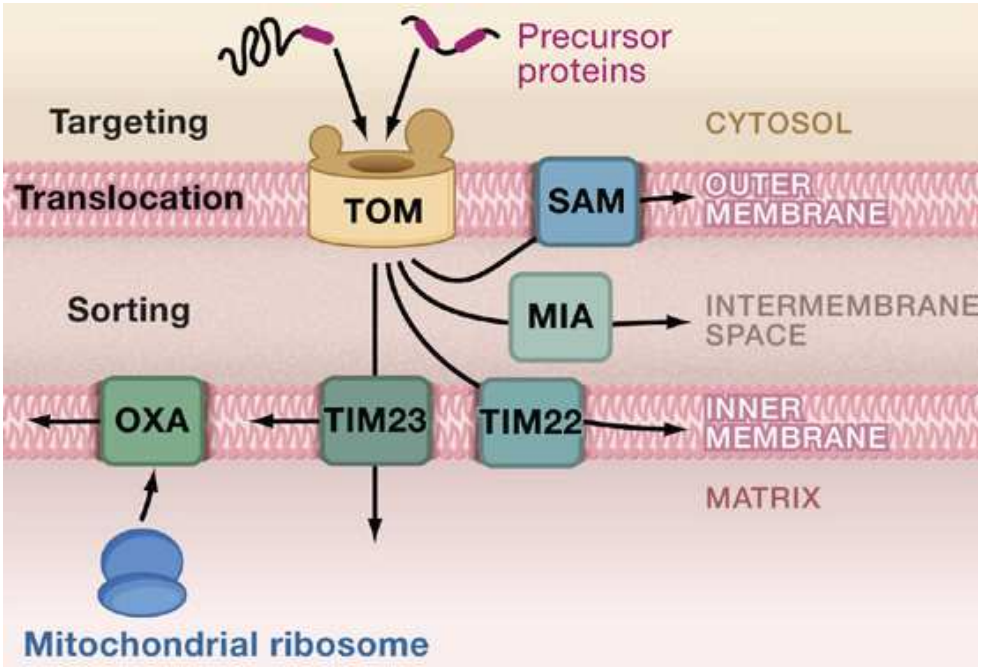

Figure 2 - Mitochondrial protein import pathways
As much as 99% of mitochondrial proteins are imported posttranslationaly from cytosol into the mitochondria via TOM and TIM translocases embedded into both mitochondrial membranes. Preproteins first cross the outer membrane through the universal TOM complex. After that, they follow one of the four principal pathways that direct them into different mitochondrial compartments. Adapted from [8].

Mitochondrial DNA

Mitochondrial DNA (mtDNA) codes for a small subset of proteins that represent about 1% of total mass of mitochondrial proteins. MtDNA was discovered in 1963 [13] and the complete sequence of human mtDNA was published in 1981 [14]. The mammalian mitochondrial genome is transmitted only through the female germ line. At cell division, mitochondria are randomly distributed to daughter cells. Every animal cell has hundreds of mitochondria and each mitochondrion contains ~2-10 mtDNA molecules. The mtDNA structure, genetic content and organization are highly conserved among mammals.

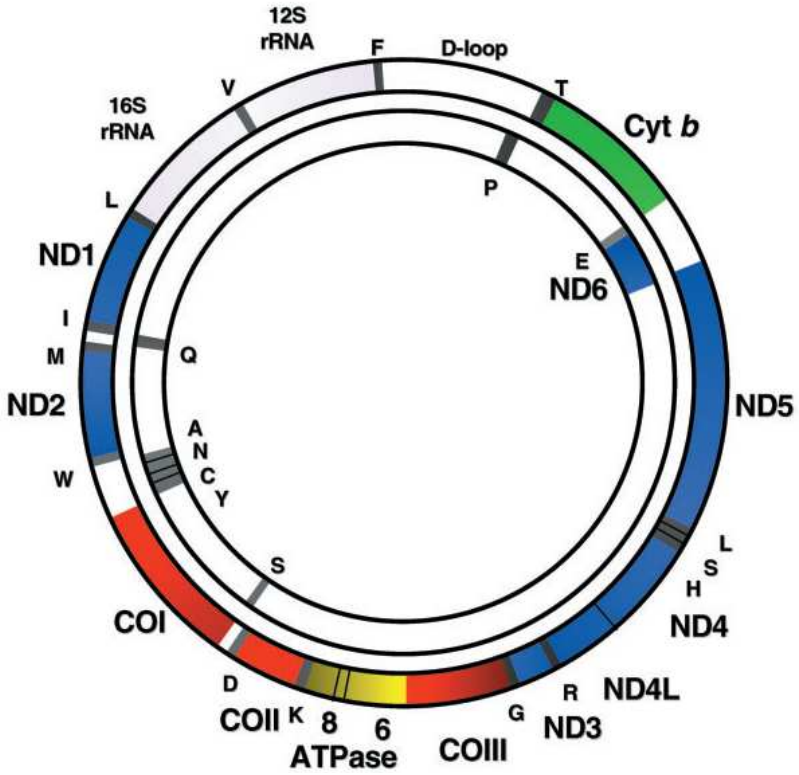

Figure 3 - The genetic map of mtDNA
Human mtDNA is a 16,5 kb circular molecule composed of two complementary, heavy and light strands. It encodes 37 genes: 2 rRNA genes (grey color) and 13 structural genes for OXPHOS subunit, in particular seven subunits of complex I (blue color), one subunit for complex III (green color), three subunits of complex IV (red color), two subunits of complex V (yellow color), which are separated by 22 tRNA genes (black color, single letters) that are essential for their translation in mitochondria. Adapted from [15].

Human mtDNA is a circular, double stranded molecule consisting of 16 569 base pairs. It encodes 37 genes: 13 structural genes for OXPHOS subunits, 2 rRNA genes and 22 tRNA important for their translation in mitochondria (Fig. 3). In particular, seven subunits of complex I, one subunit for complex III, three subunits of complex IV and two subunits of complex V are encoded by mtDNA [14]. Genes are placed on a heavy (H) and a light (L) strands of mtDNA, which differ in their G+T content. Most information is present in the H strand, including genes for two tRNA, 14 tRNA and 12 structural subunits. The L strand codes for 8 tRNA and one structural subunit. Mammalian mtDNA posses an exceptional economy of organization [16]. It lacks introns and contains only one non-coding region, which is known as displacement or D-loop. Human D-loop is ~1kb long and contains the main regulatory elements for replication and transcription. Some of the protein genes are overlapping (for example *ATP6* and *ATP8).* In many cases the termination codons are not

encoded in mtDNA, but are generated post-transcriptionally by polyadenylation of the corresponding mRNA [17]. MtDNA molecules are organized in protein-DNA semi-permanent aggregates called nucleoids [18], which protect the mitochondrial genome. The mutation rate of mtDNA is exceptionally high, 10-20 times higher than that of nDNA [19]. The reason is a lack of protective histones, limited DNA repair and closeness to damaging reactive oxygen species (ROS) generated in the inner membrane by the respiratory chain.

Mitochondrial replication

Mitochondrial replication, transcription and translation take place in mitochondrial matrix. Replication of mtDNA, in contrast to nDNA replication, is not cell cycle dependant and occurs continuously also in non-dividing cells like skeletal muscle and neurons [20]. Some mtDNA molecules pass multiple rounds of replication whereas others do not replicate.

Mammalian mtDNA replicates by an asynchronous-displacement mechanism involving two separated origins, one for each strand (O_H, O_L) [21]. Synthesis begins at the O_H origin located in the D-loop and continues along the parental L-strand producing a full daughter H-strand. After approximately two thirds of the way around the genome, the second origin O_L is reached, and DNA synthesis of the L strand initiates in the opposite direction. Mitochondrial replication occurs with the assistance of a number of nuclear proteins. Specific proteins for mitochondrial replication are polymerase γ, the only polymerase in mammalian mitochondria, Twinkle DNA helicase and mitochondrial single stranded binding protein (mtSSB). The mitochondrial polymerase γ consists of a catalytic subunit with proof-reading ability (POLG) and two same accessory subunits (POLG2), which bind DNA and increase the efficiency of POLG. Twinkle is DNA helicase that unwinds double stranded mtDNA in 5´ to 3´ direction and thus is important for mitochondrial maintenance and regulation of mtDNA copy number. MtSSB is thought to keep the integrity of single strand DNA at the replication fork and also to stimulate Twinkle and polymerase γ activity.

The mtDNA replication and/or transcription thus depend on proteins like mtSSB, Twinkle, POLG and TFAM that are components of nucleoids. Moreover, multiple repair mechanisms [22] and a balanced pool of dNTP [23] are necessary for the protection, maintenance and proper function of mtDNA.

Mitochondrial transcription

Bidirectional mtDNA transcription originates from three promoters. Two of them, the L strand promoter (LSP) and the first H strand (HSP1) promoter, are located in the D-loop. The second H strand promoter, HSP2, is located downstream of HSP1 close to the 5´ end of the 12S rRNA gene. The transcription units are polygenic multiple RNAs containing rRNA, tRNA or mRNA. The basic human mitochondrial transcription machinery is formed by mtRNA polymerase, and at least by three transcriptional factors: TFAM (mitochondrial transcriptional factor A) [24], either TFB1M or TFB2M for initiation [25], and mTERF for termination of transcription [26]. The expression of these proteins is influenced by the action of many transcriptional activators and coactivators. The PGC-1 family of regulated coactivators, consisting of PGC-1α, PGC-1β and PRC, plays a central role in a regulatory network governing the transcriptional control of mitochondrial biogenesis and respiratory function. These coactivators target multiple transcription factors. Among them, nuclear respiratory factors NRF-1 and NRF-2 have been identified to be involved in transcriptional regulation of many OXPHOS genes [27, 28]. They themselves are under the control of protein complexes responsible for regulation of gene expression through chromatin remodeling. Moreover, the expression of PGC-1 family proteins is modulated by extracellular signals controlling metabolism, differentiation or cell growth. In some cases their activities are known to be regulated by post-translational modification by the energy sensors, AMPK and SIRT1 [29].

Mitochondrial translation

Mitochondrial translation system is all encoded by nuclear genome, and it looks like the translation system in prokaryotes, rather than its eukaryotic cytoplasmic counterpart. Mitochondrial polycistronic transcripts are processed to monocistronic and then translated on mitochondrial ribosomes, bound on the matrix site of inner membrane. Moreover, the mitochondrial protein synthesis is not so well understood as the cytoplasmic one, because of the missing of proper *in-vitro* mitochondrial translation system [30]. Mitochondrial protein synthesis system is unique in many ways. Mitochondrial rRNA and tRNA species are surprisingly small; the mammalian mitoribosome has sedimentation coefficient of ~ 55S [16]. The mitochondrial mRNAs contain no or very few 5´-untranslated nucleotides [31], are uncapped [32] and contain a poly(A) tail, which is followed by stop codon or is even part of it [17]. Mitochondria have only one type of $tRNA^{met}$ for both initiation and elongation phases.

MtDNA has own genetic code, unique for mitochondria translation, with several differences from the universal genetic code [33]. In addition, the decoding mechanism allowing translations of codones is simplified and contains only 22 tRNAs [14]. Many different factors, initiation (mtIF2 and mtIF3), elongation (mtEFTu, mtEFTs, mtEFG1 and mtEFG2) and termination (mtRF1, mtRF1a and mtRRF) factors, are involved during the translation in mammals [30]. All proteins responsible for translation activation are integral or membrane bound proteins. Thus, they can promote cotranslational insertion of newly formed protein. So far, two specific translation activators, for subunits of complex IV (TACO1 and LRPPRC [34, 35]), have been found in human.

Posttranslations processes are important for maturation of some OXPHOS subunits and for the final stage of OXPHOS biogenesis. When the proteins of nuclear origin are imported to mitochondria and the mtDNA encoded proteins are translated, they all need to be incorporated into the inner mitochondrial membrane to form properly assembled OXPHOS complexes. This posttranslational process is controlled by mitochondrial chaperones, proteases and numerous assembly factors specific for particular respiratory complex [30] that are described in successive chapters.

Quality control by proteases

Up to 40% of the newly synthesized mitochondrial proteins are in excess or in non-native state, thus they are degraded into peptides by mitochondrial proteases. The key components of the protein quality control system in mitochondria are ATP-dependent proteases. Some of them also possess a chaperonin function during mitochondrial biogenesis. The ATP dependent proteases belong to a large group of proteases, which utilize the energy from ATP hydrolysis for the selective degradation of cellular proteins [36].

Three major ATP dependent proteases, Lon protease, Clp-like protease and AAA proteases are found in mammalian mitochondria [30]. Lon protease and Clp family protease are located in matrix and they not only have ability to degrade unassembled and oxidatively damaged proteins, but also have the chaperonin function. Lon is also responsible for cellular homeostasis and metabolic regulation [37]. Proteases, which degrade non assembled membrane spanning and membrane associated subunits of respiratory chain complexes and prevent their accumulation, are members of AAA protease family. These proteases are membrane embedded complexes composed of identical or closely related subunits with molecular masses of 70-80 kDa. They either expose their catalytic sites on the matrix side

(*m*-AAA) or on the inter-membrane space side (i-AAA) of the inner membrane [38]. The *m*-AAA protease affects the splicing of transcripts of mitochondrial genes of respiratory chain subunits, and controls the posttranslational assembly of respiratory complexes and the ATP synthase [39]. The *m*-AAA proteases are regulated by mitochondrial prohibitin, which can act as chaperon that stabilizes mtDNA encoded OXPHOS subunit and thus prevents their degradation by AAA proteases [40]. The prohibitin is the inner membrane bound protein complex, consisting of multiple copies of prohibitin 1 and 2 subunits (PHB1, PHB2), which are members of prohibitin protein family and are highly homologues to each other [41]. Moreover, mitochondrial prohibitin complexes control also cell proliferation, cristae morphogenesis and the functional integrity of mitochondria [42]. All the mentioned proteins are highly conserved during evolution which indicates their crucial rule in cell function.

1.1.3 Oxidative phosphorylation system

The oxidative phosphorylation (OXPHOS) system is the main source of ATP in cell. It is embedded in the inner mitochondrial membrane, and consists of five multimeric protein complexes (CI-CV) and two electron carriers (cytochrome *c* and coenzyme Q) (Fig. 4). The protein complexes of the respiratory chain and ATP synthase contain prosthetic groups and metal-involving reactive centers and they are formed during a stepwise process with the help of many chaperons and assembly factors. They are composed of ~ 85 protein subunits, 13 of which are encoded by the mitochondrial genome.

During oxidative phosphorylation, the electrons accumulated in redox equivalents NADH and $FADH_2$ enter the respiratory chain at complex I or complex II, respectively. Then, electrons are transferred by the first electron carrier coenzyme Q to complex III and from here by the second electron carrier cytochrome *c* to complex IV, where reduction of O_2 to water occurs. Transport of electrons at complexes I, III and IV leads to translocation of 4, 2 and 4 protons respectively, across the inner mitochondrial membrane from the matrix site into the intermembrane space. The flow of electrons through the respiratory chain to molecular oxygen thus generates an electrochemical proton gradient which is then utilized by ATP synthase - complex V, for ATP formation.

The respiratory chain complexes in the inner mitochondrial membrane associate into larger assembly complexes of CI, CIII and CIV called supercomplexes or “respirasom”. They are believed to facilitate a proper and efficient channeling of electrons as well as structural stabilization of respiratory complexes [43, 44].

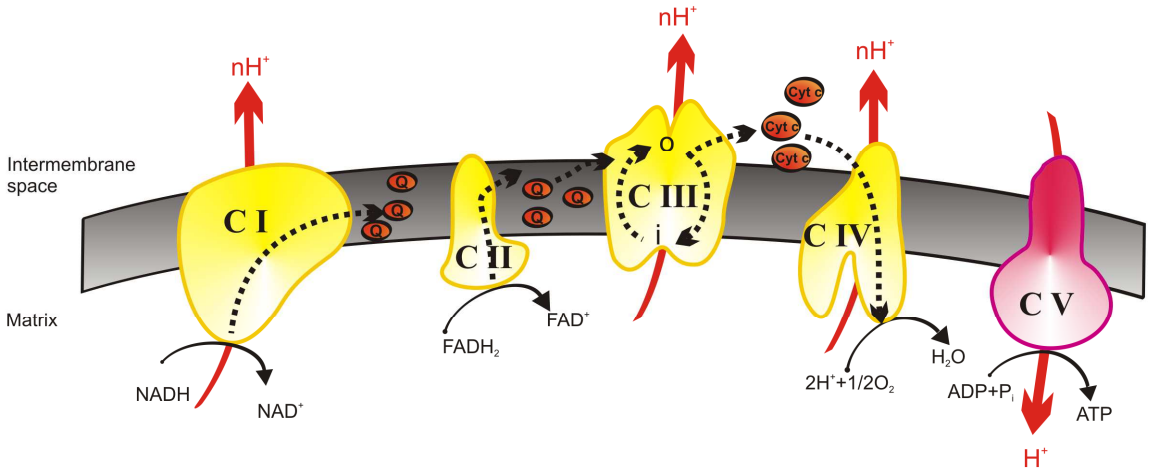

Figure 4 - The OXPHOS system
The mitochondrial OXPHOS system is embedded in the inner mitochondrial membrane and comprise of five multimeric complexes. For ATP synthesis, complex V (CV) utilizes an electrochemical proton gradient, which is generated by substrate oxidation and transport of electrons from the redox equivalents through the respiratory chain complexes CI-CIV to the molecular oxygen and by resulting proton translocation across the mitochondrial membrane.

Complex I

Complex I (NADH: coenzyme Q oxidoreductase; EC 1.6.5.3) is the largest and the least understood complex of the respiratory chain. It catalyzes the first steps of OXPHOS in which the electrons from NADH are donated via flavin mononucleotide (FMN) and series of iron-sulfur clusters to the lipid-soluble carrier ubiquinone-coenzyme Q (CoQ). This process is accompanied by the translocations of 4 protons from the matrix site to the intermembrane space for every two electrons transferred from NADH to coenzyme Q [45].

In prokaryotes, complex I is composed of a basic core of 14 structural subunits, thought to have developed from the fusion of separate modules for an electron transfer and a proton transport. During the evolution, a number of other subunits have been added to the complex, which is now comprised of 37-40 subunits in aerobic fungi and at least 45 in mammals [46-48]. In contrast, some fungi like *S. cerevisiae* and *S. pombe* have mitochondria deprived of complex I [48]. Nevertheless, the mitochondria of other fungi such as *Neurospora crassa* and *Yarrowia lipolytica*, that possess a complex I, have been successfully used to study the structure and function of this enzyme [49, 50].

Complex I is L shaped, and it can be dissociated into a soluble peripheral arm protruding to the mitochondrial matrix, and a hydrophobic arm localized in the inner mitochondrial membrane [51]. The mass of bovine complex I is approximately 1 MDa [52]. Of the 45 subunits of mammalian complex I, 14 subunits are evolutionarily conserved and form the catalytic core of the enzyme [53]. The core of complex I consists of two flavoprotein subunits (NDUFV1 and NDUFV2), five iron-sulfur protein subunits (NDUFS1, NDUFS2, NDUFS3, NDUFS7, and NDUFS8), and seven hydrophobic protein subunits (ND1 to ND6 and ND4L), that are encoded by mtDNA. The function of the remaining 31 nuclear encoded subunits is still not very well understood, but they are probably involved in complex I assembly, stability, and/or function [54].

Complex I biogenesis is thought to be initiated by the formation of a small subcomplex to which other subunits are subsequently added. Proper biogenesis and stabilization of complex I subcomplexes depends on the temporal action of the assembly factors and interactions with other OXPHOS complexes. Current experimental evidence reveals that assembly of complex I requires the assistance of at least these seven additional proteins - NDUFAF1, NDUFAF2, NDUFAF3, NDUFAF4, C8orf38, C20orf7, and Ecsit [53].

Complex II

Complex II or succinate dehydrogenase (SDH; succcinate: coenzyme Q oxidoreductase; EC 1.3.5.1) couples the oxidation of succinate to fumarate with the transfer of the electrons from $FADH_2$ to coenzyme Q pool. It is the only direct link between the Krebs cycle and electron transport chain in the mitochondrial membrane. Complex II does not contribute to the formation of electrochemical potential because it lacks the ability to translocate protons.

Complex II is the smallest complex of the respiratory chain (120 kDa). It involves five prosthetic groups and four subunits, which are all encoded by nDNA, SDH1-SDH4 in yeast and SDHA-SDHD in mammals. Two bigger hydrophilic subunits, SDHA (70 kDa) and SDHB (27 kDa), form the heterodimer localized on the matrix side of the inner mitochondrial membrane. As subunit SDHA contains covalently bound FAD and subunit SDHB contains three iron-sulphur clusters, they represent the catalytic core of the enzyme. Moreover, the binding site for succinate is localized there. Two smaller subunits, SDHC (15 kDa) and SDHD (13 kDa), anchor the catalytic heterodimer to the inner mitochondrial membrane. Subunit SDHC contains a large cytochrome *b* binding protein, and subunit SDHD contains a

small cytochrome *b* binding protein. In addition, SDHC and SDHD contain heme *b* and a binding site for ubiquinone.

Based on SDH deficiency in patients with mitochondrial dysfunction, several assembly factors for complex II have been identified, namely SDHAF1, SDHAF2 and Tcm62, but their exact function is still unknown. It has been shown that the SDHAF1 is required for the stable assembly and full function of the SDH complex [55]. The *SDHAF2* or the *SDH5* gene encodes the highly conserved (in eukaryotes and in some prokaryotes species) assembly factor important for incorporation of FAD cofactor in subunit A [56]. Correct flavination of SDH is essential for complex II activity. The Tcm62 protein appears to be also involved in SDH assembly machinery in yeast [57]. Experimental evidence suggests that Tcm62 functions in a manner related to the Hsp60 family [58].

Complex III

Complex III, or cytochrome bc_1 complex (ubiquinol: cytochrome *c* oxidoreductase; EC 1.10.2.2) is a central component of the respiratory chain, and catalyzes the transfer of electrons from coenzyme Q to cytochrome *c*, which is coupled with the generation of mitochondrial proton gradient. The transfer of every two electrons leads to the translocation of two protons. The mammalian complex III monomer has a molecular weight of about 240 kDa but it usually appears in a dimeric form.

The bovine enzyme involves 11 subunits. Only one subunit, cytochrome *b*, is encoded by mtDNA [59]. The catalytic core of the bc_1 complex consists of 3 subunits, cytochrome *b*, Rieske iron sulfur protein [2Fe-2S] (also known as ISP), and cytochrome c_1. The catalytic core also involves redox centers, localized in the two of three core subunits (ISP and cytochrome c_1), which participate in the electron transfer. The transfer of the electrons between two electron carriers proceeds in Q-cycle [60]. In addition, these three mitochondrial catalytic subunits share strong sequence, structural and functional similarities with their ancestral bacterial counterparts [60]. In mammals, the remaining non-catalytic subunits involve Core1 and Core2 subunits and six additional smaller subunits. Their function is largely unknown. These subunits are considered to be supernumerary subunits, because they are absent in bacterial equivalents of this respiratory chain complex. For example, *Paracoccus denitrificans* has only three redox subunits [61].

In *S. cerevisiae*, the non catalytic subunits are: Core1, Core2, Qcr6p, Qcr7p, Qcr8p, Qcr9p, Qcr10p [62]. In yeast, the assembly model of complex III starts with the formation of an

intermediate consisting of cytochrome *b*, Qcr7p and Qcr8p subunits. Then, Core1 and 2 subunits are added to this intermediate. Meanwhile cytochrome c_1 forms another assembly intermediate with proteins Qcr6p and Qcr9p. Then, these two subcomplexes unite to form the cytochrome bc_1 pre-complex. The following step is the assembly of the Rieske FeS protein and the subunit Qcr10p. Active complex III is formed by dimerization of two monomers [63, 64]. In humans, two factors important for complex III biogenesis are known. The first is BCS1L, a mitochondrial protein and member of the conserved AAA protein family, that is directly required for the assembly of Rieske FeS protein and Qcr10p subunit into the cytochrome bc_1 complex [65]. The second is a recently described assembly factor, TTC1, which has been identified in patient with complex III deficiency [66].

Complex IV

Complex IV or cytochrome *c* oxidase (COX; EC 1.9.3.1) is the terminal enzyme of the respiratory chain which catalyzes the reduction of molecular oxygen to water. Eukaryotic COX is a heterooligomeric complex composed of 11 subunits in *S. cerevisiae* and of 13 subunits in humans. Human COX monomer has a molecular weight of 204 kDa. It includes three large, highly hydrophobic transmembrane subunits, which are encoded by mtDNA (Cox1, Cox2 and Cox3). They represent the catalytic and structural core of the enzyme and involve all redox-active cofactors [67]. Cox1 is the largest and most conserved subunit involving heme *a*, and the binuclear active centre formed by heme a_3 and copper ion Cu_B. Another copper ion, Cu_A, is part of the Cox2 subunit. Electrons first enter the enzyme at Cu_A centre, then pass to heme *a*, and after that are channeled to the binuclear oxygen binding site on Cu_B. In the binuclear center, the molecule of oxygen is reduced to water. For the reduction of molecular oxygen, four protons are taken from matrix side, and another four protons are translocated from the matrix side to the intermembrane space, thus utilizing the free energy from the exergonic reduction of molecular O_2 to H_2O [68]. The 10 remaining smaller, nuclear encoded subunits surround the catalytic core of the enzyme and are important for the assembly/stability of the enzyme monomer or dimer. Moreover, some of them are also involved in the modulation of the enzyme's catalytic activity [69]. Catalytically active COX in the inner mitochondrial membrane is found in the dimeric form. Two monomers are bound through subunits Cox6a and Cox6b [70, 71].

COX plays an important role in the regulation of respiratory rate and ATP synthesis. This regulation is based on an allosteric inhibition of the enzyme via reversible binding of ATP to

subunit IV in mammals or subunit Va in yeast at a high intramitochondrial ATP/ADP ratio [72]. This hormonally regulated process may prevent an increase of mitochondrial membrane potential and subsequent ROS formation.

The biogenesis of COX is a relatively slow, sequential process that requires many assembly factors. According to Nijtmans et al. [73], the first step of the assembly pathway is the formation of Cox1 (S1 intermediate) which proceeds to Cox1-Cox4-Cox5a (S2 intermediate). Several factors are involved in Cox1 subunit synthesis and its incorporation into the inner membrane, namely Oxal and Cox18 translocases, or recently identified translational factors TACO1 [34] and LRPPRC [74], [75]. Addition of Cox2 results in S3 intermediate, and the formation of COX holoenzyme (S4 intermediate) is completed by addition of remaining subunits, lastly Cox6a and Cox7a/b. About 30 gene products have been identified as required for proper eukaryotic COX formation [76]. Of them, Surf1 is most likely involved in an early step of assembly during the association of Cox2 subunit with Cox1-Cox4-Cox5a subassembly. Proteins encoded by genes *COX10* and *COX15* are involved in the heme *a* biosynthesis [77, 78]. Copper metallo-chaperones Sco1, Sco2, Cox11 and Cox17 and possibly also Surf1 are necessary for incorporation of Cu (I/II) ions in to the Cu_A and Cu_B copper centers of the Cox1 and Cox2 subunits, respectively.

1.2 F_1F_o-ATP synthase

The F-ATP synthases are multisubunit complexes found in the inner membrane of the mitochondria, in the plasma membranes of bacteria and in the thylakoid membranes of chloroplasts [79].

Mitochondrial ATP synthase (EC 3.6.3.14) is a nano-size rotary engine which, under oxidative conditions, couples the proton gradient, generated by the respiratory chain enzyme to the synthesis of ATP from ADP and inorganic phosphate [80]. Historically, its structure has been described in terms of two sectors which are possible to separate under non-denaturing conditions. One sector is presented by a membrane embedded ***hydrophobic F_o*** domain, while the second domain is a matrix spanning ***hydrophilic F_1*** part. The F_o part is responsible for proton translocation which is coupled to ATP synthesis catalyzed by the F_1 part. But experimental evidences have modified this view; ATP synthase can be mechanically divided into a ***"rotor"*** part (central stalk and c-ring) and a ***"stator"*** part ($\alpha_3\beta_3$ catalytic hexamer + peripheral stalk) [81].

All F-ATP synthases have a similar gross structure and catalytic mechanism. However, the mitochondrial enzyme is the most complicated one as it gained additional subunits during evolution, mainly associated with the F_o structure, that are missing in bacteria and chloroplasts [82]. Because mitochondrial ATP synthase from yeast is highly homologous to the mammalian enzyme in subunit composition, structure, and activity, most present knowledge of the enzyme structure and function originates from experiments on ATP synthase deficient yeast strains. Nevertheless, several basic differences exist between lower and higher eukaryotes [83]. The primary difference is that subunit 9 is encoded by mtDNA in yeast, while in mammals the c subunit is of nuclear origin. This leads to a different organization of enzyme biogenesis.

1.2.1 Structure of ATP synthase complex

The ATP synthase consists of 8 different subunits in prokaryotes and 16-18 different proteins in mammals (Tab. 1). Its estimated molecular weight is 550-650 kDa, depending on the species [84]. The mammalian ATP synthase has a mass of about 650 kDa and consists of at least 16 different proteins [85]. The enzyme is built of subunits with a total composition - F_1: $\alpha_3\beta_3\gamma\delta\varepsilon$ + IF_1; F_o: abc_8defg, F_6 + A6L + OSCP [86]. The structure of an entire bovine ATP

synthase was determined at 32 Å resolution density [87]. While different organisms show variation in the subunit composition of the F_o sector, the structure of the F_1 catalytic sector is highly conserved [88].

		Bacteria		**Mitochondria**			
		E. coli		***S. cerevisiae***		***H. sapiens***	
		Subunit	**Gene**	**Subunit**	**Gene**	**Subunit**	**Gene**
F_1		α_3	*uncA*	α_3 (1)	*ATP1*	α_3	*ATP5A1*
		β_3	*uncD*	β_3 (2)	*ATP2*	β_3	*ATP5B*
		γ	*uncG*	γ (3)	*ATP3*	γ	*ATP5C1-2*
		ε	*uncC*	δ	*ATP16*	δ	*ATP5D*
		-		ε	*ATP15*	ε	*ATP5E*
	regulatory proteins			Inh1p	*INH1*	IF_1	*ATPIF1*
				Stf1p	*STF1*		
				Stf2p	*STF2*		
F_o		a	*uncB*	6	*ATP6(mt)*	a	*ATP6(mt)*
		-		8	*ATP8(mt)*	A6L	*ATP8(mt)*
		c_{10-12}	*uncE*	9_{10}	*ATP9*	c_8	*ATPG1-3*
		δ	*uncH*	OSCP	*ATP5*	OSCP	*ATP5O*
		b_2	*uncF*	b (4)	*ATP4*	b	*ATP5F1*
				d	*ATP7*	d	*ATP5H*
				h	*ATP14*	F_6	*ATP5J*
				f	*ATP17*	f	*ATP5J2*
				e	*ATP21*	e	*ATP5I*
				g	*ATP20*	g	*ATP5L, ATP5L2*
				J	*ATP18*	-	
				k	*ATP19*	-	

Table 1 - ATP synthase subunit composition overview
An overview of the subunit composition of ATP synthase of different species: E. coli, S. cerevisiae and H. sapiens [82, 89, 90].

The F_1 part of ATP synthase

The F_1 part consists of six subunits: $\alpha, \beta, \gamma, \delta, \varepsilon$ and inhibitor protein IF_1. Subunits $\gamma, \delta, \varepsilon$ [91] constitute the central stalk, while three α subunits and three β subunits form the catalytic head where ATP synthesis and hydrolysis take place. The mammalian subunit ε is the only F_1 part subunit without a homologue in chloroplasts and bacteria [92]. The counterpart of mammalian subunit δ is bacterial and chloroplast subunit ε (Tab. 1).

Bovine subunits α and β have molecular mass of 55,3 and 51,7 kDa, respectively [93]. They both bind nucleotides, but only nucleotide binding sites on the β subunit possess catalytic function. Subunits α and β are arranged alternatively around an asymmetrical antiparaller α-helical coiled coil of the γ subunit [94] (Fig. 5). Subunit γ (30,3 kDa) has two tissue-specific isoforms (heart and liver type) which are generated by alternative splicing [95]. A heterodimer, composed of δ and ε subunits, is bound on the opposite side of the γ subunit, forming the foot of central stalk [93, 96-98]. Subunit ε, which is the smallest subunit of F_1 part, is assigned the important role of foot stabilization. Mammalian ε is a 51 AA protein of 5,8 kDa that lacks a cleavable import sequence. The N-terminal region of the ε subunit is situated in the shallow cleft between the two domains of the δ subunits [97]. The central stalk is approximately 40-45 Å long and links the F_1 and F_o parts.

The rotor part of ATP synthase is composed of the central stalk and the c-ring [99]; they rotate together as a fixed ensemble during catalysis [100, 101]. The c-ring is connected to the central stalk through the δ and ε subunits [86] and the δ subunit (15,1 kDa) is essential for the mechanical coupling of the c ring to the γ subunit [102].

The inhibitor protein IF_1 is able to switch ATP synthetic activity to ATP hydrolytic activity by binding to the F_1 part. It probably contributes to the dimerization process at the F_1-F_1 interface [103]. More information on this subject will be provided in the following chapters.

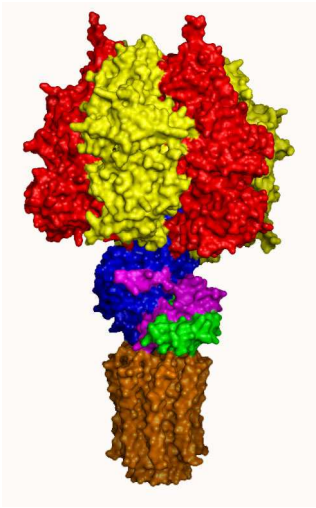

Figure 5 - The subunit organization of the mammalian F_1-c part of ATP synthase
The F_1-c part of ATP synthase is composed of the stator part, presented by the $\alpha_3\beta_3$ catalytic head (yellow and red), and the rotor part. The rotor part consists of subunits γ (blue), δ (green), ε (violet) and the c-ring (brown).

The F_o part of ATP synthase

The F_o part involves 10 subunits in mammals (*a*, b, c_8, d, e, f, g, OSCP (oligomycin sensitivity conferral protein), A6L, F_6), 12 subunits in yeast (a, b, c_{10}, d, e, f, g, i, k, OSCP, A6L, h), and 4 in bacteria (a, b_2, c_{10-12}, δ) (Tab. 1). They form two F_o domains: a peripheral stalk (or stator) and a membrane spanning part (Fig. 6).

The peripheral stalk, or stator extends from the distal point of the F_1 part, along its surface, and then down into and across the membrane part of F_o where it interacts with subunit a [104]. Its role is to prevent the $\alpha_3\beta_3$ complex from following the rotation of the central stalk and attached c-ring; moreover, it also anchors the subunit a [105].

The stator in *Escherichia coli* is formed by homodimer of subunits b and one δ subunit (homologous to OSCP). Some eubacterial species and chloroplasts contained two related subunits, b and b´ [106-108], in chloroplast known as subunit I and II [108]). In mitochondria, the peripheral stalk is formed by a single copy of subunit b (or subunit 4 in yeast), d, F_6 and OSCP [85, 105]. Bovine peripheral stalk subunits have a molecular mass of: b-24.7 kDa, d-18.6 kDa, F_6-8.9 kDa, and OSCP-20.9 kDa. The mitochondrial subunit b represents a continuous link, which extends along the catalytic head of the F_1 part to the surface of the F_o membrane part. Thus subunit b serves as a scaffold for the assembly of the other peripheral stalk subunits [109]. Subunit b protrudes into the membrane via two membrane-spanning α-helix domains at the N-terminus that make contact with subunit a [110]. The hydrophobic C-terminal portion extends into the matrix towards the top of F_1 part; binds subunits OSCP, F_6 and d in 1:1:1 ratio [85, 105]. The N-terminal part of OSCP is placed on the top of the F_1 part, and interacts with N-terminal regions of α subunits [111]. The subunit d is also important for enzyme function [112] - it is in contact with all other three subunits of the peripheral stalk [85, 105]. Subunit F_6 and its yeasts counterpart subunit h are functional homologues [113]. It seems that subunit h plays an essential role in assembly and/or catalysis, because yeast mutants devoid of subunit h are deficient in OXPHOS [113].

The membrane part consists of a ***proton channel*** formed by subunits c and a, and a number of small hydrophobic subunits, A6L, e, f and g. All species contain a single copy of subunit a, while the stochiometry of subunit c vary between organisms [81]. Moreover, the yeast protein has two additional specific subunits i and k. Since high resolution structural information on the organization of these small hydrophobic subunits is not available, the exact demarcation of the proton channel and peripheral stalk have not been determined yet [114].

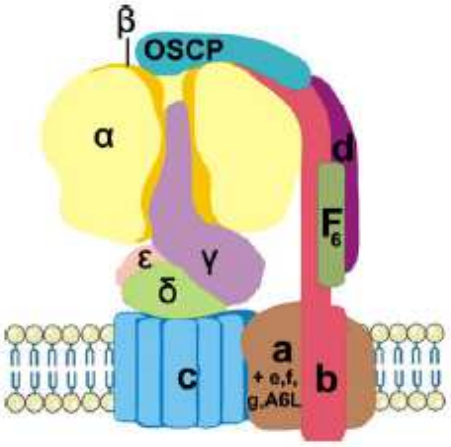

Figure 6 - The subunit organization of mammalian ATP synthase
Mammalian mitochondria ATP synthase has a mass about 650 kDa and consists of at least 16 different subunits. They form two functional domains: the catalytic F_1 part and the proton-translocating F_o part. The globular, matrix spanning F_1 part ($\alpha_3\beta_3$, IF_1) is connected by a central stalk (γ, δ, ε,) and a peripheral stalk (OSCP, d,F_6, b) to the membrane embedded F_o part (c_8, a, e, f, g and A6L). Adapted from [115]. The IF_1 is not shown, but it binds in catalytic α/β interface near the bottom of the $\alpha_3\beta_3$ hexamer.

Except for the ring of c subunits, the membrane-bound portion of a bovine ATP synthase is comprised of mtDNA encoded and hydrophobic subunit a (24,8 kDa), subunit A6L (8 kDa) and nuclear encoded subunits e (8,2 kDa), f (10,2 kDa) and g (11,3 kDa)[116, 117]. The integral membrane subunit A6L is unique for mitochondrial ATP synthase and is supposed to provide a physical link between the stator and proton channel [118].

Subunit c (in mammals) or subunit 9 (in yeasts) is a low molecular weight protein, called also proteolipid because of its extreme hydrophobicity. It has a hairpin structure consisting of two transmembrane α-helices that are separated by a small loop of polar residues extending to the matrix [119, 120]. In bovine enzyme, the c subunit is present in eight copies which form the so called c-ring [89], whereas in yeast the c-ring is formed by ten c subunits [86]. The interface between the hydrophobic c-ring and subunit a forms the proton channel across which protons pass through the inner membrane [121].

The function of the F_o part is to couple the proton translocation with the rotation of the c-ring relative to subunit a [122]. The acid residue Asp-61/Glu-59 (*E. coli/H. sapiens*), located in the second transmembrane helix of subunit c, and the basic residue Arg-210/Arg-159, in the fourth transmembrane helix of subunit a, are the key residues for proton transportation. These two residues are situated in close proximity to one another, near the middle of the lipid bilayer

at the interface of subunit c and a. The Asp-61/Glu-59 undergoes protonation and deprotonation as each subunit of the c-ring moves past a stationary subunit a (Fig. 7) [90].
In addition, Meyer et al. recently identified two small additional proteins, MLQ and AGP, associated with mammalian ATP synthase [123].

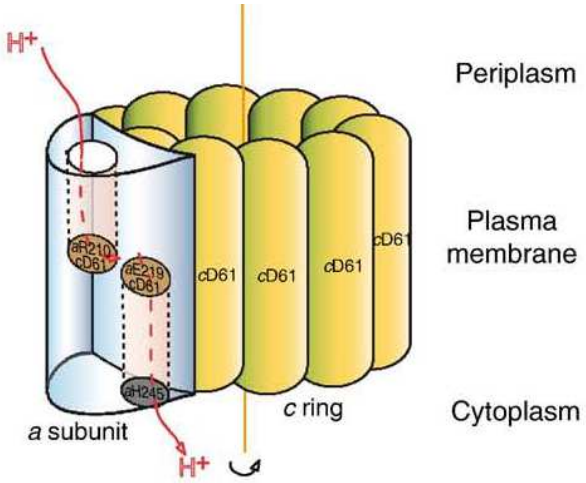

Figure 7 - The model for generating c-ring rotation by proton transport through F_o in *E. coli* [90]
The COOH-terminal α helix of each c subunit contains side chain of Asp-61 that undergoes protonation and deprotonation as each subunit of the c *ring moves past a* stationary *a subunit. The rotation of the* c *ring is proposed to be driven by* H^+ *transport at the* a/c *subunit interface. [122, 124]*

1.2.2 Supramolecular organization of ATP synthase and supercomplexes

The ATP synthase forms dimeric and oligomeric structures which are important for cristae formation. The monomer-monomer interaction is mostly mediated by subunit a and other F_o subunits, depending on the species. Moreover, the ATP synthase is also part of the ATP synthasome complex.

The F_1F_o ATP synthase is usually isolated in the form of a functional monomer, but it appears not to be the physiological form in nature. ATP synthase dimers and oligomers were first found in the mitochondria of yeast [125], later in plants [126] and mammals [127]. By electron cryo-tomography of mammalian mitochondria, it has been shown that ATP synthase molecules are organized in long ribbons of dimers [128]. It seems that ATP synthase dimers are the building blocks of the oligomeric structure [93, 127, 129, 130]. The angular

association of two monomers into the dimer seems to induce bending of the mitochondria inner membrane [128]. Experimental evidence suggests the existence of two types of dimers in oligomeric ATP synthase. The first dimeric building block is the so called “true dimer” and is characterized by a large angle (70-90°) between the two associated monomers. The second dimer type is a small angle “pseudo dimer” (35-40°) [93]. The “true dimer” is possibly formed by the association of two monomers into the dimeric building block, while “pseudo dimers” may characterize the interface between neighbouring dimers (Fig. 8). The loss of cristae structure has been revealed in cells with disturbed ATP synthase dimerization and oligomerization [131]. Thus, it seems that the oligomeric arrangement is important for biogenesis of the inner mitochondrial membrane cristae structure [103].

The most important subunit for monomer-monomer interaction is subunit a. Dimeric subunit a, together with two c rings ($c_{10}a_2c_{10}$), have been found in dimeric ATP synthase [132]. Therefore dimeric subunit a is a bridging module between two ATP synthase monomers [133]. F_o-F_o interaction is important for the formation of dimeric enzyme [129, 131]. In yeast, it involves subunits e, g and k, which are associated with ATP synthase dimers [125]. Heterodimers of the e and g subunits are important for yeast ATP synthase dimer stabilization [132] and for normal cristae structure formation [134]. The homodimers of e subunits are associated with the formation of higher oligomers [129]. Furthermore, subunits b, i and h are part of the same monomer-monomer interface and contribute to the stabilization of ATP synthase dimers [93]. However, in bovine enzyme, subunits e and g are isolated only with monomer [135]. The dimer interface of bovine ATP is formed by interaction of both ATP synthase parts (F_1 and F_o) [103]. It is postulated that the F_1-F_1 bridge is mediated by IF_1, which is important for dimer stabilization [136, 137]. This possibility was excluded for yeast [138], but confirmed in bovine [139].

The interface of ATP synthase dimers is less characterized. Possible candidates for this role are subunits e, f, g, A6L, some transmembrane helixes of subunit a, the Pi carrier, and the ADP/ATP translocator [93, 140].

F_1F_o ATP synthase holoenzyme (monomer) not only forms dimers and oligomers, but also, together with adenine nucleotide (ADP/ATP) translocator (ANT) and the Pi carrier, it forms so called ATP synthasome complex [141, 142]. These carriers supply ATP synthase with reactants-ADP and phosphate; moreover, the ADP/ATP translocator also transports ATP out of the mitochondria [141]. ATP synthasome is believed to influence an increase of ATP production by substrate-product channeling.

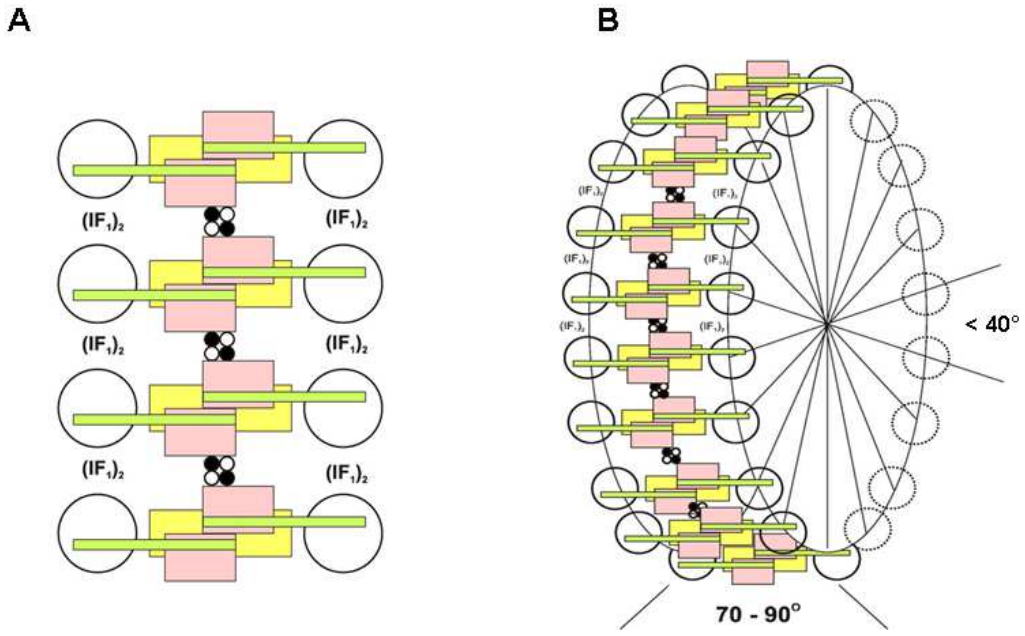

Figure 8 - Model of the oligomeric structural organization of ATP synthase
The figures show possible model of further association of dimeric mammalian ATP synthase into oligomeric structures. This model is based on cryo-electron microscopic studies of mitochondria [128] and studies of e and g subunits (white and black small circles) [129, 143, 144] IF_1 is possible linker of F_1 catalytic head. In part B, the large angles (70-90°) have been proposed to occur between "true dimers", while small angle (35-40°) are possibly between "pseudo dimers". Adapted from [133].

1.2.3 Function of ATP synthase

The main function of ATP synthase is to generate ATP. The enzyme complex uses the energy of the transmembrane proton motive force generated by respiration which is mechanically coupled to ATP synthesis by rotation of the central stalk. ATP synthase can also work in reverse mode and hydrolyze ATP to generate a proton gradient. Under mild conditions ATP synthase can be dissociated into F_1 and F_o components, retaining their functions, but only when the two parts are linked together, proton translocation and catalysis are coupled and ATP can be either synthesized or hydrolyzed.

At the beginning of ATP synthesis protons pass across the inner membrane through the proton channel located at the interface of subunit a and the c-ring. Each subunit c has a key amino acid residue localized at the midpoint of the lipid bilayer (Asp-61 in *E. coli* or Glu-59 in *H. sapiens*). The rotation of the central stalk is generated by protonation and deprotonation of each of these residues as each subunit of the c ring moves past a stationary subunit a (more on

page n. 24). Thus the flow of protons in the F_o part results in clockwise ***rotation of the* c *ring*** (as viewed from the membrane) and in rotation of the central stalk at about 100 times per second [80, 121]. This rotation causes conformational changes in the catalytic nucleotide binding site leading to ATP synthase formation. This process is called "rotary catalysis" and can be explained by the "***binding change mechanism***" first proposed by Boyer in 1975 [145]. The spherical catalytic domain is formed by $\alpha_3\beta_3$ hexamer and has three catalytic sites, which are localized on β subunits at the interface of α/β pair [94]. Each α subunit also has nucleotide binding site, which instead of a catalytic function, seems to posses a regulatory function. Each of the catalytic sites can adopt one of ***three conformation states***, each with different binding affinities for the substrate.

The three conformations of catalytic sites correspond to tight, loose and open states. All three catalytic sites pass through identical conformations, but at any time, all are in different conformations. The sequential conformation changes facilitate the binding, chemical change and release of reactants. During the synthesis of ATP, the loose site binds ADP and Pi, the tight site catalyzes ATP formation, and the open site releases ATP (Fig. 9). During hydrolysis of ATP, the reverse process happens. When the substrate is bound to the loose and open site, a 120° rotation step occurs. The reported data indicate that Mg^{2+} plays an important role in the formation of transitional states during ATP synthesis [146]. Fast ATP synthesis can take place when the tight site is already occupied and the loose site binds ADP and Pi, whether or not the open site has nucleotide present [147]. This process is called bi-site activation.

Three molecules of ATP are produced during one 360° rotation, which requires the translocation of one proton for Arg-61 or Glu-59 by each subunit c in the ring. The number of c subunits forming the c-ring varies from eight to fifteen among different species (mammals, yeast, eubacteria, plant chloroplasts) [86, 89, 148, 149]. Thus, the bioenergetic cost of making ATP ranges from 2.7-3.5 protons translocated per ATP. From the sequence alignment of c subunits, it seems that ATP synthase in all vertebrates and probably all or most invertebrates contain c_8 in the c-ring. Thus, they belong to a group of the most efficient organisms that have ever been found [89]. The presence of another additional protein "coupling factor B" (20,4 kDa) was shown to be essential for energy transduction by the enzyme [150].

When the cell lacks oxygen (for example during ischemia), the mitochondrial ATP synthase switches its activity from ATP synthesis to ATP hydrolysis. It means that ATP synthase operates in reverse mode and catalyzes the hydrolysis of ATP to ADP and phosphate. The

function of ATP synthase thus maintains a normal mitochondrial membrane potential necessary for protein import across the inner membrane [151].

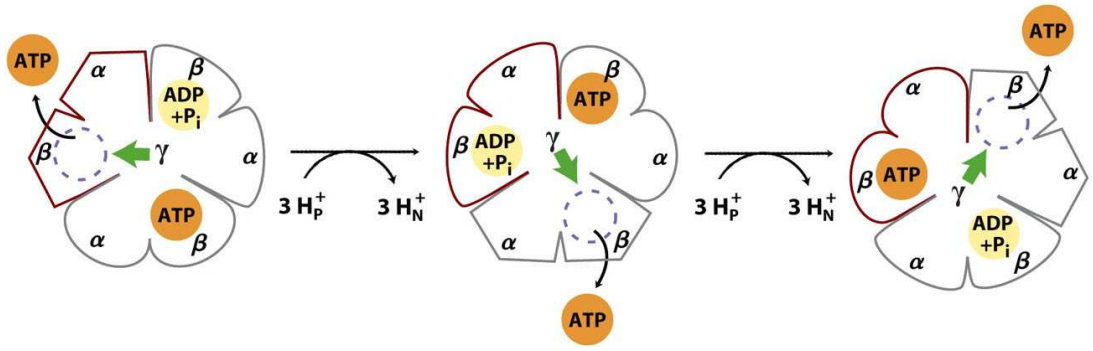

Figure 9 - The binding-change mechanism of the ATP synthesis
Proton translocation cause a 120° rotation of the central stalk, resulting in the conformation change of the nucleotide binding site (here, the central stalk is represented by the γ subunit shown as a green arrow). The catalytic head has three nucleotide binding sites, one for each α/β pair, which differ in binding affinity for the substrate. During the synthesis of ATP all three catalytic sites pass through identical conformations, but at any time all are in a different one. Nucleotide binding sites convert from the loose conformation state (ADP and Pi are bound) to a tight conformation state (ATP is formed) and then, finally, to an open conformation state (ATP is released). Adapted from [3].

The natural inhibitor protein IF_1 controls this process in mitochondria. Its binding is dependent on pH value; its inhibitor capacity increases below neutrality [152]. Above a pH value of 6,5 IF_1 forms a tetramer and below this pH a dimer [137]. The dimeric form is active and it is able to bind two F_1 domains at the same time. ATP hydrolytic activity in yeast is under the control of two inhibitory proteins, Inh1 and Stf1, which have different binding affinities [153]. The action of these inhibitory proteins is modulated by two additional factors, Stf1 and Stf2 [154].

1.2.4 Biogenesis of ATP synthase

Biogenesis of mammalian ATP synthase depends on expression of 16 genes encoding structural subunits, the genes encoding mitochondrial biogenesis factors and the genes encoding factors specific for ATP synthase biogenesis. *De novo* formation of ATP synthase is a stepwise process requiring the assistance of many assembly factors, and depends on the coordinated expression of nuclear and mitochondrial genomes. Most genes encoding

structural ATP synthase proteins and all genes important for the biogenesis of ATP synthase are of a nuclear origin, except two subunits (in higher eukaryotes) of F_o which are encoded by mtDNA.

In mammals subunits a (subunit 6) and A6L (subunit 8) are encoded by mtDNA. However, in yeast and plants subunit c (subunit 9) is also encoded by mtDNA [155, 156]. Therefore, the *ATP9* gene in higher eukaryotes was transferred to the nucleus. An interesting situation exists in filamentous fungi (like *Neurospora crassa*) which have mitochondrial and nuclear copies of the *ATP9* gene [157, 158]. These organisms perhaps represent an intermediate evolutionary stage during which the *ATP9* gene had been successfully transferred to the nucleus, but for unclear reasons maintains a functional mitochondrial copy [159, 160].

Many nuclear proteins, which are not detected in mature ATP synthase, are molecular chaperons facilitating post-translation stages of the ATP synthase assembly pathway [82]. There are also other factors that influence enzyme expression at different levels (Tab. 2). The assembly process of ATP synthase from individual subunits in mammals is not yet fully understood. The most valuable information regarding ATP synthase biogenesis and assembly in eukaryotes results from studies on mutated yeasts, even though there are several differences between yeasts and mammals. Moreover, several biogenesis factors were described after the pathogenic mutation in their genes was found. ATP synthase assembly (Fig. 10) starts with the formation of the F_1 catalytic part, which then associates with the c-ring followed by binding of the other F_o subunits. It appears that the last step of monomer assembly is incorporation of two mtDNA encoded subunits, a and A6L [83, 140]. In yeast, an assembly complex of ATP6 and ATP8 subunits has been found before incorporation into the full ATP synthase [161]. The final steps of ATP synthase biogenesis include formation of dimers and of oligomers (see more in chapter 1.2.2).

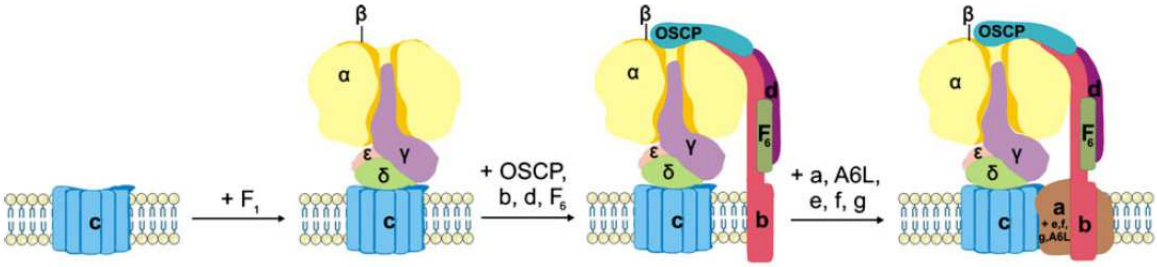

Figure 10 - The scheme of mammalian ATP synthase assembly
The current working model of mammalian F_1F_o ATP synthase step-wise formation process [115].

Recent studies show possible mechanisms that in case of impaired F_1 formation prevent the formation and accumulation of an ATP6-ATP9 ring intermediate capable of dissipating the membrane potential. In *S. cerevisiae* a properly formed F_1 catalytic part is required for translation of ATP6 and ATP8 subunits. Mutations that prevent the formation of the F_1 result in aggregations of α and β into inclusion bodies. Mutants lacking α subunits contain aggregated β subunits and vice versa [162-164]. However, in mammals the mechanism of protecting from proton leakage across inner membrane is based on fast proteolyses of formed, but non assembled, subunits [164].

Ancillary factors of ATP synthase biogenesis

Three genes encoding specific assembly factors are known to be important for formation of $\alpha_3\beta_3$ hexamer in yeast: *ATP11, ATP12 and FMC1*. Atp11p and Atp12p (ATPAF1 and ATPAF2) were first reported as proteins necessary for F_1 part assembly in *S. cerevisiae* [162]. It has been demonstrated that Atp11p interacts with the β subunit, and Atp12p binds selectively to the α subunit [165]. These two factors are essential for the correct assembly of functional $\alpha_3\beta_3$ oligomers. ATP11 and ATP12 are now the only known assembly factors found both in yeasts and mammals. Other ancillary factors that have been found in *S. cerevisiae* are absent in higher eukaryotes and also in bacteria. The third assembly factor for yeast's F_1 part is encoded by the *FMC1* gene. The FMC1 protein was proposed to be involved in correct folding of Atp12p [114].

Most of known ancillary factors involved in biogenesis of the F_o part affect stability and/or translation of mitochondrial ATP6, ATP8 and ATP9 mRNA (Tab. 2). Three nuclear factors are important for expression of the ATP9 gene. AEP1/NCA1 facilitates translation of ATP9 [166, 167], AEP2/ATP13 promote stability and processing of ATP9 mRNA [168]. Moreover, ATP25 is not only a necessary stabilization factor specific for ATP9 mRNA, but is also important posttranslationaly because it is involved in assembly of the c subunit into the c-ring [169], whereas Atp10p and ATP23 are required for incorporation of subunit a into c-ring [162]. In particular, ATP23 is necessary for the processing of subunit 6 into a mature protein. In humans, there is a partial homologue of ATP23 but its function is unclear as human subunit 6 lacks the N-terminal cleavable presequence [170].

Recently, a new protein has been found to be lacking in patients with isolated ATP synthase deficiency [171]. It is called TMEM70, and it is an inner membrane protein [172], which contains the conserved domain DUF1301 and two putative transmembrane regions (Fig. 11).

The TMEM70 protein precursor consists of 260 amino acids (29 kDa), and is then processed into a mature form of 179 amino acids (21 kDa) [172]. Surprisingly, phylogenetic analysis revealed that TMEM70 is found in higher eukaryotes and plants only, and not in yeast and fungi. The mRNA levels of TMEM70 in human cells and tissues are very low, which is characteristic of assembly factors ATPAF1 and ATPAF2, thus it seems that TMEM70 plays a regulatory role in biogenesis of ATP synthase. Further studies are needed for better understanding of TMEM70 role and function.

Gene	Function	Target	Mammals	Yeast
ATP11	chaperone	F_1 subunit β	+	+
ATP12	chaperone	F_1 subunit α	+	+
TMEM70	perhaps chaperone	unknown	+	
FMC1	co-chaperone	ATP12p/F_1 subunit α		+
NAM1	mRNA processing	subunit a and A6L		+
AEP3, NCA2, NCA3	mRNA stability/translation	subunit a and A6L		+
AEP1, AEP2/ATP13	translation	subunit c		+
ATP10	chaperone	subunit a		+
ATP22	translation	subunit a		+
ATP23	processing/assembly	subunit a	?	+
NCA1, ATP25	mRNA stability	subunit c		+

Table 2 - Proteins involved in ATP synthase biogenesis in yeast and mammals
During the biogenesis of ATP synthase many ancillary factors are required. Only assembly factors ATP11 and ATP12 are involved in ATP synthase biogenesis in yeast and mammals. While many factors specific for ATP synthase biogenesis in lower eukaryotes are known, until now only one specific factor for ATP synthase biogenesis in higher eukaryotes had been identified (TMEM70). This can be due to the different genetic origin of subunit c. A homologue of ATP23 was found in humans, but its function here is unknown [82, 171].

Tissue specific regulation of ATP synthase content

The changes in ATP synthase biogenesis intensity are caused by regulations at both transcriptional and translational levels [173]. It is possible that the amount of ATP synthase is controlled by the availability of subunit c. This was observed in thermogenic brown adipose tissue (BAT), where the amount of ATP synthesis was physiologically reduced due to repressed transcription of subunit c [174]. The regulation of ATP synthase content by expression of subunit c was later demonstrated in other tissues [175, 176]. Mammalian c subunit is encoded by three different genes (*ATPG1* - isoform P1, *ATPG2* - isoform P2 and *ATPG3* - isoform P3) that are translated with different mitochondria import sequences but

upon their removal yield the same product-mature c subunit protein [177]. While isoform P2 and P3 are important for the maintenance of a basal level of the c subunit, P1 isoform gene expression is regulated in response to different stimuli. It seems that the amount of P1 c subunit isoform determines the final amount of ATP synthase [178].

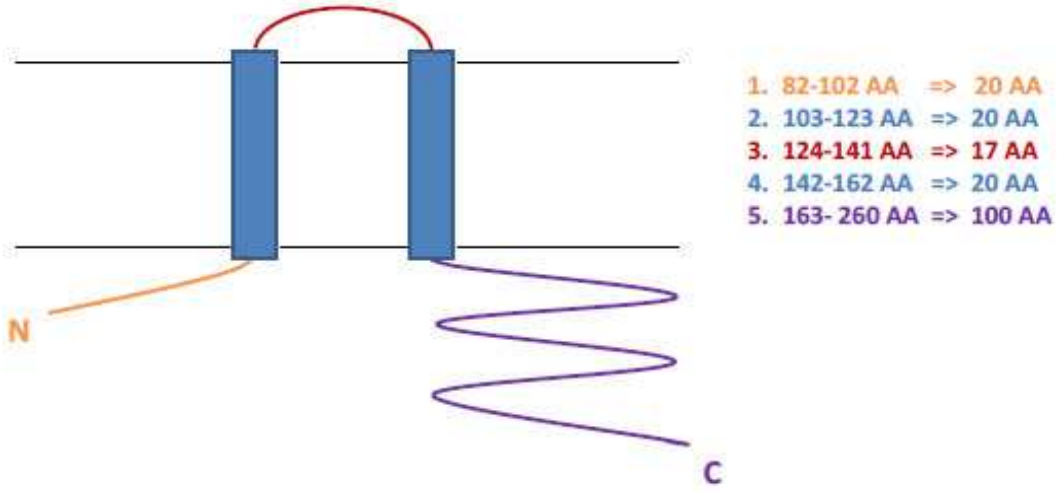

Figure 11 - The predicted topology of TMEM70
It has been predicted that the TMEM70 protein has two transmembrane domains. The transmembrane topology of the protein has not been demonstrated yet. The mature TMEM70 protein consists of 179 amino acid which correspond to 21 kDa [172].

1.3 Mitochondrial diseases

Mitochondrial disorders belong to the group of highly diverse, mostly inherited metabolic diseases caused by impairments of respiratory chain system. The first case was discovered already 50 years ago by researchers at the Karolinska Institute and Stockholm University in Sweden [179]. Because mitochondrial diseases mostly affect brain and skeletal muscle, they are also known as mitochondrial encephalomyopathies. Their estimated prevalence in the population is approximately 1 per 5000 neonates [180]. Mitochondrial diseases are unique for the fact that respiratory chain is the only metabolic pathway in the cell encoded by two genomes, as few but essential components of respiratory chain are encoded in mitochondrial DNA. Thus, mitochondrial diseases uniquely display both the Mendelian and mitochondrial - maternal inheritance, and structural changes in respiratory chain proteins are caused either by mutation in nuclear genes or by mutation in mitochondrial DNA.
The first mutations in mtDNA causing mitochondrial encephalomyopathy were found in 1988 [181, 182]. Since then, during last decade of the 20th century, human mtDNA was studied extensively and around 300 pathogenic mutations associated with maternally inherited syndromes were identified (see MITOMAP; http://www.mitomap.org/MITOMAP). After that, the research became much more focused on mutation in nuclear encoded mitochondrial proteins thus reflecting the fact that mtDNA encodes only 13 structural respiatory chain subunits, while the other approximately 1500 mitochondrial proteins constituting mitochondrial proteome are encoded by nDNA. Indeed, they include not only all remaining ~80 subunits of mitochondrial respiratory chain but also all components of mtDNA transcription, replication and protein synthesis machineries. Therefore, it is not surprising that maternally inherited disorders represent only 15-20% of all human mitochondrial diseases [183].

Mitochondrial diseases of nuclear origin often affect only one complex of respiratory chain. Mitochondrial deficiencies involving several respiratory chain complexes are more common in mtDNA mutations; however, they can be also caused by mutations in nuclear genes involved in mtDNA maintenance or mitochondrial protein synthesis or respiratory chain assembly. Mitochondrial diseases affect particularly tissues and organs with high-energy demands, such as brain, heart, skeletal muscles or sensory organs. Hence, the frequent clinical symptoms of mitochondrial disorders are neuromuscular symptoms, which include skeletal muscle weakness, exercise intolerance, seizures, cardiomyopathy, sensori-neural hearing loss,

optic atrophy, retinitis pigmentosa, ophtalmoplegia, hypothyroidism, gastrointestinal reflux, renal dysfunction and immunodeficiency [184]. Nevertheless, diagnosis of mitochondrial diseases cannot be based only on clinical features because of their diverse and often non specific nature [185]. Furthermore, numerous studies indicate that mitochondrial dysfunction of primary or secondary origin may play a role in the pathogenesis of complex and age-related diseases such as diabetes, various neurodegenerative and cardiovascular diseases, and cancer [186].

Following chapters are focused on mitochondrial diseases caused by frequent mutations in mitochondrial and nuclear genomes, specifically those affecting respiratory chain electron transport complexes and those affecting the key enzyme of mitochondrial energy provision-mitochondrial ATP synthase.

1.3.1 Pathogenic mutations in mtDNA

Coexistence of mutated and wild-type mtDNA in the cell is known as heteroplasmy [187]. The severity of maternally inherited mitochondrial disorders depends on the percentage of mutated DNA in the cell. The level of heteroplasmy can differ within cells and tissues. The OXPHOS system is affected by mtDNA mutation only if the level of heteroplasmy exceeds a certain threshold when the disease symptoms, biochemical and/or clinical begin to manifest. Threshold levels have been shown to vary, usually within the range of 50-60% for mtDNA deletions to >90% for some tRNA point mutations [188]. In addition, the threshold levels differ in different tissues as those with high energy demands, such as brain or heart muscle, are more sensitive than others [189]. The state when the cell contains only mutated mtDNA is called homoplasmy. Generally, the homoplasmic mutations are less pathogenic than the heteroplasmic mutations.

In humans, mtDNA is transmitted from mother to offspring through egg cytoplasm. Despite the high mtDNA copy number in the oocytes, a rapid shift of mtDNA variants between generations genotype have been observed. This has led to a bottleneck theory explaining the segregation of mutated and wt mtDNA during transmission because of low number of mtDNA copies [190]. Experimental evidence has revealed that the bottleneck occurs during postnatal folliculogenesis [191]. Moreover, it is known that some patients with mtDNA related mitochondrial disorders present different disease symptoms during their life span.

MtDNA replication is not cell cycle dependent, which means that a single mtDNA molecule can replicate more than once or not at all during one cell cycle. Thus, non-equal and random segregation of mtDNA during mitotic cell division can cause abrupt changes in proportion to the amount of mutated mtDNA in daughter cells. In addition, there is evidence suggesting an accumulation of mutated mtDNA over time in post-mitotic tissues. In contrast to transmission to offspring, it is rather slow and time-age dependent. This process may be regulated in some cell types by Gimap3, an outer mitochondrial membrane GTPase [192].

Mutation in mtDNA can be divided into two groups: (i) mutations or rearrangements in tRNAs or rRNAs that affect mitochondrial protein synthesis, and (ii) mutations in mRNAs for individual respiratory structural subunits [193]. Most of the sequence variants of mtDNA found among individuals are nonpathogenic polymorphisms which do not lead to the development of mitochondrial disorders. About 60 positions from 16 569 can differ in unrelated individuals. Described hypervariable regions of mtDNA are useful for purposes of forensic identification [194] and evolutionary biology [195]. On the other hand, high levels of mtDNA polymorphisms complicate the identification of mutations found in patients with disease. Mapping the natural variation of mtDNA sequences across populations led to the determination of several haplogroups characteristic for different ethnic groups that are associated with predilection to or protection from various clinical phenotypes [195].

Mutations or rearrangements in mitochondrial tRNAs or rRNAs

Only around 23% of mtDNA coding capacity belongs to the protein synthesis genes (14% to 2 rRNA and 9% for 22 tRNA). However, approximately 65% of mtDNA related disorders are caused by mutations in genes for mitochondrial translation, mostly in tRNA genes [196]. Mutations in these genes lead to the impaired translation of all mtDNA encoded structural subunits and result in generalized OXPHOS deficiencies. The two most common syndromes are MELAS and MERRF. The first, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes, affects children or young adults after normal previous development. It is mostly due to mutation in the $tRNA^{Leu}$ (A3243G) gene, but around a dozen other mutations have been identified in patients with MELAS [197]. The second, myoclonus epilepsy with ragged red fibers syndrome [198] is associated with three mutations in the $tRNA^{Lys}$ gene (A8344G, T8356C, G8363A) and one mutation of the $tRNA^{Phe}$ gene [199]. Not surprisingly, syndromes associated with tRNA mutations can affect every system in human body.

Mutations in mtDNA - encoded subunits of respiratory chain electron transport complexes

Defects in protein coding genes most frequently impair respiratory chain complex I - NADH dehydrogenase (CI). They usually manifest as Leber hereditary optic neuropathy, LHON syndrome - bilateral optic atrophy, which causes acute or subacute loss of vision in young adults, usually males. To date, the pathogenic mutations leading to CI deficiency have been described in all 7 mtDNA encoded CI structural subunits [54]. They associate with a wide range of phenotypes. About half of the inherited mutation in mtDNA CI genes are homoplasmic and result in LHON syndrome [200]. Most common are mutations in ND4 (G11778A), ND1 (G3460A) and ND6 (T14484C) subunits. However, mutations responsible for more severe infantile encephalopathies are usually heteroplasmic. For example, a number of mutations in ND1, ND3, ND4, ND5 and ND6 lead to dystonia or Leigh syndrome. Mutations in ND1 and ND5 have been reported to cause MELAS syndrome as well [201-203]. Moreover, the mutation G3376A may results in LHON/MELAS [204], while the mutation A13045C can lead in LHON/MELAS/Leigh [205] syndromes overlap.

Numerous mutations also affect respiratory chain complexes III and IV (CIII, CIV), but their incidence is much lower. More than thirty different mutations in cytochrome b are responsible for CIII deficiencies (see MITOMAP; http://www.mitomap.org/MITOMAP). They are characterized by exercise intolerance, episodic myoglobinuria, hypertrophic cardiomyopathy, WPW syndrome and mitochondrial encephalopathy [206]. Moreover, mutations in the cytochrome *b* gene are mostly *de novo* sporadic mutations or somatic and heteroplasmic mutations. Although heterogeneous phenotypes in CIII mtDNA defects have been observed, the predominant clinical manifestation is the progressive exercise intolerance started in different age [200].

Mutations in Cox1, Cox2 and Cox3 are mostly heteroplasmic and, in contrast to nuclear COX disorders, disease onset is in late childhood or adulthood [207]. The clinical manifestations of Cox1-3 mutations are the most variable among mutations in the mtDNA genome. The phenotypes vary from myopathy and exercise intolerance to severe early onset multisystem disease or Leigh syndrome [208-212].

1.3.2 Pathogenic mutations in the nuclear genome

Mitochondrial diseases of a nuclear origin can be caused by mutations that affect number of systems, mainly (i) the structural subunits of respiratory chain or their assembly factors, (ii) proteins required for the integrity and replication of mtDNA, (iii) mitochondrial translation machinery, (iv) mitochondria protein import, (v) inner membrane lipid milieu, and (vi) mitochondrial dynamics [193, 213].

Mutations in the structural subunits of respiratory chain and mutations in their assembly factors

Disorders caused by mutation in the structural subunits of the respiratory chain system or in proteins needed for their proper assembly and function, are frequently inherited in recessive fashion. It means that both parents are heterozygous carriers of same mutation.

In case of complex I, pathogenic mutations have been found in the following structural subunits: NDUFV1, NDUFV2, NDUFS1, NDUFS2, NDUFS3, NDUFS8, NDUFS4, NDUFS6, NDUFA1, NDUFA2 and NDUFA11 (references see in [54]). Moreover, mutations leading to CI deficiencies were found in several assembly factors: NDUFAF1 [214], NDUFAF2 [215], NDUFAF3 [216], NDUFAF4 [217], C8orf38 [218], C20orf7 [219] and ACAD9 [220, 221]. Patients with mitochondrial diseases caused by a nuclear origin defect of CI have severe clinical manifestations during infancy and early childhood, frequently resulting in premature death [222].

In case of complex II, mutation in the assembly factor SDAHF1 for complex II leads to infantile leukoencephalopathy [55]. The patients with mutations in assembly factor SDAHF2 [56] and in structural subunits SDHB, SDHC, SDHD [58] suffer from familial paraganglioma syndrome. Mutations in structural subunit SDHA may cause Leigh syndrome [223]. Nevertheless, CII diseases are very rare and thought to account for only 2-4% of the respiratory chain deficiencies [224].

Also mutations in complex III structural genes are rare and have been found only in *UQCRQ* and *UQCRB* genes. Re-arrangement of the *UQCRB* gene causes hypoglycemia and lactic acidosis [225], while missense mutation in the *UQCRQ* gene results in severe psychomotor retardation and mildly elevated lactate [226]. However, more than 20 pathogenic mutations

have been found in gene encoding BCS1 protein. BCS1 is the inner membrane assembly factor for CIII which belongs to the AAA+ protein family and is required for the incorporation of Rieske protein and Qcr10p subunits into the complex III [65]. Recently, a new assembly factor TTC19 has been identified in patient with complex III deficiency [66]. Mutations in CIII assembly factors result in a variety of phenotypes that range from less severe Björnstad syndrome to a fatal complex deficiency manifested by neonatal onset encephalopathy alone or with additional liver failure and tubulopathy [227]. Another mutation can cause the severe syndrome GRACILE [228].

Of the nuclear structural subunits forming complex IV, a mutation causing isolated deficiency of COX was identified only in Cox6b1 subunit [229]. However, most isolated COX deficiencies are due to the mutations in complex IV assembly factors. Autosomal recessive mutations in ancillary factors *SURF1, SCO1, SCO2, COX10, COX 15, LRPPRC* and *TACO1,* which are important for the formation of functional COX enzyme, have been described in humans [34, 35, 230-234]. They are associated with different clinical symptoms, including encephalomyopathies such as Leigh syndrome, fatal cardiomyopathy, hepatic failure and leukodystrophy [235, 236]. Recently, mutation in another factor C12orf62, important for Cox1 synthesis and assembly, has been reported [237]. Complex V mutation will be discussed in the following chapters.

Mutations in proteins required for the integrity and replication of mtDNA

MtDNA replication, maintenance, and integrity involve many proteins encoded by the nuclear genome. Defects in these proteins often cause Mendelian disorders characterized by the qualitative (multiple deletions) or quantitative (depletion) alteration of mtDNA [238]. Multiple DNA deletions have been found in SANDO patients with mutations in POLG and in patients with autosomal dominant mutations in the genes responsible for maintenance of mtDNA integrity, such as *TWINKLE, ANT1, POLG2* and *OPA1* [239, 240]. This group of diseases may result in an autosomal dominant form of progressive external ophtalmoplegia (PEO) with a variety of other symptoms and signs [184]. Autosomal recessive mtDNA depletion syndrome caused by a mutation in the genes involved in mtDNA biogenesis or maintenance of dNTP pools results in a reduction of mtDNA content. Mutations have been found in at least nine genes: *DGUOK, POLG, TK2, RRM2B, TYMP, MPV17, SUCLA2, SUCLG1*, and *C10orf2* (*TWINKLE*). Some of these mutations are tissue-specific. Mutations in POLG belong to the most common mutations causing mitochondria disorders of a nuclear

origin. So far, more than 100 mutations have been reported. The clinical manifestation of POLG mutations can be very heterogeneous [241]. It can result in an autosomal recessive form of Alpers syndrome [242] and an autosomal dominant form or recessive form of PEO [243, 244]. Mutation in thymidine kinase (TK2) and RRM2B results in early onset myopathy with or without renal proximal tubulopathy [245, 246]. Severe infantile disease, which include liver failure and encephalopathy, are caused by the molecular defects in *MVP17* (mitochondrial inner membrane protein with unknown function) [247], *DGUOK* [248] and *TWINKLE* genes [249]. Mutations in *SUCLA2* and *SUCLG1* cause myopathy, lactic acidosis, encephalopathy and a mild elevation of methylmalonic acid [250-252]. Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is associated with mutation in thymidine phosphorylase (TYMP). Its deficiency affects particularly smooth muscle and the brain [253]. Dysfunction of TYMP results in the accumulation of thymidine and deoxyuridine and thus to deoxynucleotide pool imbalances [254].

Defects in mitochondrial translation machinery

Approximately 150 nuclear factors are required for mitochondrial translation [255]. This includes the nuclear proteins of the mitochondrial ribosome, proteins encoding the mitochondrial aminoacyl-tRNA synthetase, tRNA modification enzymes, and translation factors. Mutations in these factors lead to early onset mitochondrial disease with an often severe outcome. The disease's clinical presentation is heterogeneous (lethal neonatal acidosis, infantile encephalomyopathy, hypertrophic cardiomyopathy with encephalomy- opathy and leukoencephalopathy with brain stem and spinal cord involvement and lactic acidosis [236]). Most patients have a combined respiratory chain deficiency. The number of patients with pathogenic mutations in translation machinery is small, suggesting that the nuclear defects of mitochondrial translation are either under diagnosed or intrauterine lethal [256].

Pathogenic mutations have been found in following genes and corresponding proteins: two mitochondrial ribosome proteins MRPS16 and MRPS22 [257, 258]; genes for mitochondrial translation elongation factors EFG1, EFTu, EFTs and C12orf65 [259-263]; tRNA modifying genes PUS1 and TRMU [264-266]; mitochondrial tyrosyl transfer RNA synthetase gene (YARS2) and mitochondrial aspartyl and arginyl transfer RNA synthetase (DARS2, RARS2) [267-269].

Defects of mitochondria protein import system

The next group of nuclear mitochondrial diseases is associated with the impairment of mitochondrial protein import from cytoplasm, where their synthesis takes place, across outer and inner mitochondrial membrane via TIM/TOM complexes. Several mutations in targeting sequences, which prevent individual proteins from reaching their destination, have been reported. However, only a few disorders that lead to the impairment of general protein machinery are known, perhaps because the resulting changes in mitochondrial function would be lethal [193, 270]. For example, mutations in the *TIMM8A* gene, a component of the intermembrane space import machinery, cause X-linked Mohr-Tranebjaerg syndrome [271], and mutations in the gene for importing chaperonin HSP60 cause an autosomal dominant form of hereditary spastic paraplegia [272], or an autosomal recessive form of neurodegenerative disorders [273].

Defects of inner membrane lipid milieu

Alteration of the lipid milieu of the inner mitochondrial membrane is mainly caused by Barth syndrome. This X-linked recessive disorder is the result of mutation in the Tafazine gene (TAZ), which encodes phospholipid acyltransferase. This protein is probably involved in acyl-specific remodeling of cardiolipin, because affected patients have reduced concentrations and an altered composition of cardiolipin [274]. Patients suffer from mitochondrial myopathy, hard failure, growth retardation and leucopenia [275].

Impairment of mitochondrial dynamics

Since mitochondria are dynamic organelles, they move, fuse, and divide. Alterations in mitochondrial dynamics result mainly in neurological diseases which affect either the central or the peripheral nervous system. Mutations have been found, for example, in *OPA1*, *MFN2*, *KIF5A* and various other genes which encode mitochondrial motility proteins. The *OPA1* gene encodes guanosine triphosphate (GTP), which is involved in mitochondria fusion, and its impairment leads to autosomal dominant optic atrophy [276]. Moreover, mutation in the *OPA1* gene also affects mitochondrial intergenomic signaling [277]. Additionally, mutation in the *MFN2* gene that encodes mitochondrial fusion protein mitofusin 2 results in Charcot-Marie-Tooth neuropathy [278]. Lastly, mutation in gene *KIF5A* for kinesin, which moves

mitochondria along microtubules, is associated with autosomal dominant hereditary spastic paraplegia [213, 279].

1.3.3 Genetic defects of ATP synthase

ATP synthase disorders belong to a group of the most deleterious mitochondrial diseases affecting paediatric population. Two main types of ATP synthase dysfunction, which differ in pathogenic mechanism, biochemical phenotype, and clinical manifestation [83], have been described. The first group represents qualitative defects of ATP synthase caused by mutation in mtDNA encoded subunits that mostly lead to unchanged amount of non-functional enzyme. More specifically, these defects impair the F_o proton channel and thus prevent ATP synthesis. However, ATP synthase hydrolysis remaine unchanged. The second group represents quantitative defects caused by mutations in the nuclear genome which result in a selectively reduced amount of the functional enzyme. In these cases, ATP synthase content is selectively decreased to <30% of control values and the enzyme activity is profoundly reduced.

ATP synthase defects due to mutations in mtDNA

Mitochondrial encoded *ATP6* and *ATP8* genes overlap by 46 nucleotides at the 3´ end of *ATP8* gene and the 5´ end of the *ATP 6* gene. Thus the precursor transcript is a bi-cistronic mRNA which encodes two genes with two different reading frames. ATP synthase diseases of mitochondrial origin can be caused by mutation in both ATP6 and ATP8 (A6L) subunits. However, the mutations in the *ATP6* gene are much more frequent.

The most common *ATP6* mutations are T8893G (C) missence mutations. The mutation T8893G causes the conversion of leucine to arginine at position 156. Patients with this mutation are heteroplasmic; typically having 70%-90% mutated mtDNA. The frequent phenotypic manifestation of this disease is a NARP syndrome. However, when the percentage of mutated mtDNA exceeds 90-95%, patients are usually affected with more severe MILS - maternally inherited Leigh syndrome [280, 281]. A similar dependence on mutation load was found in the case of the milder T8893C mutation, where leucine converts to proline [282, 283]. Mutation T8993G results in decreased ATP production, but in normal ATP hydrolytic activity [284-286]. The affected, highly conserved Leu156 is situated in C-terminal region of ATP6 subunit (subunit a, or F_o-a) important for coupling of proton translocation to a rotation of c-ring which drives the ATP synthesis in the F_1 catalytic part of ATP synthase [287]. The

amino acid exchange affects the rotation of the c-ring while the proton flux through F_o is slower, but not blocked [287]. The altered function of ATP synthase proton channel is the reason for severely decreased ATP synthesis [288]. Disturbed interaction between mutated subunit ATP6 and c-ring results in instability of ATP synthase complex [289] that manifests as incomplete subcomplexes of ATP synthase resolved by BN-PAGE system [174].
Less frequent are mutations T9176G (C), affecting leucine at position 217 [290] and the T8851C mutation, affecting tryptophan at position 109 [291]. All cause striatal necrosis syndromes. Mutation m.9035T>C [292], m.9185T>C and m. 9191T>C [293] lead to a NARP-MILS phenotype.
A completely different pathogenic mechanism is represented by a rare 2bp deletion of TA at position 9205 and 9206 (ΔTA9205) that affects the STOP codon of the *ATP6* gene and the cleavage site between the RNA of *ATP6* and *Cox3* [294]. Thus this mutation does not lead to amino acid exchange but significantly prevents the synthesis of ATP6 subunit and consequently it leads to assembly of incomplete ATP synthase unable to synthesize ATP. Patients with these mutations have also a decreased amount of COX due to the impairment of COX biogenesis [295].

The first mutation found in the *ATP8* gene has been reported [296] in 2008. A patient with this mutation has homoplasmic nonsense mutation m.8529G→A (p.Trp55X) in A6L subunit. This mutation results in the improper assembly and reduced activity of ATP synthase. Then, second missense mutation m.8528 T>C (p.Trp55Arg) have been found in four unrelated patients [297]. Both of these mutations affect the same amino acid and result in cardiomyopathy. Another mutation affecting the *ATP8* gene is de novo mutation m.8411A>G, which leads to a leukodystrophy phenotype [298].

ATP synthase defects due to mutations in nDNA

Autosomally transmitted isolated deficiencies of ATP synthase typically affect paediatric population and are characterized by early onset and an often fatal outcome. In 1999, the first patient with ATP synthase deficiency of a nuclear origin was reported. From that point on, the number of patients diagnosed with the same symptoms kept growing, but the affected gene was unknown [299-301]. Then in 2004, the first mutation responsible for ATP synthase disorder of a nuclear origin was reported in the gene for ATP12 assembly factor [302]. However, mutation was found only in one case and was absent in numerous other patients. In

2008, we and our collaborators succeeded to uncover a mutation in the gene for putative mitochondrial protein TMEM70 using gene expression analysis [303] and whole genome homozygosity mapping [171]. *TMEM70* mutation was then found in most other ATP synthase deficient patients. Furthermore, within Czech-Austrian collaboration, in 2010 we also succeeded to identify the first mutation in a structural subunit of ATP synthase. One patient carried the mutation in the epsilon subunit of F_1 part of ATP synthase [299]. So far, mitochondrial disorders of ATP synthase of a nuclear origin have been shown to result from mutations in three disease-causing genes: *TMEM70* and *ATPAF2* encoding specific biogenesis factors of ATP synthase and *ATP5E* gene encoding the structural subunit epsilon of the enzyme.

The homozygous missense mutation p.Trp94Arg in the gene for **assembly *factor ATP12*** (*ATPAF1*) resulted in a severe decrease of complex V amount and activity [302]. The substitution of tryptophan to arginine does not alter its electrostatic properties, but may change the dynamic properties of the ATP12 protein [304]. The ATP12 protein is the chaperone important for the assembly of the β subunit into the F_1 oligomer [305]. The patient suffered severe neonatal encephalopathy, dysmorphic features, severe development delay with seizures, a failure to thrive, and died at the age of 14 months.

A homozygous substitution c.317-2A>G in the splicing site of the second intron of the ***TMEM70 gene*** leads to aberrant splicing and loss of *TMEM70* transcript. The mutation was found in 25 homozygous patients mostly of Roma ethnic origin, only few had consanguineous parents. All these patients showed a profound decrease of ATP synthase amount (30%) and activity (10-30%), therefore the presence of TMEM70 protein must be essential for ATP synthase biogenesis.

The identification of at least eight other pathogenic mutations, which lead to the isolated defect of this enzyme, confirms the crucial role of TMEM70 in ATP synthase biogenesis (Tab. 3). Two patients with heterozygous mutations c.317-2A>G and heterozygous frameshift mutations 118_119insGT, which encodes a truncated TMEM70 protein, Ser40CysfsX11, [171, 306] have been reported. These patients came from non-consanguineous and non-Roma families. Another patient inherited the already known splice site mutation c.366T>A from one parent, and previously unclassified missense variant c.494G>A from both of them. Next, four new mutations in six patients from 4 consanguineous unrelated families of Arab Muslim origin have been identified. One patient has homozygous point mutations c.366T>A, which

leads to a truncated protein due to the premature stop codon. Two brothers were homozygous for the splice site mutation c.316+1G>T in the second exon. The next patient had homozygous point mutation c.238C>T in the second exon and the other two patients carried 2 bp deletions c.578_579delCA resulting in a 197 AA truncated protein [307]. Homozygous mutation c.211-450_317-568del leads to the deletion of exon 2 and part of intron 1 and 2 (2290bp), which results in a truncated protein of 71 AA [308]. A second deletion found in the *TMEM70* gene leads also to loss of exon 2, which is due to 1353bp deletion (g.2436-3789) [309].

In collaboration with Paracelsus Medical University in Salzburg, we described for the first time that a mutation in ***ATP5E gene*** for a structural subunit of ATP synthase can cause ATP synthase deficiency [310]. Analysis of patient fibroblasts uncovered the homozygous missense mutation c.35A>G in the second exon of the nuclear gene *ATP5E*, which encodes epsilon (ε) subunit.

This leads to the amino acid exchange p.Tyr12Cys corresponding to Tyr11, which is highly conserved among eukaryotes. This mutation causes a decrease of oligomycin-sensitive ATP synthase activity, a decrease of mitochondrial ATP synthesis to 60-70 % and a reduced amount of fully assembled ATP synthase of a normal size containing the mutated ε subunit. Also, the content of all F_1 and F_o ATP synthase subunits were similarly reduced, except for the F_o-c subunit, which was found to be accumulated in a detergent-insoluble form. Moreover, down-regulation of the expression of ε subunit in HEK293 cells by shRNA led to decreased activity and protein content in mitochondrial ATP synthase, decreased content of the F_1 (α, β, ε) and F_o (a, d) subunits, and caused accumulation of subunit c. Thus, these findings revealed that the ε subunit is necessary for assembly and/or stability of the F_1 catalytic part of the mammalian ATP synthase and it is also important for incorporation of the hydrophobic subunit c into the F_1-c oligomer during ATP synthase biogenesis.

Metabolic consequences of mitochondrial disorders caused by mutations in the nuclear genes important for ATP synthase biogenesis typically present with diminished biosynthesis of this enzyme, and thus in ATP synthase deficiency which limits the production of ATP. The impairment of ATP synthesis prevents discharge of mitochondrial proton gradient and leads to high values of mitochondrial membrane potential $\Delta\Psi_m$, resulting in increased ROS production [311]. It has been shown that high levels of $\Delta\Psi_{m,}$ above 140 mV, cause an exponential increase of mitochondrial ROS production [312, 313].

Mitochondria are considered a major producer of a reactive oxygen species (ROS) within mammalian cells. The ROS are produced as a consequence of the reaction of unpaired electrons from the OXPHOS and molecular oxygen. Thus, superoxide ions, highly reactive free radical species, are formed. The respiratory complexes I and III are considered to be two

Mutation	Place	Inheritance	Number of patients	Origin	Reference
c.317-2A>G	exon 2	homozygote	>30	Roma/non Roma	[314]
c.317-2A>G/ c.118_119insGT	exon 2, 1	compound heterozygote	2	non Roma/Croatian	[306, 314]
c.317-2A>G **c.494-G>A**	exon 2 exon 2	heterozygote homozygote	1	non Roma	[315]
g.2436-3789 deletions of 1353 BP	exon 2	homozygote	1	Iraqi	[309]
c.366 T>A	exon 2	homozygote	1	Arab Muslim	[307]
c.316+1G>T	exon 2	homozygote	2	Arab Muslim	[307]
c.238C>T	exon 2	homozygote	1	Arab Muslim	[307]
c.578_579delCA	exon 3	homozygote	2	Arab Muslim	[307]
c.211-450_317-568del	intron 1, 2 exon 2	homozygote	1	not reported	[308]

Table 3 - On overview of identified mutation in the *TMEM70* gene

main superoxide sources [316, 317]. Oxidative stress is a deleterious process that can be an important mediator of damage to cell structures, including lipids and membranes, proteins, DNA, and above all, mtDNA. Thus, mitochondria represent not only a major source of ROS generation, but also a major target of ROS induced damage. However, reactive oxygen species are "two-faced" products. In moderate concentrations they act as molecular signals that regulate a series of physiological processes [318].

Thus, the two main pathogenic consequences of ATP synthase diseases are energy deprivation and high ROS generation. These metabolic changes might affect nucleo-mitochondrial signaling and biogenesis of other complexes in the mitochondrial respiratory chain. Experiments performed by our group revealed an increase in the protein amounts of complex III (to 122-153% of controls) and complex IV (to 150-262% of controls) in 10 patients with the c.317-2A>G mutation in the *TMEM70* gene. However, the increase of these complexes has been found only at protein level, not at mRNA level. Thus, it seems that compensatory increases of complex III and complex IV protein amounts are due to adaptive regulation of mitochondrial biogenesis which occurs during posttranslational events [319].

Clinical manifestation of ATP synthase diseases due to nuclear genetic origin

The most common mutation in the TMEM70 protein is homozygous mutation c.317-2A>G, which results in disease with a neonatal onset, severe outcome and involvement of tissues with high energy demand; many of the affected patients died in early childhood [301, 314]. Most patients suffer namely from lactic acidosis, 3-methyl-glutaconic aciduria, hypertrophic cardiomyopathy, variable CNS involvement and psychomotor retardation. For a more detailed clinical presentation of 25 patients with c.317-2A>G mutation see [171, 314]. Patients with compound heterozygous mutations c.317-2A>G and 118_119insGT, or c.317-2A>G and c.494-G>A also showed symptoms of early onset, hypertrophic cardiomyopathy and growth retardation, but the course of disease was milder allowing almost normal or improving psychomotor development. Six patients of Arab Muslim origin harboring homozygous mutation (c.366 T>A, c.316+1G>T, c.238C>T, c.578_579delCA) [307] manifested similar clinical features as the group of patients with the c.317-2A>G mutation. Moreover, they also suffered from early onset cataract, gastrointestinal dysfunction, congenital hypertonia and a fetal onset of the diseases [115].

Up until now, only one patient with a mutation in assembly factor ATP12 and one patient with a mutation in ε subunit have been reported. In contrast to patients with *TMEM70* mutations the clinical symptoms of the patient with ATP12 mutation revealed pronounced brain atrophy but no cardiomyopathy. Also the presentation of the patient with a mutation in the epsilon subunit was different. Apart from early onset, 3-metylglutaconic aciduria and neonatal lactic acidosis, similar to patients with TMEM70 mutations, the course of disease was milder and major symptoms were exercise intolerance and weakness, a strongly shortened walking distance and a severe peripheral neuropathy with the loss of tendon reflexes. This patient survived to adult hood.

2. AIMS OF THE THESIS

The aims of my thesis were to identify genetic defects and characterize pathogenic mechanism of mitochondrial encephalo-cardiomyopathies caused by isolated deficiency of ATP synthase. The thesis was based on previous studies of our department that described the first case of early-onset severe isolated defect of ATP synthase that was caused by a mutation in an unknown nuclear gene. Moreover, a unique cohort of patients with highly similar enzyme defects was diagnosed in collaboration with other research groups within several years.

Thus the specific aims of the thesis were to attempt to identify the affected gene and to learn more about molecular mechanism of these specific disorders of mitochondrial biogenesis. The thesis program represented a part of a broad, collaborative work that succeeded in identifying two new disease-causing genes. In this joint research, the effort was focused on the following point:

(i) Complementary studies with patient cell lines proving the mutation in candidate *TMEM70* gene and characterization of affected TMEM70 protein.

(ii) Testing the hypothesis that isolated defect of ATP synthase can be “compensated” at the level of biogenesis of other respiratory chain complexes.

(iii) Biochemical analysis of changes in structure, function and biogenesis of ATP synthase in patient with mutation in *ATP5E* encoding F_1 epsilon subunit.

(iv) Modeling the dysfunction of epsilon subunit by knockdown of *ATP5E* gene in HEK293 cell line.

3. SUMMARY OF THE RESULTS

This thesis consists of five publications. The first three are concerned with the TMEM70 protein dysfunction responsible for the most frequent isolated deficiency of ATP synthase leading to a severe mitochondrial disease. Article number one reports identification of the first mutation in the *TMEM70* gene and discovery of specific ancillary role of TMEM70 protein in biosynthesis of mitochondrial ATP synthase. The second paper presents successive biochemical and morphological characterization of mitochondrial localization and expression of the TMEM70 protein. The third paper reveals the compensatory-adaptive consequences of c.317-2A>G mutation in the *TMEM70* gene at the level of respiratory chain enzymes. Then, the other two publications deal with discovery of mutated epsilon subunit of ATP synthase as a cause of mitochondrial disease. The fourth paper reports on the first patient with a mutation in the *ATP5E* gene for epsilon subunit of the F_1 part of ATP synthase, and the fifth paper reveals the consequences of down-regulation of this subunit by RNAi in the HEK293 cell line.

1) TMEM70 mutations cause isolated ATP synthase deficiency and neonatal mitochondrial encephalocardiomyopathy

Cízková A, Stránecký V, Mayr JA, Tesarová M, Havlícková V, Paul J, Ivánek R, Kuss AW, Hansíková H, Kaplanová V, Vrbacký M, Hartmannová H, Nosková L, Honzík T, Drahota Z, Magner M, Hejzlarová K, Sperl W, Zeman J, Houstek J, Kmoch S.
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Dysfunction of ATP synthase can be caused by mutations in mtDNA or in nuclear genes. In the time when this article was published (autumn 2008) several mutations in mtDNA encoded subunits leading to the maternally transmitted disorders of ATP synthase were known. In contrast, affected genes responsible for ATP synthase deficiency of nuclear origin were practically unknown, but the number of diagnosed patients was increasing. Only one patient had the mutation in ATP synthase assembly factor ATP12. Investigation of other cases excluded mutations in any of the 16 genes encoding enzyme subunits. However, through gene expression analysis and whole genome homozygosity mapping mutation in putative mitochondrial protein TMEM70 of about 30 kDa was uncovered. Complementation experiments revealed that ATP synthase deficiency can be rescued by the wild type *TMEM70* gene. The homozygous substitution in the splice site of the second exone (317-2A>G) resulting in aberrant splicing and loss of TMEM70 transcript was found in 23 of 25 patients

and was absent in controls. One patient with a truncated TMEM70 protein was heterozygote for this mutation and for frameshift mutation 118_119insGT. In the last patient, no *TMEM70* mutation was found. Most patients were of Roma ethnic origin and suffered from neonatal lactic acidosis, hypertrophic cardiomyopathy and/or variable CNS involvement and 3-methylglutaconic aciduria. Phylogenetic analysis showed that the TMEM70 protein is present only in multicellular eukaryotes and plants and thus represents the first ancillary factor of ATP synthase specific for higher eukaryotes.

2) Expression and processing of the TMEM70 protein

Hejzlarová K, Tesarová M, Vrbacká-Cízková A, Vrbacký M, Hartmannová H, Kaplanová V, Nosková L, Kratochvílová H, Buzková J, Havlícková V, Zeman J, Kmoch S, Houstek J. Biochimica et Biophysica Acta. 2011 Jan;1807(1):144-9. Epub 2010 Oct 16.

The purpose of the second article was to gain more information about a new factor of ATP synthase biogenesis, TMEM70. It is the third assembly-ancillary factor described in mammals in addition to ATP11 and ATP12. However, little is still known about its function and localization. Here, we report that TMEM70 is synthesized as a 29 kDa precursor, which is then imported to mitochondria and processed into a 21 kDa mature protein. The mitochondrial TMEM70 localization was proved by MS analysis, antibody detection in isolated mitochondria, and by morphological analysis of cultured fibroblasts. Moreover, fractionation of isolated mitochondria indicated that TMEM70 is part of the mitochondrial inner membrane. By 2D electrophoretic analysis (BN/SDS PAGE) and WB we demonstrated that TMEM70 may be found in dimeric form and that the TMEM70 protein is not associated with some of the ATP synthase subunits.

Our MS analysis revealed very low cellular content of the TMEM70 protein which is in agreement with available expression profile data reporting extremely low levels of *TMEM70* transcript in human cells and tissues. Notably, *TMEM70* transcripts vary slightly among different tissues. Low cellular abundance, low transcript levels, and no tissue-specificity are characteristics for general biogenesis factors.

With a prepared anti-TMEM70 antibody we confirmed that the normal TMEM70 protein is absent in mitochondria with homozygous 317-2A>G mutation and no aberrant protein with lower molecular weight has been identified. However, a small amount of fully functional ATP synthase found in patients with a loss of *TMEM70* transcripts indicate that TMEM70 is not absolutely essential for the biogenesis of ATP synthase.

3) Compensatory upregulation of respiratory chain complexes III and IV in isolated deficiency of ATP synthase due to TMEM70 mutation

Havlícková-Karbanová V, Cízková-Vrbacká A, Hejzlarová K, Nusková H, Stránecký V, Potocká A, Kmoch S, Houstek J.
Biochimica et Biophysica Acta. Jul;1817(7):1037-43. Epub 2012 Mar 10

In this paper, we used the unique cohort group of 10 patients with the same genetic defect of homozygous 317-2A>G mutation in the *TMEM70* gene to further investigate a possible common compensatory mechanism in reaction of cells to altered mitochondrial energy provision. The dysfunction of ATP synthase leads to low ATP production, elevated mitochondrial membrane potential, and increase of ROS production. We wanted to find out how this metabolic disbalance influences the OXPHOS system. Thus, we performed a quantitative analysis of respiratory chain complexes and intramitochondrial proteases trough SDS-PAGE/WB or BN-PAGE/WB analysis. In homogenates of the patient's fibroblasts we found that the average content of ATP synthase decreased to 18% of controls amount. Conversely, the average content of respiratory enzymes III and IV increased to 133% and 163% of the control amounts, respectively. We did not find a significant change in the amount of any analyzed protease (Lon, paraplegin and prohibitins 1 and 2). The mtDNA copy number was unchanged as well. Furthermore, correlation with whole genome expression profiling determined in investigated fibroblasts did not show parallel consistent changes in the OXPHOS mRNA levels of subunits or specific assembly factors of respiratory chain complexes. The results indicate that ATP synthase deficiency leads to compensatory changes that are mainly due to posttranscriptional regulation of biogenesis of mitochondrial respiratory chain complexes.

4) Mitochondrial ATP synthase deficiency due to a mutation in the ATP5E gene for the F_1 epsilon subunit

Mayr JA, Havlícková V, Zimmermann F, Magler I, Kaplanová V, Jesina P, Pecinová A, Nusková H, Koch J, Sperl W, Houstek J.
Hum Mol Genet. 2010 Sep 1; 19(17):3430-9. Epub 2010 Jun 21.

This publication results from collaboration with Paracelsus Medical University in Salzburg.
In the group of analyzed patients with an isolated defect of ATP synthase there was one patient with a distinct clinical phenotype and no mutation in *TMEM70* gene. An Austrian girl with neonatal-onset lactic acidosis, 3-methylglutaconic aciduria and no-cardiac involvement, suffered from exercise intolerance, mild mental retardation and developed peripheral

neuropathy. As a genetic cause of the disease we identified the homozygous missense mutation A>G c.35 in second exon of the *ATP5E* gene, encoding epsilon subunit of the ATP synthase. It is the first mutation identified in the structural subunit of ATP synthase. It leads to amino acid exchange (pTyr12Cys) affecting highly conserved amino acid among eukaryotes Tyr11 located at the N-terminus which is involved in the formation of ε-δ heterodimer. Biochemically, the patient fibroblasts showed a decrease of both ATP synthase activities by 60-77% compared with the controls and an equally reduced, but fully assembled ATP synthase containing mutated epsilon. Moreover, protein content analysis revealed a decrease of either F_1 (α, β, ε) or F_o (a, d, OSCP, F6) ATP synthase subunits, except subunit c, which was found to accumulate in detergent-insoluble form. The protein content of respiratory chain complexes I, II, III and IV were normal or slightly increased in respect to the controls. Furthermore, the data from the pulse-chase experiment of metabolic labeling with ^{35}S-methionine indicates a decrease of *de novo* synthesis of ATP synthase. Thus, it seems that the isolated defect of ATP synthesis is caused by the impairment of the biogenesis of ATP synthase, which leads to a small amount of functional enzyme. The study of this patient case shows that presence of the epsilon subunit is important for proper assembly of ATP synthase.

5) Knockdown of F_1 epsilon subunit decreases mitochondrial content of ATP synthase and leads to accumulation of subunit c

Havlíčková V, Kaplanová V, Nůsková H, Drahota Z, Houštěk J.
Biochim Biophys Acta. 2010 Jun-Jul;1797(6-7):1124-9. Epub 2009 Dec 21.

The last article completes previous publication about the importance of epsilon subunit. The epsilon subunit is the smallest and functionally less characterized subunit of the F_1 ATP synthase part, which lacks the N-terminal cleavable presequence and does not have a homolog in bacterial and chloroplast enzymes. To find out more about the role of the mammalian epsilon subunit, we down regulated expression of the *ATP5E* gene by RNAi. Silencing of the *ATP5E* gene in human HEK293 cell line leads to a decrease of activity and protein content of mitochondrial ATP synthase complex and ADP-stimulated respiration to approximately 40% of control amounts. In *ATP5E* silenced cell lines, a decreased amount of the ε subunit was accompanied by a decreased content of the F_1 subunits α and β and as well as of the F_o subunits a and d, while the content of F_o subunit c was not affected. In addition, we found the accumulated subunit c to be present in fully assembled ATP synthase complex or in subcomplexes of 200-400 kDa, which contained neither F_1 subunits α or β, nor the F_o subunits a, b or d. Thus, down-regulation of the epsilon subunit leads to a biochemical

phenotype that is very similar to the phenotype of patient with the c.35A>G mutation. Both our studies show that the ε subunit is necessary for assembly and/or stability of the F_1 catalytic part of the mammalian ATP synthase, and that it is also important for incorporation of the hydrophobic subunit c into the F_1-c oligomer during ATP synthase biogenesis.

Contributions of dissertant to these publications

The presented results were achieved in a team effort; the dissertant contribution on presented articles is as follows:

1) Complementation studies of patient's fibroblasts with vectors containing the *wtTMEM70* gene (cell culturing, transfection and ELFO/WB analysis).
2) Antibody testing and purification, transfection of HEK293 with a *TMEM70 GFP* plasmid.
3) The determination of compensatory changes in OXPHOS at protein level and analysis of the mtDNA/nDNA copy number (cell culturing, ELFO/WB analysis, QT-RT PCR).
4) The determination of the protein content of different subunits and analysis of native complexes of ATP synthase and respiratory chain enzymes (cell culturing, ELFO/WB analysis), functional analysis of ATP synthase (oxygraphy, enzyme assays).
5) Preparation of the cells with stable down-regulation of the epsilon subunit and their analysis (vector purification, transfection, cell culturing, ELFO/WB analysis, QT-RT PCR).

4. CONCLUSIONS

From the results of this thesis it can be concluded that:

1)

- *TMEM70* was identified as a new disease-causing nuclear gene responsible for most cases of isolated ATP synthase deficiency. The most frequent is homozygous mutation 317-2A>G that results in aberrant splicing and loss of *TMEM70* transcript and prevents synthesis of TMEM70 protein.
- TMEM70 protein is a novel ancillary factor important for the proper assembly of ATP synthase in higher eukaryotes.
- TMEM70 is synthesized as a 29 kDa precursor, which is imported to mitochondria and processed into a 21kDa mature protein localized in the inner mitochondria membrane.
- A decreased content of ATP synthase and resulting metabolic imbalance leads to the compensatory upregulation of complex III and VI due to adaptive mechanisms originating at a posttranscriptional level, without changes in mtDNA content or the content of intramitochondrial proteases.

2)

- *ATP5E* gene was identified as the third disease-causing gene responsible for ATP synthase deficiency.
- The homozygous missense mutation in epsilon subunit (c.35A>G) was found as a cause of phenotypically rare and mild form of mitochondrial disease. It is also the first mutation in a structural subunit of ATP synthase that has been reported.
- By RNAi interference, stable HEK293 cell lines with down-regulated expression of the *ATP5E* gene were prepared.
- The cells with a c.35A>G mutation and the cell with down-regulated synthesis of the epsilon subunit manifested with similar decrease in ATP synthase complex to 30-40%. Correspondingly all F_1 and F_o subunits were reduced, except for the F_o-c subunit, which was found accumulated in a detergent-insoluble form.
- Our results showed the ε subunit is necessary for assembly and/or stability of the F_1 catalytic part of the mammalian ATP synthase, and it is also important for incorporation of the hydrophobic subunit c into the F_1-c oligomer during ATP synthase biogenesis.

The results of this thesis contributed to better understanding of molecular genetic mechanisms responsible for inborn mitochondrial diseases caused by a dysfunction of ATP synthase and further improved our knowledge of the components and events of ATP synthase biogenesis.

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6. SUPPLEMENTS: ARTICLES 1-5

ARTICLE 1

nature genetics

TMEM70 mutations cause isolated ATP synthase deficiency and neonatal mitochondrial encephalocardiomyopathy

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We carried out whole-genome homozygosity mapping, gene expression analysis and DNA sequencing in individuals with isolated mitochondrial ATP synthase deficiency and identified disease-causing mutations in *TMEM70*. Complementation of the cell lines of these individuals with wild-type *TMEM70* restored biogenesis and metabolic function of the enzyme complex. Our results show that TMEM70 is involved in mitochondrial ATP synthase biogenesis in higher eukaryotes.

Mitochondrial ATP synthase, a key enzyme of mitochondrial energy provision, catalyzes synthesis of ATP during oxidative phosphorylation. ATP synthase is a 650-kDa protein complex composed of 16 types of subunits; 6 form the globular F_1 catalytic part and 10 form the transmembraneous F_o part with two connecting stalks[1]. Two mammalian ATP synthase subunits, ATP6 and ATP8, are encoded by mtDNA; all the others are encoded by nuclear DNA. Biogenesis of ATP synthase is a stepwise process requiring a concerted action of assembly factors. Several of these factors have been described in yeast (for example, ATP10, ATP11, ATP12, ATP22, ATP23 and FMC1)[2], but only three have been found in mammals—homologs of F_1-specific factors ATP11 and ATP12 (refs. 2–4) essential for assembly of F_1 subunits α and β, and a homolog of the F_o-related ATP23 with unclear function in mammals[5].

Inherited disorders of ATP synthase belong to most deleterious mitochondrial diseases, which typically affect the pediatric population[6]. Maternally transmitted ATP synthase disorders are caused by heteroplasmic mutations of *MT-ATP6* (ref. 7) and rarely of *MT-ATP8* (ref. 8). These defects impair the energetic function of the F_o proton channel and thus prevent ATP synthesis, although the rate of ATP hydrolysis and the concentration of the enzyme complex remain largely unchanged. In contrast, ATP synthase defects of nuclear genetic origin (MIM604273) are characterized by selective decrease of ATP synthase concentrations (to <30%) and a profound loss of both synthetic and hydrolytic activities[9]. Most affected individuals show neonatal lactic acidosis, hypertrophic cardiomyopathy and/or variable central nervous system involvement and 3-methylglutaconic aciduria. The disease outcome is severe, and half of affected individuals die in early childhood[10]. During the last decade, an increasing number of affected individuals, mostly of Roma (Gypsy) ethnic origin, have been reported[10–13], but a mutation affecting the F_1-specific factor ATP12 was only found in one case[11]. To identify the genetic defect in the other affected individuals with isolated deficiency of ATP synthase we used Affymetrix GeneChip Mapping 250K arrays and genotyped eight index affected individuals, their healthy siblings and parents from six families (**Supplementary Methods** and **Supplementary Fig. 1** online) and performed linkage analysis (**Supplementary Fig. 2** online) and homozygosity mapping (**Fig. 1a** and **Supplementary Fig. 3** online). To prioritize candidate genes, we intersected the mapping information with Agilent 44K array gene expression data[13]. This analysis illuminated a single gene, *TMEM70*, as it has previously been localized in a top-candidate region on chromosome 8 (**Fig. 1a**), showed reduced transcript amount in fibroblast cell lines from affected individuals (**Fig. 1b**,**c**,**d**) and encodes what has been characterized as a mitochondrial protein[14]. Through sequence analysis of genomic DNA (**Supplementary Table 1** online), we identified in affected individuals a homozygous substitution, 317-2A>G, located in the splice site of intron 2 of *TMEM70* (NM-017866; **Fig. 1e**), which leads to aberrant splicing and loss of *TMEM70* transcript (**Fig. 1b**,**d**). We carried out PCR-RFLP analysis in investigated families and proved autosomal recessive segregation of the mutation, as all the affected individuals were homozygous, all parents were heterozygous and unaffected siblings showed either the wild-type or heterozygous genotype. We screened for the 317-2A>G mutation among 25 individuals with low ATP synthase content being studied in our institutions, and found 23 who were homozygous for the mutation (**Supplementary Table 2** online). In an additional single heterozygous individual, P27, we identified on the second allele the frameshift mutation 118_119insGT (**Supplementary Fig. 4** online), which encodes a truncated TMEM70 protein, Ser40CysfsX11. We did not find any mutation in affected individual P3, in whom *TMEM70* transcript amount was also unchanged (**Fig. 1c**). We did not find any of the identified mutations in 100 control individuals.

To prove that TMEM70 is necessary for the biogenesis of the ATP synthase, we carried out RT-PCR analysis of several human tissues (**Fig. 1d**) and found no evidence of distinct *TMEM70* splicing variants

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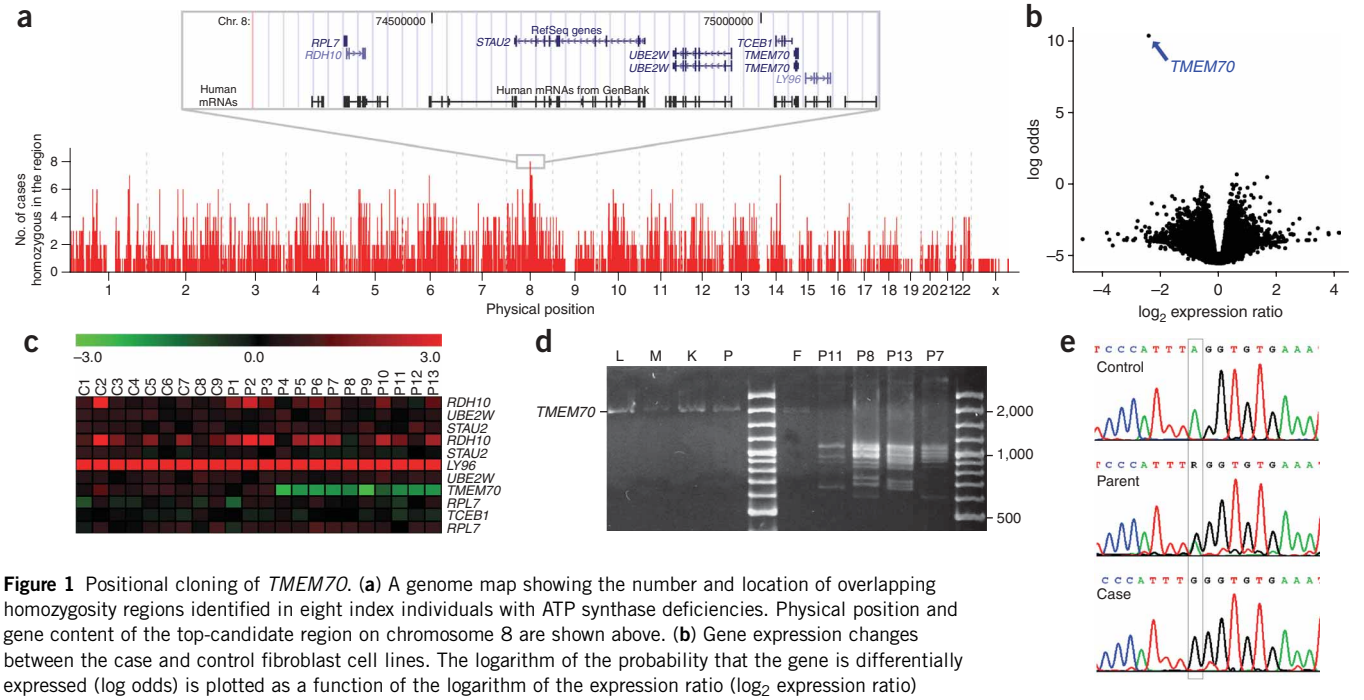

Figure 1 Positional cloning of *TMEM70*. (**a**) A genome map showing the number and location of overlapping homozygosity regions identified in eight index individuals with ATP synthase deficiencies. Physical position and gene content of the top-candidate region on chromosome 8 are shown above. (**b**) Gene expression changes between the case and control fibroblast cell lines. The logarithm of the probability that the gene is differentially expressed (log odds) is plotted as a function of the logarithm of the expression ratio ($\log_2$ expression ratio) between the case and control samples. (**c**) Expression matrix of the genes located in the candidate region showing reduced *TMEM70* transcript amount in all but one case (P3) with a nuclear defect. Normal *TMEM70* transcript amount is present in cases P1 and P2, as these individuals had the mt9205ΔTA microdeletion and not the *TMEM70* mutation. The results are shown as $\log_2$ ratios of gene expression signal in each sample to that of a common reference sample. (**d**) *TMEM70* cDNA analysis. L, M, K, P and F lanes show presence of a single RT-PCR product in control human liver, muscle, kidney, pancreas and fibroblasts, respectively. Abnormal RT-PCR products were observed in fibroblasts from affected individulas (lanes P11, P8, P13 and P7). (**e**) Chromatograms of *TMEM70* genomic DNA sequence showing 317-2A>G substitution.

reported in genomic databases. We cloned *TMEM70* cDNA into the pEF-DEST51 expression vector and transfected skin fibroblast cell lines of several affected individuals (**Fig. 2a**). We found that transfected cells increased the amount of both F_1 and F_o structural subunits of ATP synthase (**Fig. 2b**) and produced normal concentrations of the full size, assembled ATP synthase complex (**Fig. 2c**). Consequently,

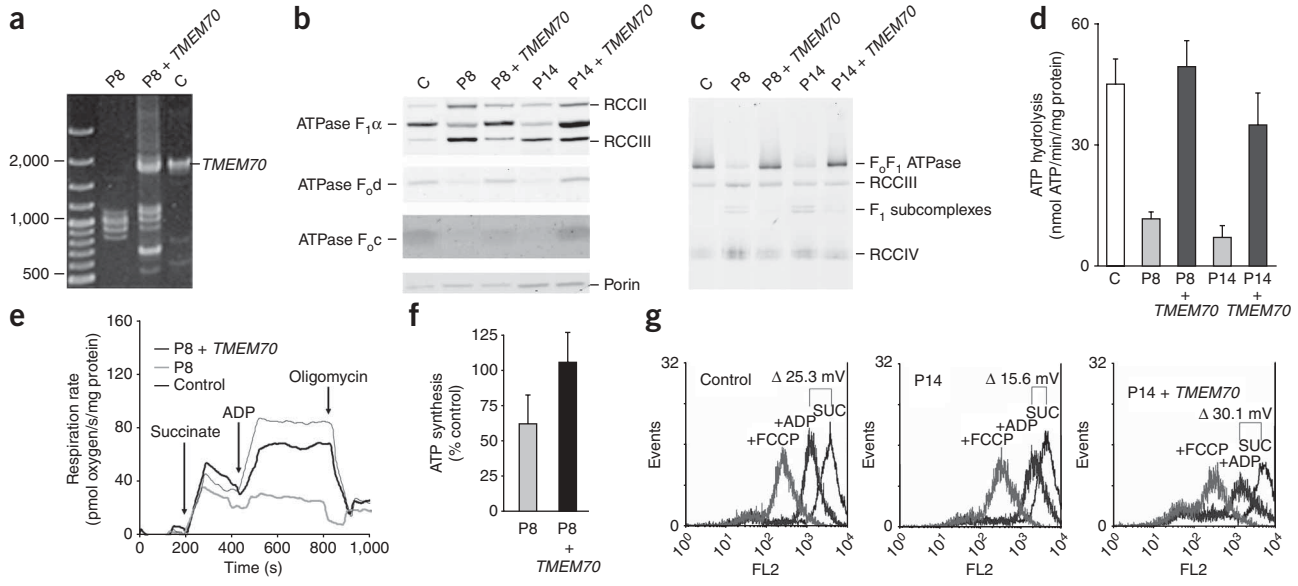

Figure 2 *TMEM70* complementation of ATP synthase deficiency. (**a**) *TMEM70* cDNA is present after transfection. (**b**) SDS-PAGE protein blot of fibroblasts shows a specific increase of the content of ATP synthase subunits relative to the respiratory chain complexes and porin. (**c**) BN-PAGE protein blot of mitochondria shows increase of the full-size assembled ATP synthase 650-kDa complex relative to respiratory chain complexes. (**d**) Oligomycin-sensitive ATP synthase hydrolytic activity is restored. (**e**) ADP stimulation is enhanced in digitonin-permeabilized cells. (**f**) Analysis of ATP formation shows restoration of mitochondrial ATP synthesis. (**g**) TMRM cytofluorometric measurements in permeabilized cells show restoration of the ADP-induced drop of mitochondrial membrane potential at state 4. Data in **d** and **f** are shown as mean ± s.d.; $n = 3$.

the vector restored oligomycin-sensitive ATP hydrolysis (**Fig. 2d**), ADP-stimulated respiration (**Fig. 2e**), mitochondrial ATP synthesis (**Fig. 2f**) and ADP-induced decrease of mitochondrial membrane potential (**Fig. 2g**).

TMEM70 contains the conserved domain DUF1301 and two putative transmembrane regions. Using phylogenetic analysis, we found *TMEM70* homologs in genomes of multicellular eukaryotes and plants, but not in yeast and fungi (**Supplementary Fig. 5** online). This indicates that the evolution of TMEM70 may be an important factor accounting for differences in the ATP synthase assembly process in higher eukaryotes, yeast and bacteria[2,3].

We have identified TMEM70 as a protein involved in the biogenesis of the ATP synthase in higher eukaryotes and shown that its defect is relatively frequent among individuals, particularly Romanies, with mitochondrial energy provision disorders. Existence of the prevalent mutation and co-occurrence of cases with severe and milder phenotypes, probably representing varying quality and functionality of individual nonsense-mediated RNA decay systems, open a way for investigation of translational bypass therapy in this group of individuals.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

A.C., H.Hartmannová and L.N. carried out DNA and gene expression analysis and TMEM70 cloning. V.S. and R.I. were responsible for genotyping, gene expression analysis and bioinformatics. J.A.M. carried out biochemical diagnosis and DNA analysis. A.W.K. did genotyping and homozygosity mapping. M.T. and H.Hansíková carried out biochemical diagnosis, cell culturing and transfections. V.H., J.P. and V.K. carried out transfections, complementation studies, ELFO/WB analysis and bioinformatics. M.V., Z.D. and K.H. were responsible for functional studies. T.H. and M.M. were responsible for family ascertainment and sample collection, and J.Z. and W.S. handled diagnosis and clinical characterization. S.K. and J.H. initiated and coordinated the study and wrote the manuscript.

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ARTICLE 2

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Expression and processing of the TMEM70 protein

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ABSTRACT

TMEM70 protein represents a novel ancillary factor of mammalian ATP synthase. We have investigated import and processing of this factor in human cells using GFP- and FLAG-tagged forms of TMEM70 and specific antibodies. TMEM70 is synthesized as a 29 kDa precursor protein that is processed to a 21 kDa mature form. Immunocytochemical detection of TMEM70 showed mitochondrial colocalization with MitoTracker Red and ATP synthase. Western blot of subcellular fractions revealed the highest signal of TMEM70 in isolated mitochondria and mitochondrial location was confirmed by mass spectrometry analysis. Based on analysis of submitochondrial fractions, TMEM70 appears to be located in the inner mitochondrial membrane, in accordance with predicated transmembrane regions in the central part of the TMEM70 sequence. Two-dimensional electrophoretic analysis did not show direct interaction of TMEM70 with assembled ATP synthase but indicated the presence of dimeric form of TMEM70. No TMEM70 protein could be found in cells and isolated mitochondria from patients with ATP synthase deficiency due to TMEM70 c.317-2A>G mutation thus confirming that TMEM70 biosynthesis is prevented in these patients.

1. Introduction

Biogenesis of eukaryotic ATP synthase is a stepwise process, in which 16 different subunits assemble the structure of the enzyme consisting of the F_1 catalytic part and the membranous F_o part connected together by two stalks [1,2]. The biosynthesis of individual subunits and formation of the ATP synthase holoenzyme depend on several specific helper proteins that are partly common to, and partly unique to, higher and lower eukaryotes. Several yeast-specific factors (NCA1-3, NAM1, AEP1-3 ATP22 and ATP25) are involved in mRNA stability, translation and processing of mtDNA encoded subunits ATP6 and ATP9 [1,3–5] or their assembly (ATP10, ATP22). Additional factor ATP23 [5,6], the metalloprotease with chaperone activity is implicated in processing of ATP6 and its association with ATP9 oligomer. There exists mammalian ortholog of ATP23 which contains a HEXXH motif of the protease active site, but its function is unknown. The only two yeast factors that are found in mammals [1,7,8], having identical function are the F_1 chaperones, ATPAF1 and ATPAF2, interacting with F_1 subunits β and α. Both are absolutely essential for assembly of the functional $\alpha_3\beta_3$ heterooligomer. The FMC1, the third factor involved in F_1 assembly in yeast at high temperature [9] is again specific for yeast. Till now, only one essential ancillary factor, the TMEM70 protein, has been found in mammals being absent in yeast and fungi [10,11]. The mutations in *TMEM70* gene were found to be responsible for isolated deficiency of ATP synthase leading to a severe mitochondrial disease [10,12]. The enzyme defect was rescued by the wtTMEM70. The TMEM70 protein was identified as a putative mitochondrial protein that fulfils the criteria of MITOCARTA [13,14]. The biological roles, as well as biogenesis of this protein remain unknown. In this study we attempted to use tagged forms of TMEM70 and specific antibodies for characterization of expression, processing and localization of this factor.

2. Materials and methods

2.1. Cell cultures

Human embryonic kidney cells (HEK293, CRL-1573, ATCC) were grown in high-glucose DMEM medium (PAA) supplemented with 10% (v/v) fetal calf serum (PAA) at 37 °C in 5% CO_2 in air. Fibroblasts were grown in DMEM medium (Sigma) containing 10% fetal calf serum (Sigma), penicillin (100 U/mL) and streptomycin (100 μg/mL), at 37 °C in 5% CO_2 in air. Confluent cells were harvested by trypsinization

Abbreviations: DDM, dodecyl maltoside; F_1, catalytic part of ATP synthase; F_o, membrane embedded part of ATP synthase; PDH, pyruvate dehydrogenase

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and washed twice with PBS (8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, 0.20 g/L KH_2PO_4).

2.2. Expression vectors

TMEM70 cDNA clone MHS1011-60493 was obtained from Open Biosystems. Following the sequence verification, the insert was transferred into the mammalian expression vector pEF-DEST51 using the Gateway technology (Invitrogen). Resulting plasmids TMEM70-pEF-DEST51 were propagated in *Escherichia coli*, isolated and fully sequenced before the transfection. *TMEM70-Flag* cDNA expression vector—the full-length human *TMEM70* coding sequence was amplified from the IMAGE clone 3631570 and inserted into the C-FLAG fusion mammalian expression vector pCMV-Tag4 (Stratagene). The fidelity of the construct was confirmed by sequencing. *TMEM70-GFP* cDNA expression vector [13] was kindly provided by Dr V.K. Mootha.

2.3. Transfections

Vectors were transfected into the fibroblast or HEK293 cell lines (2 μg of DNA/5×10^5 cells) using Nucleofector device and NHDF nucleofection kit (Amaxa/Lonza), following the standard protocol. For the transient expression of the TMEM70-FLAG fusion protein, cell transfection was carried out with Express-In Transfection Reagent (Open Biosystems). Transfected cell lines were cultured for 48 h on BD Falcon 4-well CultureSlides.

2.4. Isolation of mitochondria

HEK293 cells (1×10^7) were harvested by trypsinization, washed twice in PBS, re-suspended in isotonic STE buffer (250 mM sucrose, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.2% (v/v) Protease Inhibitor Cocktail (PIC, Sigma P8340)), and disrupted on ice using Dounce homogenizer. Homogenate was centrifuged for 15 min at 600*g* and 4 °C, the post-nuclear supernatant was centrifuged for 25 min at 10,000*g* and 4 °C. The resulting supernatant corresponding to the cytoplasm fraction was collected and the mitochondrial pellet was washed by centrifugation with STE buffer.

Fibroblast mitochondria were isolated by the method utilizing the hypotonic shock cell disruption [15]. To avoid proteolytic degradation, the isolation medium (250 mM sucrose, 40 mM KCl, 20 mM Tris–HCl, 2 mM EGTA, pH 7.6) was supplemented with the 0.2% PIC. The isolated mitochondria were stored at −80 °C.

Rat liver mitochondria for import studies were isolated by the method of Enriquez et al. [16].

2.5. Submitochondrial fractionation

Fractionation of mitochondria from HEK293 cells was carried out according to Satoh et al. [17] with slight modifications. Briefly, isolated mitochondria were re-suspended in STE buffer at final concentration 1 mg/mL and disrupted by repeated freezing-thawing 3 times followed by sonication on ice for 5 s at 20% amplitude and 0.5 cycle using an UP 200S Ultrasonic Processor (Hielscher, Germany). Unbroken mitochondria were removed by centrifugation at 10,000*g* for 10 min. The soluble mitochondrial proteins and membranes were separated by centrifugation of the supernatant at 100,000*g* for 35 min. The pellet was re-suspended in 100 mM sodium carbonate, pH 11.5, and incubated at 4 °C and continuous vortexing for 30 min followed by centrifugation at 100,000*g* and 4 °C for 40 min. Supernatant containing membrane associated proteins was collected and the pellet was re-suspended in STE buffer. All collected fractions were kept at −80 °C until analysis.

2.6. TMEM70 antibodies

The cDNA sequence encoding 50–260 AA of human TMEM70, corresponding to expected mature part of the protein was cloned into pMAL-c2 expression vector (New England Biolabs). The construct with the correct sequence was introduced into the *E. coli* MAX Efficiency DH5αF'IQ cells and the fusion protein MBP-TMEM70 was expressed upon IPTG induction. Fusion protein was isolated from sonicated and detergent solubilized cell lysate (20 mM Tris–HCl, 0.2 M NaCl, 1 mM EDTA, 1% Nonidet P-40) using amylose resin column (New England Biolabs). The protein was eluted from the column by 50 mM maltose and directly used for rabbit immunization (Open Biosystems).

2.7. Electrophoresis and Western blot analysis

SDS-PAGE, two-dimensional BN/SDS-PAGE and Western blot analysis were performed by standard protocols as previously [18,19] using specific primary antibodies against GFP (Santa Cruz Biotechnology), FLAG (Sigma), cytochrome *c* oxidase subunit Cox1, ATPase β subunit, PDH E1 α subunit (Mitosciences), MtHSP70 (Alexis Biochemicals), α-Tubulin (Cell Signaling), a mixture of antibodies to respiratory chain proteins (MS603 to ATPase α, Core2, NDUFA9, SDH70, Cox4; Mitosciences), or the polyclonal rabbit antibody to TMEM70. The immunoblots were detected with peroxidase-conjugated secondary antibodies and SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) using VersaDoc 4000 Imaging System (Bio-Rad), or with fluorescent secondary antibodies (Alexa Fluor 680, Molecular Probes) on an Odyssey infrared imaging system (LI-COR).

2.8. Immunocytochemistry

Fibroblast cells were grown on glass chamber slides (BD Falcon 4-well CultureSlides). After 48 h, the cells were washed with PBS, fixed and permeabilized for 10 min with methanol at −20 °C or with paraformaldehyde for 10 min at 4 °C. After blocking unspecific sites with 5% FBS, cells were incubated overnight at 4 °C with indicated antibody in 5% FBS followed by 60 min incubation at 37 °C with fluorophore-conjugated secondary antibody (1 μg/mL, Molecular Probes). The following primary antibodies were used: mouse monoclonal (MS503, Mitosciences) or polyclonal [20] to F_1 β subunit, mouse monoclonal to FLAG (Sigma), or rabbit polyclonal antibody to TMEM70.

When using MitoTracker Red (Molecular Probes), cells were incubated in 300 nM prewarmed medium solution of MitoTracker Red at 37 °C for 15 min, washed with fresh prewarmed medium, fixed and permeabilized. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Prepared slides were mounted in fluorescence mounting medium Immu-Mount (ShandonLipshaw) and analyzed by confocal microscopy (Nikon Eclipse TE2000, Leica AOBS—Acusto-Optical Beam Splitter) and/or epifluorescent microscopy (widefield epifluorescent microscope Nikon Eclipse E400).

2.9. Mitochondrial import

Protein precursor was synthesized in the presence of ^{35}S-methionine using TNT T7 Quick Coupled Transcription/Translation System (Promega) with plasmid vector or PCR product as a DNA template, according to manufacturer's recommendation. Translation product was centrifuged at 13,000*g* for 2 min and supernatant was used for import to isolated rat liver mitochondria or human HEK293 mitochondria.

The import reaction was carried out in 50 μL of a medium containing 25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM KH_2PO_4, 0.05 mM EDTA, 5 mM $MgCl_2$, 10 mM Tris–HCl, 1 mg BSA/mL,

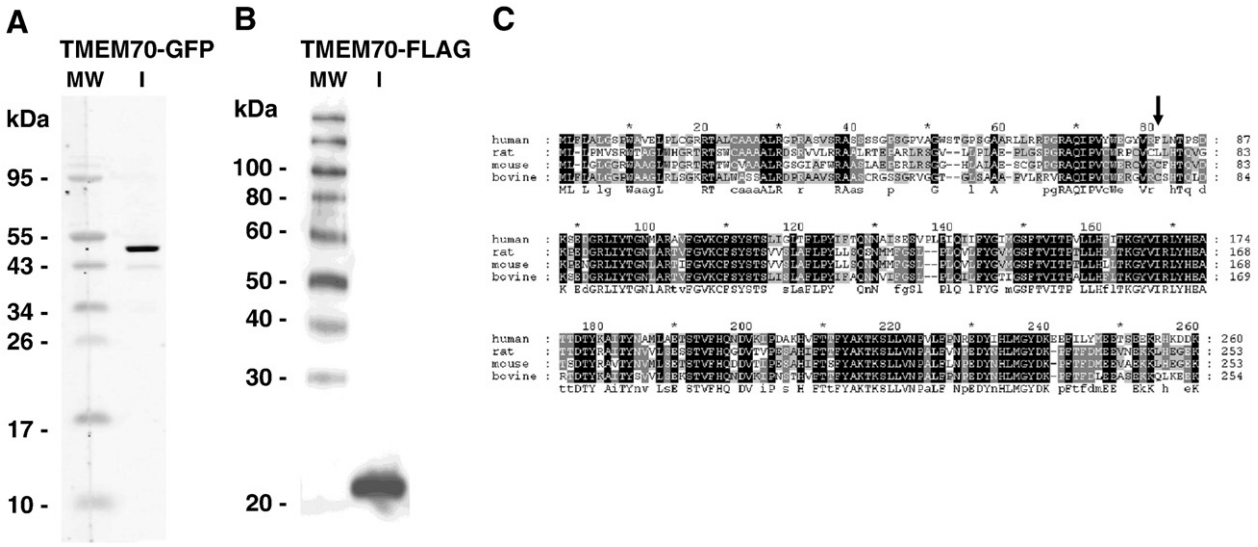

Fig. 1. The size of mature TMEM70. Western blot analysis of TMEM70-GFP (A) and TMEM70-FLAG (B) expressed in fibroblast and HEK293 cells. (C) Alignment of human (NCBI GI: 34147498), rat (157823940), mouse (15030135) and bovine (148878159) TMEM70 protein sequence; arrow indicates predicted cleavage site of the TMEM70 precursor protein.

1 mM methionine, pH 7.4, [21] and 2 mg protein/mL of freshly isolated mitochondria. Incubation with ^{35}S labeled-translation product was performed for 30 min at 30 °C, in presence or absence of 4 μM FCCP. Indicated samples were treated with 0.16 mg trypsin/mL for 20 min on ice. Then 4 μM PMSF (phenylmethanesulfonyl fluoride) was added to all samples and mitochondria were sedimented and washed twice by centrifugation at 13,000*g* for 2 min at 4 °C. Samples were analyzed by SDS-PAGE and radioactivity was detected using BAS-5000 system (Fuji).

2.10. Ethics

The project was approved by the Scientific Ethics Committees of the 1st Faculty of Medicine of Charles University in Prague and Institute of Physiology, Academy of Sciences of the Czech Republic. Patient participation in the project was made on a voluntary basis after oral and written information and consent according to the Helsinki V Declaration.

3. Results and discussion

As shown in Fig. 1A, the *TMEM70-GFP* construct is well expressed in human fibroblasts or HEK293 cells yielding a protein band of about 46 kDa. The *TMEM70* gene encodes 260 amino acids protein of expected MW of 29.0 kDa and the tagged TMEM70-GFP protein of 260 + 238 amino acids has calculated MW of 55.9 kDa. The difference between calculated and observed size of the TMEM70-GFP is ~10 kDa and corresponds well with predicted cleavable N-terminal sequence of 81 amino acids (Fig. 1C). Similar experiment using *TMEM70-FLAG* construct (Fig. 1B) revealed the size of expressed protein of approximately 22 kDa and thus both tagged forms of the TMEM70 supported the conclusion that this protein is synthesized as a precursor that is processed into a ~21 kDa mature form of 179 AA.

To obtain specific antibodies to TMEM70 protein, we have expressed mature part of TMEM70 in a form of a fusion protein with maltose binding protein (MBP-TMEM70) in *E. coli*. Resulting protein was isolated and used for immunization. As shown by SDS-PAGE/WB analysis in Fig. 2, the anti-TMEM70 antibody recognized a

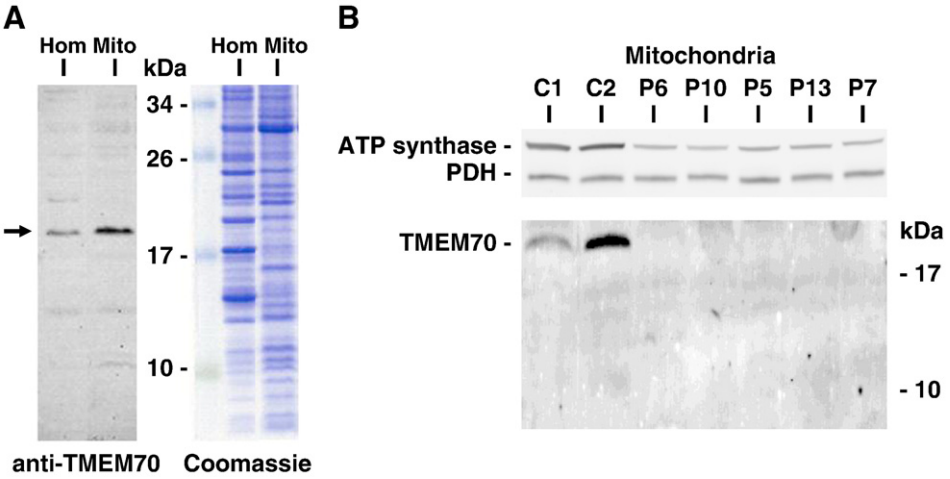

Fig. 2. Mature TMEM70 of ~21 kDa is absent in patients with ATP synthase deficiency. (A) Western blot detection of TMEM70 by polyclonal antibody in human heart homogenate and mitochondria. (B) Western blot detection of ATP synthase (β subunit), PDH (E1 α subunit) and TMEM70 in fibroblast mitochondria from control (C1, C2) and indicated patients (P) with *TMEM70* c.317-2A>G mutation.

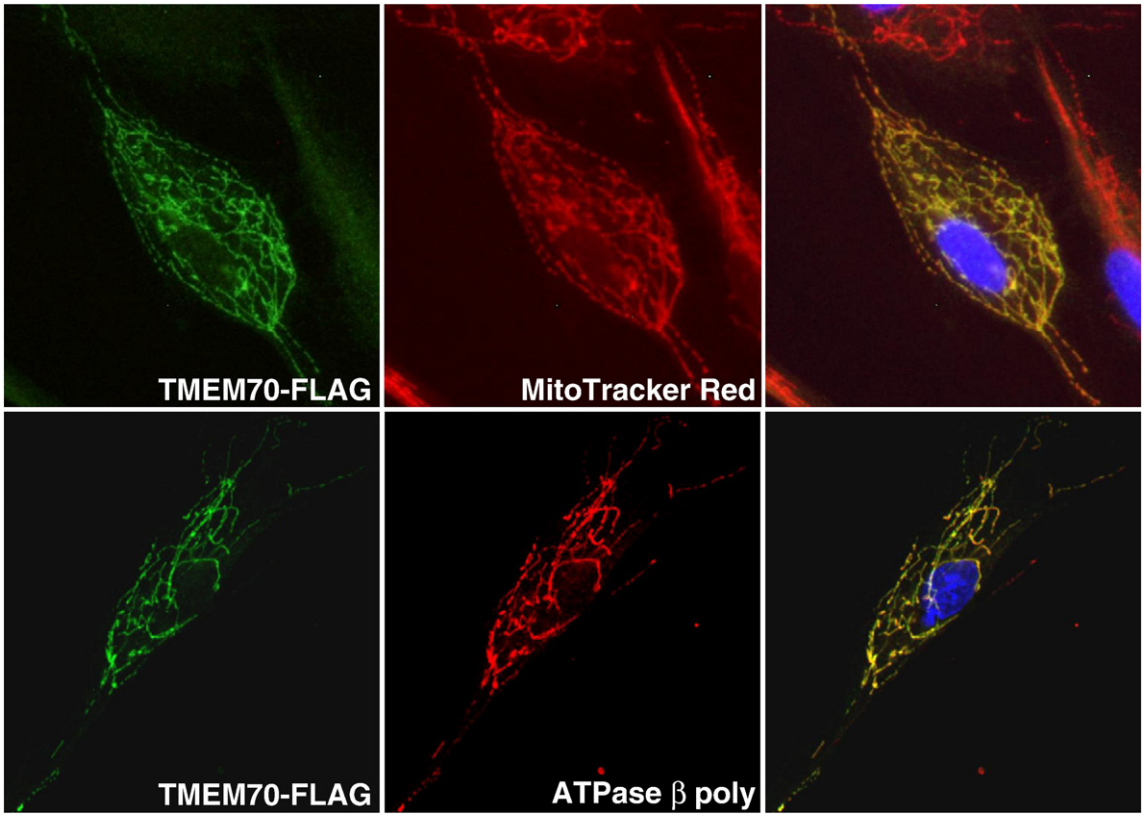

Fig. 3. Subcellular localization of TMEM70-FLAG with respect to MitoTracker Red or ATP synthase in human fibroblasts. Last column represents an overlay of the first two columns and shows cell nuclei stained with DAPI (blue).

20 kDa band in tissue homogenate of control human heart that was concentrated in isolated mitochondria (10,000*g*). The immunoreactive band was absent in fibroblast mitochondria (Fig. 2B) of the patients with ATP synthase deficiency due to the homozygous *TMEM70* mutation c.317-2A>G. This mutation at the second intron of TMEM70 gene has been shown to results in aberrant splicing and loss of *TMEM70* mRNA [10]. WB with anti-TMEM70 antibody thus confirmed previous conclusion that normal TMEM70 protein is absent in patient mitochondria. There was no immunoreactive band at lower molecular weight region indicating that no aberrant TMEM70 protein is produced in patient cells.

The presence of TMEM70 protein was also verified by mass spectrometry. LC-MS/MS analysis performed on mitochondria, whole tissue homogenates or 100,000*g* microsomal fraction from human heart and mouse heart or liver did not detect the TMEM70 protein. However, when a targeted approach was used (Supplementary Fig. S1), based on the knowledge of the retention time, precise mass and fragmentation spectrum of the human TMEM70 protein (MS/MS analysis of MBP-humanTMEM70 fusion protein), TMEM70 HVFTTFYAK tryptic peptide that is not present in any other human protein was found in the approximately 18–23 kDa region sample of isolated human heart mitochondria. MS analysis thus confirmed mitochondrial location of TMEM70 and further indicated that the cellular content of the TMEM70 is very low.

This conclusion is also supported by existing expression profile data (http://biogps.gnf.org/), which show that the level of *TMEM70* transcripts is extremely low in human cells and tissues (Supplementary Fig. S2), being several orders of magnitude lower than the level of mRNAs for structural subunits of ATP synthase (e.g. *ATP5B* mRNA for F_1 β subunit). The *TMEM70* mRNA levels are thus similarly low as the levels of transcripts of *ATPAF1* and *ATPAF2* assembly factors [1], the content of which is very small in mammalian tissues (Supplementary Fig. S2 and [22]). Furthermore, *TMEM70* transcripts show very small variation among various tissues, similarly as *ATPAF2*, characteristic for housekeeping genes. All these data thus support the view that the TMEM70 protein, ancillary factor of ATP synthase biogenesis is a low abundant mitochondrial protein, not exerting tissue-specificity.

Cellular localization of TMEM70 protein was further analyzed at a morphological level in cultured fibroblasts. For experiments the cells transfected with tagged *TMEM70* or control cells expressing the *wtTMEM70* were used. As shown in Fig. 3, in cells transfected with

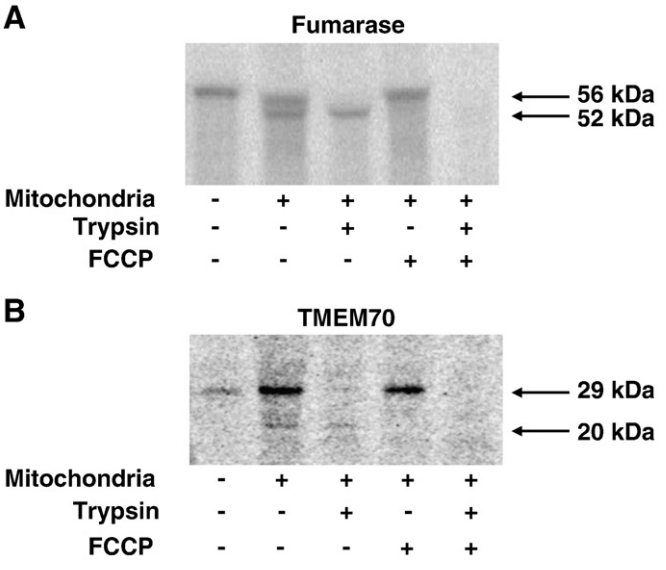

Fig. 4. Import of TMEM70 to mitochondria. *In vitro* translated human fumarase (A) and TMEM70 (B) were processed and imported to isolated rat liver mitochondria. Upon the import, the mature forms of both proteins resisted to trypsin and their import was prevented by uncoupler of oxidative phosphorylation FCCP.

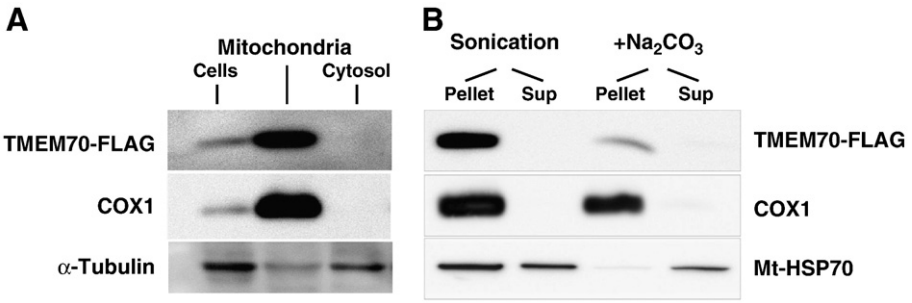

Fig. 5. TMEM70 is a membrane bound mitochondrial protein. HEK293 cells were transfected with *TMEM70-FLAG* and (A) cell homogenate, isolated mitochondria and cytosolic fractions were analyzed. (B) Isolated mitochondria were sonicated and extracted with Na_2CO_3 and 100,000*g* pellet and supernatant were prepared. Western blot was performed with indicated antibodies.

TMEM70-FLAG the tagged TMEM70 signal detected by anti-FLAG antibody colocalized with the signal of MitoTracker Red and with the signal of mitochondrial ATP synthase, detected with antibody to β subunit of the F_1 catalytic part, in accordance with previously demonstrated mitochondrial localization of TMEM70-GFP [13]. Antibody to TMEM70 protein also confirmed mitochondrial localization in control cells, but it was less specific in immunocytochemical experiments (not shown).

To assess further the basic properties of TMEM70 import to mitochondria, we have synthesized radioactive TMEM70 *in vitro* using coupled transcription-translation system and followed its import to isolated mitochondria. For comparison, we analyzed import of matrix located fumarase, as an example of mitochondrial protein that is synthesized with cleavable N-terminal sequence. As shown in Fig. 4A, the import analyses with isolated liver mitochondria showed a 56 kDa precursor and a 52 kDa mature form of fumarase, with expected sensitivity to trypsin and/or uncoupler. Analysis of TMEM70 protein revealed ~29 kDa band produced by *in vitro* translation and additional band of ~20 kDa present in the mitochondrial pellet. The 29 kDa band was sensitive to protease indicating its extramitochondrial localization typical for a precursor form. The 20 kDa band was resistant to protease but it disappeared in the presence of uncoupler, thus confirming the intramitochondrial localization of the TMEM70 mature form. Interestingly, there was only small amount of the mature 20 kDa protein found relative to the amount of precursor added. These data indicate that upon cleavage of the N-terminal part, the mature TMEM70 is rather labile at the conditions of *in vitro* import assay, or that additional cellular components are required for its import *in vivo*. However, similar pattern was observed when using mitochondria of human origin isolated from HEK293 cells or in co-translational import assay.

To characterize further the mitochondrial localization of TMEM70, we have investigated HEK293 cells expressing the *TMEM70-FLAG* construct, which is only slightly larger than the *wtTMEM70*. When we have isolated mitochondria from the cells expressing the TMEM70-FLAG protein, we found that the TMEM70-FLAG was fully recovered in mitochondria while it was absent in the cytosolic fraction (Fig. 5A). Then we fractionated the isolated mitochondria by sonication and treated the mitochondrial membranes with Na_2CO_3. Analysis of 100,000*g* pellet and supernatant by SDS-PAGE and WB (Fig. 5B) fully recovered the TMEM70-FLAG in the sediment, similarly as cytochrome *c* oxidase (COX), indicating its localization in the mitochondrial membrane. Efficiency of the treatments was verified by antibody to matrix located HSP70 that was quantitatively recovered in the soluble fractions.

Finally, to search for native conformation of TMEM70, we have extracted fibroblast mitochondria with mild detergent dodecyl maltoside (DDM) and analyzed the solubilized proteins by two-dimensional BN-PAGE/SDS-PAGE and WB. As demonstrated in Fig. 6, TMEM70 was found as two spots of identical mobility in the second

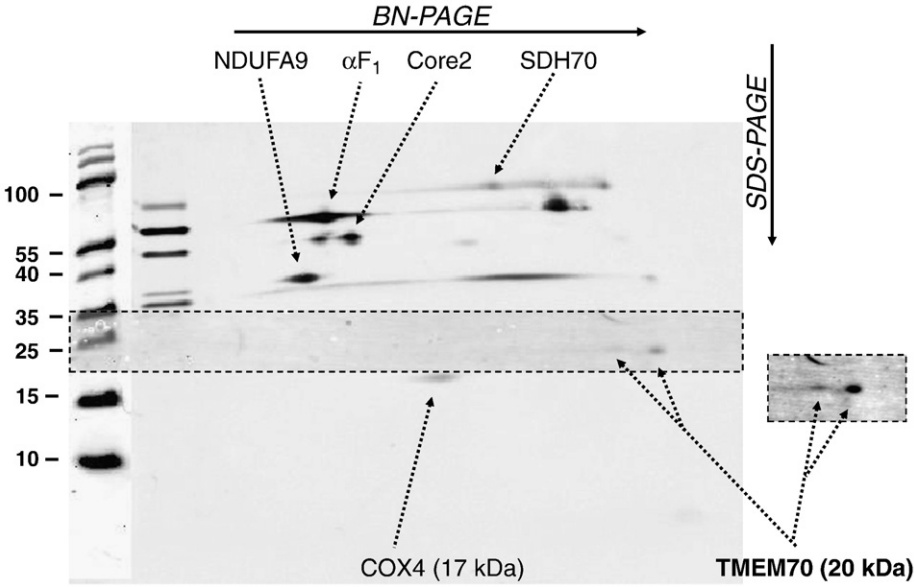

Fig. 6. DDM-solubilized mitochondrial proteins reveal a dimeric form of TMEM70. Fibroblast mitochondria were solubilized with DDM (1 g/g protein) and 30,000*g* supernatant was analyzed by two-dimensional electrophoresis (BN/SDS-PAGE) and WB using antibodies to indicated proteins. Dashed line frames the region probed with anti-TMEM70 antibody (higher intensity signal shown on the right).

dimension, corresponding to 21 kDa. Their mobility in the first dimension BN-PAGE gel was approximately 20 and 40 kDa. Parallel detection of ATP synthase and respiratory chain complexes did not reveal any association of TMEM70 with the assembled ATP synthase complex or free α or β subunits and indicated that TMEM70 exists in monomeric and dimeric forms when solubilized with detergent DDM.

Taken together, our experiments convincingly demonstrated that TMEM70 is a membrane bound 21 kDa mitochondrial protein that is synthesized as a 29 kDa precursor. TMEM70 is firmly associated with inner mitochondrial membrane and it does not interact directly with the ATP synthase complex. Very low cellular content of this protein, analogous to low content of ATPAF1 and ATPAF2 chaperones [23] supports the view of a regulatory — catalytic role of TMEM70 in ATP synthase biogenesis. It also indicates that putative interacting partner of TMEM70 might be similarly low-abundant protein(s), as are for example F_1 assembly intermediates [24]. A larger form of TMEM70 on two-dimensional gels appears to be a dimer according to calculated molecular weight, but we cannot exclude that it represents TMEM70 interacting with some other protein, although it cannot be the large ATP synthase subunits. Absence of TMEM70 in patient cells containing small amounts of functional ATP synthase complex [10,11] indicates that TMEM70 is not absolutely essential for ATP synthase biogenesis. Further studies are needed to elucidate the biological role of this factor. A unique specificity of TMEM70 to higher eukaryotes prevents the use of yeast cells, but the studies utilizing overexpression of TMEM70, various tagged forms as well as crosslinking and preparation of antibodies allowing for specific immunoprecipitation may represent a perspective strategy.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbabio.2010.10.005.

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Compensatory upregulation of respiratory chain complexes III and IV in isolated deficiency of ATP synthase due to *TMEM70* mutation

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ABSTRACT

Early onset mitochondrial encephalo-cardiomyopathy due to isolated deficiency of ATP synthase is frequently caused by mutations in *TMEM70* gene encoding enzyme-specific ancillary factor. Diminished ATP synthase results in low ATP production, elevated mitochondrial membrane potential and increased ROS production. To test whether the patient cells may react to metabolic disbalance by changes in oxidative phosphorylation system, we performed a quantitative analysis of respiratory chain complexes and intramitochondrial proteases involved in their turnover. SDS- and BN-PAGE Western blot analysis of fibroblasts from 10 patients with *TMEM70* 317-2A>G homozygous mutation showed a significant 82–89% decrease of ATP synthase and 50–162% increase of respiratory chain complex IV and 22–53% increase of complex III. The content of Lon protease, paraplegin and prohibitins 1 and 2 was not significantly changed. Whole genome expression profiling revealed a generalized upregulation of transcriptional activity, but did not show any consistent changes in mRNA levels of structural subunits, specific assembly factors of respiratory chain complexes, or in regulatory genes of mitochondrial biogenesis which would parallel the protein data. The mtDNA content in patient cells was also not changed. The results indicate involvement of posttranscriptional events in the adaptive regulation of mitochondrial biogenesis that allows for the compensatory increase of respiratory chain complexes III and IV in response to deficiency of ATP synthase.

1. Introduction

Isolated deficiency of ATP synthase belongs to autosomally transmitted mitochondrial diseases that typically affect paediatric population and present with early onset and often fatal outcome [1]. Nuclear genetic origin of ATP synthase deficiency was first demonstrated in 1999 [2] and up to now more than 30 cases have been diagnosed. Within the last few years, mutations in *ATP12* (*ATPAF2*) [3] and *TMEM70* [4] genes, encoding two ATP synthase ancillary factors have been identified as a cause of the disease. Most recently we have found that a mutation in *ATP5E* gene coding for ATP synthase F_1 epsilon subunit can also downregulate enzyme biogenesis resulting in a mitochondrial disease [5]. While *ATP12* and *ATP5E* mutations remain limited to one unique described case, mutations in *TMEM70* were present in numerous patients [4,6–9], thus representing the most frequent cause of ATP synthase deficiency. Up to now at least 8 different pathogenic mutations have been found in *TMEM70* gene [4,8–10]; however, most of the patients are homozygous for *TMEM70* 317-2A>G mutation thus forming a unique cohort of cases with an isolated defect of the key enzyme of mitochondrial ATP production, harboring an identical genetic defect.

TMEM70 is a 21 kDa mitochondrial protein of the inner mitochondrial membrane [11] synthesized as a 29 kDa precursor. It functions as an ancillary factor of mammalian ATP synthase biogenesis [12], and is uniquely specific for higher eukaryotes [4,13]. Its absence caused by the homozygous substitution in *TMEM70* gene (317-2A>G) results in an isolated decrease of the content of fully assembled ATP synthase and reduction of enzyme activity to less than 30% of control values. The clinical presentation of affected patients includes the early onset, lactic acidosis, frequent cardiomyopathy, variable CNS involvement and 3-methylglutaconic aciduria [1,2,4,14].

Diminished phosphorylating capacity of ATP synthase, with respect to respiratory chain capacity, results in low ATP production and insufficient discharge of mitochondrial proton gradient. Elevated levels of mitochondrial membrane potential ($\Delta\Psi_m$) thus stimulate mitochondrial ROS production and the overall metabolic disbalance is characterized by insufficient energy provision and increased oxidative stress in ATP synthase-deficient patient cells [1,15].

Abbreviations: OXPHOS, oxidative phosphorylation; ATP synthase, mitochondrial F_oF_1 ATPase; DDM, dodecyl maltoside; COX, cytochrome *c* oxidase; RT-PCR, real-time PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

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Assuming that these metabolic changes may influence the nucleo-mitochondrial signaling, in the present study we tested whether the patient cells may respond to ATP synthase deficiency and consequent metabolic disbalance by changes in biogenesis of mitochondrial OXPHOS system. To investigate possible compensatory/adaptive changes, we performed quantitative analysis of mitochondrial respiratory chain complexes I–V and intramitochondrial proteases (Lon protease, paraplegin, and prohibitins), quantified mtDNA content and compared the protein analysis data with the data from gene expression profiling analyses.

2. Materials and methods

2.1. Patients

Fibroblast cultures from 10 patients with isolated deficiency of ATP synthase (P4–P13 [4]) and 3 controls were used in this study. All the patients showed major clinical symptoms associated with ATP synthase deficiency and harbored a homozygous substitution 317-2A>G in gene *TMEM70* [4]. Relevant clinical, biochemical and molecular data on individual patients included in this study were described previously (see [2,4,6,14,16]).

2.2. Cell culture and isolation of mitochondria

Fibroblast cultures were established from skin biopsies and were grown at 37 °C in 5% (v/v) CO_2 atmosphere in high-glucose Dulbecco's modified Eagle's medium (DMEM, PAA) supplemented with 10% (v/v) fetal calf serum. When indicated, cultivation was also performed in DMEM without glucose (Sigma) that was supplemented with 5.5 mM galactose and 10% dialyzed fetal calf serum. Cells were harvested with 0.05% trypsin and 0.02% EDTA and washed twice with phosphate-buffered saline (PBS, 8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4). The protein content was measured by Bio-Rad Protein Assay (Bio-Rad Laboratories), using BSA as standard.

Mitochondria were isolated as in [5] by hypotonic shock cell disruption [17].

2.3. mtDNA quantification

Genomic DNA was isolated by QIAamp DNA Mini kit (Qiagen). To quantify the mtDNA content, we selected two mitochondrial target sequences—16S rRNA and D-loop, and GAPDH as a nuclear target. RT-PCR (LightCycler 480 instrument, Roche Diagnostics) was performed with SYBR Green Master kit (Roche) using the following primers—16S (5′ -3′): F-CCAAACCCACTCCACCTTAC, R-TCATCTTTCCCTTGCGGTA; D-loop: F-CACCATCCTCCGTGAAATCAA, R-GCGAGGAGAGTAGCACTCTTGTG; GAPDH: F-TTCAACAGCGACACCCACT, R-CCAGCCACTACCAGGAAAT [18]. The mtDNA content was calculated from threshold cycle (C_T) ratio of C_{TmtDNA}/C_{TnDNA}.

2.4. Electrophoresis and immunoblot analysis

Tricine SDS polyacrylamide gel electrophoresis (SDS-PAGE) [19] was performed on 10% (w/v) polyacrylamide slab minigels (Mini Protean, Bio-Rad). The samples were incubated for 20 min at 40 °C in 2% (v/v) mercaptoethanol, 4% (w/v) SDS, 10 mM Tris-HCl (pH 7.0) and 10% (v/v) glycerol. Bis-Tris blue-native electrophoresis (BN-PAGE) was performed on 4–13% polyacrylamide minigels [20]. Fibroblasts were solubilized by dodecyl maltoside (DDM, 2 g/1 g of protein) for 15 min at 4 °C in 1.75 mM 6-aminohexanoic acid, 2 mM EDTA, 75 mM Bis-Tris (pH 7). Samples were centrifuged for 20 min at 30000 *g* and 4 °C, Coomassie Brilliant Blue G-250 (8:1, DDM:dye) and 5% glycerol were added to the supernatants and electrophoresis was run for 30 min at 45 V and then at 90 V at 4 °C.

The separated proteins were blotted onto PVDF membranes (Immobilon-P, Millipore) by semi-dry electro transfer for 1 h at 0.8 mA/cm^2. The membranes were blocked with 5% (w/v) non-fat milk in TBS, 0.1% (v/v) Tween-20 and then incubated for 2 h or overnight with subunit specific antibodies. We used monoclonal antibodies from Mitosciences against complex I (NDUFA9-MS111, NDUFS3-MS112), complex II (SDH70-MS204), complex III (Core1-MS303, Core2-MS304), complex IV (Cox1-MS404, Cox2-MS405, Cox4-MS408, Cox5a-MS409), complex V-ATP synthase (F1-β-MS503), and against porin (MSA03). For detection of proteases, the polyclonal antibodies to Lon (kindly provided by Dr. E. Kutejova), paraplegin (kindly provided by Dr. T. Langer), prohibitin 1 (Lab Vision/NeoMarkers) and prohibitin 2 (Bethyl, A300-657A) were used. Quantitative detection was performed using infrared IRDye®-labeled secondary antibodies (goat anti-mouse IgG, Alexa Fluor 680 (A21058) and goat anti-rabbit IgG, Alexa Fluor 680 (A21109), Invitrogen) and Odyssey Infrared Imager (Li-Cor); the signal was quantified by AIDA 3.21 Image Analyzer software (Raytest).

2.5. Gene expression analysis

RNA isolations and RNA quality control were performed as previously described [4]. Total RNA (500 ng) was reverse transcribed, labeled and hybridized onto Agilent 44 k human genome microarray using Two-color Microarray Based Gene Expression Analysis Kit (Agilent). Patient samples and controls (Cy5-labeled) were hybridized against common Cy3-labeled reference RNA isolated from HeLa cell lines. The hybridized slides were scanned with Agilent scanner with PMT gains adjusted to obtain highest intensity unsaturated images. Gene PixPro software (Axon Instruments) was used for image analysis of the TIFF files generated by the scanner. Comparative microarray analysis was performed according to MIAME guidelines [21]. Normalization was performed in R statistic environment (http://www.r-project.org) using Limma package [22], a part of Bioconductor project (http://www.bioconductor.org). Raw data from individual arrays were analyzed as one color data and processed using loess normalization and normexp background correction. Quantile was used for normalization between arrays. Linear model was fitted for each gene given a series of arrays using lmFit function. The empirical Bayes method was used to rank differential expression of genes using eBayes function. Multiple testing correction was performed using the method of Benjamini and Hochberg [23].

2.6. Data accession

Expression data reported in this study are stored and available in Gene Expression Omnibus repository under accessions GPL4133 and GSE10956.

2.7. Protein/transcript correlation

Gene expression signals were background corrected, log2 transformed and normalized using the quantile normalization method. Relative protein levels (ratio to porin) were mean centered, averaged and log2 transformed. For all possible pairs of genes and proteins, we calculated the Pearson correlation coefficient and its significance levels using correlation test function in R statistical language.

2.8. Ethics

This study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Committees of Medical Ethics at both collaborating institutions. The informed consent was obtained from parents.

3. Results

3.1. Changes in the content of mitochondrial OXPHOS complexes and mtDNA

To analyze possible changes in mitochondrial OXPHOS system, we determined by SDS-PAGE and Western blotting the protein content of individual OXPHOS complexes in homogenates of fibroblasts from 10 patients with ATP synthase deficiency caused by *TMEM70* mutation and from 3 healthy controls. We used monoclonal antibodies to selected subunits of ATP synthase (subunit β), complex I (NDUFA9, NDUFS3), complex II (SDH70), complex III (Core1, Core2) and complex IV (Cox1, Cox2, Cox4, and Cox5a). The signal of each subunit was normalized to the signal of porin and expressed as percentage of controls. As shown in Fig. 1, the average content of ATP synthase decreased to 18% of the controls. In contrast, the content of respiratory chain complexes I, II, III, and IV accounted for 115%, 125%, 133%, and 163% of the controls, using antibodies to NDUFA9, SDH70, Core1, and Cox5a, respectively. Analogous differences were observed when the immunodetection data were calculated per mg of protein (102%, 123%, 150%, and 170% of the controls). A similar pattern of changes was found when using antibodies to other subunits of these complexes (Table 1A). The calculation from each subunit signal data thus revealed a significant increase to 124–133% of the control in complex III subunits and to 150–262% in complex IV subunits. Only detection of the subunit Cox4 behaved differently than other complex IV subunits, and the change of its content was small and insignificant.

The changes in complex IV and complex III content revealed by SDS-PAGE were also observed at the level of assembled OXPHOS complexes resolved by BN-PAGE of DDM-solubilized mitochondrial proteins (Fig. 2, Table 1B). Cytochrome *c* oxidase detected with Cox1 antibody was increased to 184% of the control. The bc_1 complex detected with Core 1 antibody was increased to 153% of the control. There was also a tendency of increase in the complex I content but the difference was not significant due to a large variation of values. The content of complex II that accounted for 101% of the control was the least varying one. When the relative ratio of complexes III, IV and V to complex II was calculated and compared to control, the complex V was decreased to 11% and complexes III and IV were increased to 130% and 181%, respectively.

We have also performed cultivation of fibroblasts in galactose medium lacking glucose to increase their dependence on oxidative metabolism. As a result, the growth of patient fibroblasts with *TMEM70* mutation progressively declined and after 2–3 passages they stopped growing. SDS-PAGE and Western blotting did not reveal any significant change in respiratory chain complexes I–IV, due to the change in cultivation conditions, there was also no change in the low content of complex V (not shown).

Table 1
Protein content of OXPHOS subunits (A), OXPHOS complexes (B) and mitochondrial proteases (C) in fibroblasts of patients with *TMEM70* mutation expressed in % of control. Data are mean ± SD and *p*-values are determined by *t*-test (*p*-values <0.05 are in bold).

A

Complex	Subunit	% control ± SD	*p*-value
ATP synthase	$F_1\beta$	18 ± 5.5	**9.4121E−08**
Complex I	NDUFA9	115 ± 29.2	0.4623
Complex I	NDUFS3	110 ± 35.2	0.7369
Complex II	SDH70	125 ± 28.1	0.2747
Complex III	Core 1	133 ± 20.2	**0.0309**
Complex III	Core 2	124 ± 16.6	**0.0477**
Complex IV	Cox1	150 ± 21.1	**0.0084**
Complex IV	Cox2	262 ± 74.0	**0.0052**
Complex IV	Cox4	107 ± 30.0	0.7243
Complex IV	Cox5a	163 ± 21.6	**0.0003**

B

Complex	Antibody to	% control ± SD	*p*-value
ATP synthase	$F_1\beta$	11 ± 4.8	**0.0001**
Complex I	NDUFA9	126 ± 54.4	0.3150
Complex II	SDH70	101 ± 14.9	0.9799
Complex III	Core 1	153 ± 70.6	**0.0463**
Complex IV	Cox1	184 ± 83.6	**0.0207**

C

Protein	% control ± SD	*p*-value
Lon protease	136 ± 61	0.4516
Paraplegin	104 ± 27.6	0.9793
Prohibitin 1	99 ± 36.1	0.2763
Prohibitin 2	90 ± 10.7	0.8514

When determining the content of mtDNA in patient and control fibroblasts, we found that the mtDNA copy number reflected by mtDNA/nDNA ratio is not influenced by the *TMEM70* mutation (Table 2).

3.2. Changes in mitochondrial proteases

When the ATP synthase assembly is impaired due to *TMEM70* mutation, the unused subunits of ATP synthase have to be degraded by mitochondrial quality control system [24]. Therefore, we analyzed the protein content of several components of mitochondrial proteolytic machinery in fibroblast mitochondria by Western blotting using polyclonal antibodies to matrix Lon protease that degrades misfolded, unassembled and oxidatively damaged matrix proteins [25], paraplegin, subunit of the inner membrane mAAA protease that degrades membrane spanning and membrane associated subunits of respiratory chain complexes [26] and prohibitins 1 and 2—regulatory proteins of AAA proteases [27]. As shown in Fig. 3, the changes in the

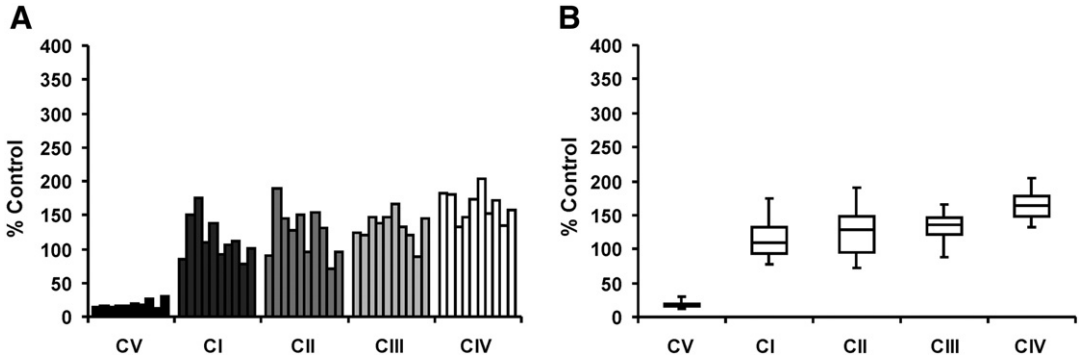

Fig. 1. The protein content of respiratory chain complexes in fibroblasts with *TMEM70* mutation. SDS-PAGE and Western blot analysis of fibroblasts were performed using subunit specific monoclonal antibodies. Detected signals were normalized to mitochondrial marker porin and expressed in % of control values. A—Values for each patient cell line. B—Statistical analysis of 10 patient cell lines, box plot represents maximum, Q3, median, Q1, and minimum.

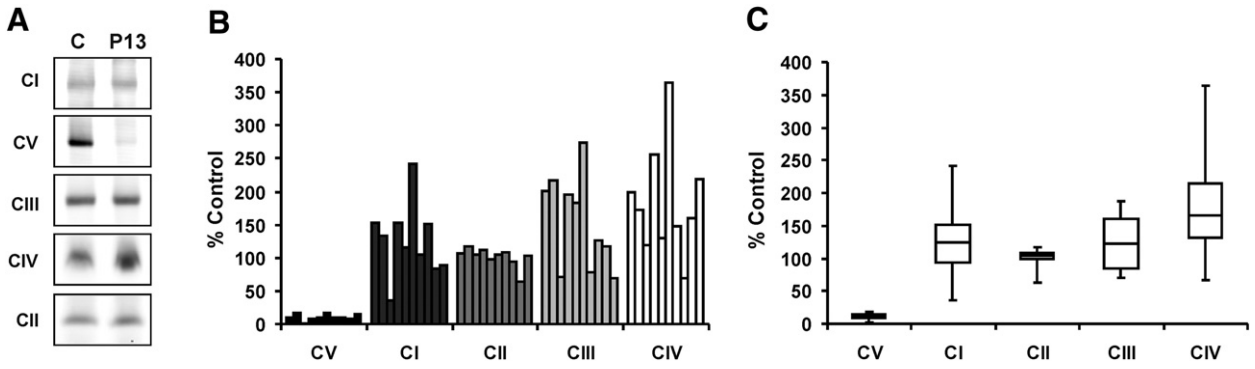

Fig. 2. The content of native respiratory chain complexes in fibroblasts with *TMEM70* mutation. BN-PAGE and Western blot analysis of DDM-solubilized fibroblasts were performed using subunit specific monoclonal antibodies. A—Analysis of control and P13 patient fibroblasts is shown. Detected signals were expressed in % of control values. B—Values for each patient cell line. C—Statistical analysis of 10 patient cell lines, box plot represents maximum, Q3, median, Q1, and minimum.

content of mitochondrial ATP-dependent proteases and prohibitins did not reveal significant differences in patient cells as compared with the controls. In case of Lon protease we observed only a moderate increase of about 30%; however, this difference was also not statistically significant.

3.3. Changes in mRNA expression profiles

To assess putative changes in transcriptional activity of genes involved in mitochondrial biogenesis, we analyzed mRNA expression profiles using whole genome (44 k) array. From the whole dataset, 51% gene spots provided a signal of sufficient quality to be used for microarray analysis. Out of this subset, there were 2700 genes upregulated and 525 downregulated in patient cells as compared to controls at unadjusted $p<0.05$. At adjusted $p<0.01$, 104 and 2 genes were upregulated and downregulated, respectively.

The generalized upregulation of transcriptional activity in patient cells indicates their tendency to increase the levels of majority of transcripts. However, only a small number of the significantly upregulated genes were associated with OXPHOS metabolism. No significant differences in expression levels of the genes encoding ATP synthase subunits and ATP synthase assembly factors were found; the only exception was *TMEM70* gene whose transcript in patient cells was decreased by a factor of 4.6 (adj. $p<0.001$). Furthermore, in case of other genes encoding OXPHOS subunits only the expression of 6 genes differed from the controls (unadjusted $p<0.05$, Table 3). Specifically, 3 subunits of complex I, 1 subunit of complex II, and 2 subunits of complex III, revealed slight (1.2–1.6 fold change) but non-consistent changes, with up- and downregulation observed for genes encoding different subunits of the same complex.

Among differentially expressed genes two pro-mitochondrial regulatory genes, the *TFAM* and *PPRC1* participating in mitochondrial biogenesis were 1.3 fold upregulated, while the COX-specific SCO2 assembly factor was downregulated. The expression profiling did not reveal any changes in mitochondrial proteases or other components of the mitochondrial quality control system.

3.4. Correlation of expression profiling and protein amount

In the investigated group of OXPHOS genes (structural subunits or specific assembly factors) we did not find any correlation between mRNA and protein levels. Even in case of complexes IV and III, with significantly increased protein content, no parallel significant changes in mRNA levels were found in corresponding nuclear or mtDNA encoded genes. The results indicate that ATP synthase deficiency-induced changes in respiratory chain complexes are not related with corresponding changes in the transcriptional activity of the genes involved in OXPHOS biogenetic machinery.

Table 2
The content of mtDNA in patient fibroblasts, determined as a ratio between mtDNA (16S or D-loop) and nDNA (GAPDH), expressed in % of control.

mtDNA gene/nDNA gene	% control ± SD
16S/GAPDH	100.21 ± 1.41
D-loop/GAPDH	101.00 ± 1.95

4. Discussion

The aim of our study was to investigate possible compensatory/adaptive changes of mitochondrial OXPHOS system in a unique group of fibroblast cell lines from 10 patients with an identical mutation in *TMEM70* gene, downregulating specifically the ATP synthase (complex V) content and function. We found a pronounced and significant increase in cellular protein content of the subunits of respiratory chain complex III and complex IV, in accordance with our previous analysis of one of the patients [16]. The increase of complex IV subunits was found in mtDNA encoded subunits Cox1 and Cox2 and in nuclear encoded subunit Cox5a, ranging 150–262% of the control, whereas Cox4 protein showed only a small increase. Complex III was increased in Core1 and Core2 subunits and accounted for 125–133% of the control. BN-PAGE analyses further showed that these changes reflected a corresponding increase to 153% and 184% in assembled respiratory complexes III and IV, respectively.

Changes in the content, morphology, or cellular localization of mitochondria, as well as secondary changes in the content of some of mitochondrial OXPHOS complexes are often observed in mitochondrial disorders. Good examples are ragged-red muscle fibers of MERRF patients with mtDNA 8344A>G mutation in the $tRNA^{Lys}$ gene [28], mitochondrial cardiomyopathies [29] or AZT-induced mtDNA depletion [30]. However, little is known about the underlying adaptive mechanisms.

The pronounced isolated defect in one OXPHOS complex induced by identical homozygous autosomal mutation, such as ATP synthase deficiency studied here, represents an interesting model for investigation of possible adaptive changes. Despite methodical limitations of patient cell culture studies (differences in patients' age, number of passages in cell culture), the upregulation of complex IV and complex III could be demonstrated as an apparent consequence of complex V deficiency in patients with *TMEM70* 317-2A>G homozygous mutation [4], leading to the absence of the TMEM70 protein [11]. Interestingly, when we analyzed fibroblasts with ATP synthase deficiency due to mutation in *ATP5E* gene, we also observed elevated contents of complexes III and IV [5]. On the other hand, in case of ATP synthase deficiency due to *ATP12* mutation, BN-PAGE and in gel enzyme activity staining revealed unchanged content of respiratory chain complexes I, II and IV [3]. However, for each of these two mutations (*ATP5E*,

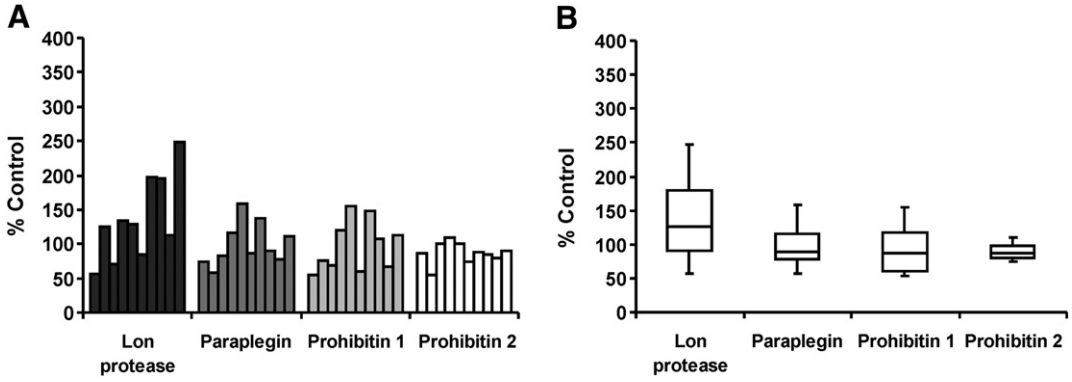

Fig. 3. The protein content of mitochondrial proteases in fibroblasts with *TMEM70* mutation. The content of Lon protease, paraplegin subunit of mAAA protease, prohibitin 1 and prohibitin 2 was analyzed in fibroblasts mitochondria by SDS-PAGE and WB using polyclonal antibodies. Detected signals in patient fibroblasts were normalized to protein content and expressed in % of controls. A—Values for each patient cell line. B—Statistical analysis of 10 patient cell lines, box plot represents maximum, Q3, median, Q1 and minimum.

ATP12) there has been only one case described so far, and, thus, these observations can hardly be generalized.

Mitochondrial OXPHOS complexes are formed independently by biogenetic processes with the help of numerous, complex-specific helper proteins [31,32]. The decreased content of ATP synthase could therefore influence biogenesis and assembly of other respiratory complexes only indirectly, possibly *via* changes in membrane potential ($\Delta\Psi_m$), adenine nucleotides levels or mitochondrial ROS production [1,15]. High values of $\Delta\Psi_m$, increased ROS production as well as low ATP production are also hallmarks of ATP synthase dysfunction due to mtDNA mutations in *ATP6* gene encoding subunit a of ATP synthase. The most pathogenic is T8993G missense mutation resulting in numerous NARP/MILS cases at high mutation load [33,34]. Pathogenic mechanism of T8993G mutation has been intensively studied in different tissues, fibroblasts and derived cybrids and while some authors observed increase of the respiratory chain complexes [35], others found no significant changes [36] or even decrease [34]. It is possible that this variability may reflect varying heteroplasmy of *ATP6* mutation; however, a nuclear genetic background of different patients should also be considered [37]. Our data also showed a pronounced variation of the adaptive responses at the level of complex IV and complex III in individual cell lines with homozygous *TMEM70* mutation, which may reflect differences in nuclear genome of individual cases, determining their potential to respond to underlying ATP synthase deficiency.

The extent of adaptive response could depend on the relative contribution of OXPHOS system to the overall energetics of fibroblasts, which is small as fibroblasts are largely glycolytic cells. However, we were not able to further increase upregulation of respiratory chain complexes in fibroblasts with *TMEM70* mutation by cultivating them in galactose medium in order to increase their oxidative metabolism. In fact, the viability of fibroblasts with *TMEM70* mutation was strongly impaired and they stopped growing in galactose medium. This would indicate that the lack of ATP synthase prevents sufficient ATP production when glycolysis was inhibited and that observed compensatory upregulation of respiratory chain complexes was, as expected, energetically unproductive.

It would be interesting to see whether the variation of data in fibroblast cell lines associates with the *in vivo* impairment of mitochondrial energetics and consequently with the clinical state of individual cases. Nevertheless, it is very problematic to link the changes in respiratory chain complexes in fibroblasts with the clinical presentation of *TMEM70* mutation as previous clinical studies of large number of patients revealed no real differences in the disease onset and severity of clinical symptoms and indicated that management of intensive care after the birth is crucial for patients' survival beyond the neonatal period [6].

Studies in yeast represent efficient strategy to investigate pathogenic mechanisms of human mitochondrial diseases, in particular various types of ATP synthase disorders [38]. Di Rago and colleagues created a yeast model of *ATP6* mutations and recent studies in *Saccharomyces cerevisiae* demonstrated both in ATP synthase-deficient or in oligomycin-inhibited cells that the content of complex IV selectively and rapidly decreases, due possibly to translational downregulation of Cox1 subunit caused by high $\Delta\Psi_m$ [39]. This is in sharp contrast with upregulation of complex IV in patient cells with *TMEM70* mutation. Apparently, the yeast and mammalian/human cells respond differently to dysfunction of ATP synthase and consequent increase of $\Delta\Psi_m$, due to differences in the mechanism and regulation of synthesis and assembly of mtDNA encoded Cox1. While Cox1 is synthesized in a precursor form in *S. cerevisiae* and its translation and processing depend on MSS51 [40], human Cox1 is not processed and its translation is controlled by two factors, TACO1, a specific translational activator that might have evolved in concert with the loss of the mitochondrial mRNA regulatory sequences that occurred with the extreme reduction in the size of

Table 3
OXPHOS genes expressed differently in patient and control cells (unadjusted $p<0.05$).

Gene ID	Gene	Gene name	M	Fold change	*p*-value
NM_004548	*NDUFB10*	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10, 22 kDa	−0.32	1.3	0.051
NM_005006	*NDUFS1*	NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75 kDa	0.28	1.2	0.039
NM_015965	*NDUFA13*	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 13	−0.31	1.2	0.046
NR_003266	*LOC220729*	Succinate dehydrogenase complex, subunit A	0.64	1.6	0.001
NM_001003684	*UCRC10*	Ubiquinol-cytochrome c reductase, complex III subunit X	−0.45	1.4	0.012
NM_003366	*UQCRC2*	Ubiquinol-cytochrome c reductase core protein II	0.34	1.3	0.025
NM_005138	*SCO2*	SCO cytochrome oxidase deficient homolog 2 (yeast)	−0.26	1.2	0.042
NM_017866	*TMEM70*	Transmembrane protein 70	−2.20	4.6	<0.001
NM_015062	*PPRC1*	Peroxisome proliferator-activated receptor gamma, coactivator-related 1	0.38	1.3	0.005
NM_003201	*TFAM*	Transcription factor A, mitochondrial	0.39	1.3	0.007

the metazoan mitochondrial genome [41] and C12orf62, a vertebrate specific, small transmembrane protein that is required for coordination of the Cox1 synthesis with the early steps of COX assembly [42].

The important finding of our study is that the observed adaptive response of mitochondrial biogenesis is probably enabled by post-transcriptional events that allow for an increased biosynthesis of two respiratory chain complexes without correspondingly increased mRNAs. Our attempts to link protein changes to transcriptional profiles did not show any direct correlation. Besides very low *TMEM70* transcript, only 9 genes encoding OXPHOS biogenesis proteins were differentially expressed, but only with a low significance, including two pro-mitochondrial regulatory genes participating in mitochondrial biogenesis, *viz TFAM* and *PPRC1* (PGC-1 related co-activator) that were 1.3-fold increased. As neither the mRNAs for multiple regulatory and/or assembly factors, nor for structural subunits of complexes III and IV were consistently increased, our data suggest that posttranscriptional, possibly translational regulation may be responsible for the adaptive changes observed. It is tempting to speculate that $\Delta\Psi_m$ and/or ROS may be the signals activating/stimulating this process and that the targets might be the factors such as the transcriptional activator TACO1 [41], metazoan specific LRPPRC protein implicated in regulation of stability and handling of mature mitochondrial mRNAs as part of a ribonucleoprotein complex [43], processing of ribosomal MRPL32 protein by mAAA protease [44] or alike, and that the observed adaptive responses may include increased stability of the mRNA coding for some subunits of upregulated OXPHOS complexes, or a longer half-life of the corresponding protein subunits, or both.

The pronounced isolated deficiency of one key complex of mitochondrial energy provision also represents an interesting model to study the function of mitochondrial biogenesis quality control system that determines the fate of all newly synthesized mitochondrial proteins and directly modulates mitochondrial translation [24,45]. Our analysis of mitochondrial proteases revealed that the elimination of unassembled subunits of ATP synthase is associated neither with the increased mitochondrial content of these proteases nor with upregulation of the respective transcripts. The degradation of excess subunits can be apparently maintained by a normal, steady state level of mitochondrial proteases. A moderate increase was only found in case of Lon protease.

Normal levels of transcripts for ATP synthase subunits observed in patient cells indicate that enzyme subunits are synthesized, whereas Western blot analysis showed that the unused subunits are effectively degraded by mitochondrial surveillance system. ATP synthase is one of the most abundant proteins of mammalian mitochondria and represents several percents of the total mitochondrial protein. However, based on analysis of mitochondrial proteases as well as expression profiling data, it appears that the capacity of mitochondrial quality control system is fully sufficient to degrade the orphan subunits of ATP synthase in cells with *TMEM70* mutation, which has been demonstrated by rapid degradation of newly synthesized beta F_1 subunit observed in patient fibroblasts [2]. This is in accordance with the view that up to 30% of newly synthesized nascent mitochondrial proteins are rapidly degraded owing to folding errors [46,47]. Efficient removal of excess subunits was also described in complex I disorder due to ND1 mutation [48], or in SDH deficiency in yeasts caused by the lack of SDH5 ancillary factor [49]. The efficacy of mitochondrial quality control and protein degradation pathway is also apparent from tissue specific downregulation of ATP synthase in brown fat, where the lack of the subunit c leads to a 10-fold decrease of ATP synthase complex without any accumulation of unassembled subunits, despite the fact that their mRNA levels are the highest among mitochondria-rich mammalian tissues [50]. Under conditions of high excess of unfolded proteins degraded to peptides, increase in the transcription of HSP60 or mtHSP70 can be triggered by upregulation of bZIP transcription factor ZC376.7 [51,52]. However, based on our expression profiling data, this does not seem to be the case in patient cells with *TMEM70* mutation.

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ARTICLE 4

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Mitochondrial ATP synthase deficiency due to a mutation in the *ATP5E* gene for the F_1 ε subunit

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F_1F_o-ATP synthase is a key enzyme of mitochondrial energy provision producing most of cellular ATP. So far, mitochondrial diseases caused by isolated disorders of the ATP synthase have been shown to result from mutations in mtDNA genes for the subunits ATP6 and ATP8 or in nuclear genes encoding the biogenesis factors TMEM70 and ATPAF2. Here, we describe a patient with a homozygous p.Tyr12Cys mutation in the ε subunit encoded by the nuclear gene *ATP5E*. The 22-year-old woman presented with neonatal onset, lactic acidosis, 3-methylglutaconic aciduria, mild mental retardation and developed peripheral neuropathy. Patient fibroblasts showed 60–70% decrease in both oligomycin-sensitive ATPase activity and mitochondrial ATP synthesis. The mitochondrial content of the ATP synthase complex was equally reduced, but its size was normal and it contained the mutated ε subunit. A similar reduction was found in all investigated F_1 and F_o subunits with the exception of F_o subunit c, which was found to accumulate in a detergent-insoluble form. This is the first case of a mitochondrial disease due to a mutation in a nuclear encoded structural subunit of the ATP synthase. Our results indicate an essential role of the ε subunit in the biosynthesis and assembly of the F_1 part of the ATP synthase. Furthermore, the ε subunit seems to be involved in the incorporation of subunit c to the rotor structure of the mammalian enzyme.

INTRODUCTION

Mitochondrial diseases caused by inborn defects in the mitochondrial ATP synthase (F_1F_o-ATP synthase, complex V) have been found less frequently than defects of the respiratory chain complexes; they are severe and affect predominantly the pediatric population (1).

The mitochondrial ATP synthase is a multisubunit complex composed of 16 different subunits (2) that form the globular, catalytic F_1 part connected by two stalks with the proton-translocating, membrane-spanning F_o part. Two subunits from the F_o part are encoded by the mitochondrial genome, subunits a and A6L (subunits 6 and 8) (3), whereas all the other ATP synthase subunits are encoded by nuclear genes. In addition, several other nuclear encoded proteins are specifically involved in the function and biogenesis of this enzyme. ATP synthase is regulated by the coupling factor B (4); two associated proteins MLQ (C14orf2) and AGP (5) are possibly involved in the formation of ATP synthase dimers and at least four other proteins, ATPAF1 (ATP11), ATPAF2 (ATP12), ATP23 and TMEM70, are supposed to take part in the biosynthesis and assembly of ATP synthase (6–9).

Mutations in both genomes were found to be responsible for different types of ATP synthase disorders. Maternally transmitted dysfunction of ATP synthase, known since 1990 (10), is mainly caused by mtDNA missense mutations in the *ATP6* gene (subunit a) (11). Several mutations of varying pathogenicity were described (see www.mitomap.org) to alter the function of the F_o proton channel and to result in the loss of ATP synthetic activity, whereas the hydrolytic activity and the amount of the enzyme are mostly retained.

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Patients present with neuropathy, ataxia, retinitis pigmentosa, maternally inherited Leigh syndrome, or bilateral striatal necrosis (11). More rarely, ATP synthase dysfunction can be caused by the lack of ATP6 protein due to altered processing of polycistronic *ATP8/ATP6/COX3* transcript (12,13) or by a mutation in the *ATP8* gene (14).

A distinct group of inborn defects of ATP synthase is represented by the enzyme deficiency due to nuclear genome mutations characterized by a selective inhibition of ATP synthase biogenesis (15). Biochemically, the patients show a generalized decrease in the content of ATP synthase complex which is <30% of the control. The insufficient ATP synthase phosphorylating capacity with respect to the respiratory chain results in an impaired energy provision and an increased ROS production due to an elevated mitochondrial membrane potential (1,16). The isolated ATP synthase deficiency appears to be a rather frequent mitochondrial disease, and more than 40 patients are known today. Most cases present with neonatal-onset hypotonia, lactic acidosis, hyperammonemia, hypertrophic cardiomyopathy, and 3-methylglutaconic aciduria. About a half of the patients die within few months or years (17–20). Since the demonstration of the nuclear gene involvement by cybrid complementation in 1999, pathogenic mutations were found in two genes, both coding for ancillary factors of ATP synthase biogenesis. In 2004, the first genetic defect was described in *ATPAF2* (*ATP12*), coding for a specific assembly factor of F_1 subunit α (21). The patient was homozygous for TGG-AGG missense mutation in exon 3 changing Trp94 to Arg, and presented with degenerative encephalopathy characterized by cortical and subcortical atrophy. Interestingly, this phenotype of marked brain atrophy differed significantly from other patients who lacked the *ATPAF2* mutation and presented with cardiomyopathy (18). In 2008, the expression profiling and homozygosity mapping identified a mutation in the second intron of *TMEM70* encoding a 30 kDa mitochondrial protein of unknown function, and ATP synthase-deficient patient fibroblasts were complemented by the wild-type TMEM70 protein (19). This protein turned out to be a novel ancillary factor of ATP synthase biosynthesis, interestingly, the first one specific for higher eukaryotes. The homozygous *TMEM70* c.317-2A>G mutation leading to aberrant splicing and to a loss of *TMEM70* transcript was found in 24 patients, and an additional patient was compound heterozygous for the c.317-2A>G and c.118_119insGT frameshift mutations (19). Since then, the *TMEM70* mutations were found in several other cases, constituting the most frequent cause of ATP synthase deficiency (20,22).

Nevertheless, one of the patients we analyzed had a distinct clinical phenotype of early-onset lactic acidosis, 3-methylglutaconic aciduria, no cardiac involvement, mild mental retardation, development of a severe peripheral neuropathy and survival to adulthood. In this case, neither *TMEM70* nor *ATPAF2* were affected, indicating the presence of a different genetic defect (P3 in reference 19).

RESULTS

Low ATP synthase activity in patient fibroblasts

As stated in our previous reports (18,23), the mitochondria of patient fibroblasts showed a normal activity of respiratory chain enzymes, but a decreased activity of mitochondrial ATP synthase (Table 1). A further analysis of patient fibroblasts showed a pronounced decrease of ATP synthase hydrolytic activity sensitive to oligomycin and aurovertin (67 and 77% decrease with respect to the control, respectively) (Fig. 1A). Likewise, the mitochondrial synthesis of ATP in the digitonin-permeabilized cells supplemented with ADP and respiratory substrates, using either pyruvate and malate or succinate, was decreased by 74 and 71% compared with the control, respectively (Fig. 1B). Therefore, a similar decrease in the synthetic and hydrolytic activity of ATP synthase was observed in the patient fibroblasts. Both types of measurements fully confirmed the presence of isolated defect of mitochondrial ATP synthase.

Table 1. Respiratory chain enzyme activities in frozen mitochondria isolated from the patient and control fibroblasts

Enzyme activities (mU/mg protein)	Patient	Controls
Citrate synthase	335	225–459
Complex I	16	15–52
Complex I + III	123	73–279
Complex II	98	64–124
Complex II + III	142	137–267
Complex III	861	208–648
Complex IV	370	202–403
Oligomycin-sensitive ATPase	<10	43–190

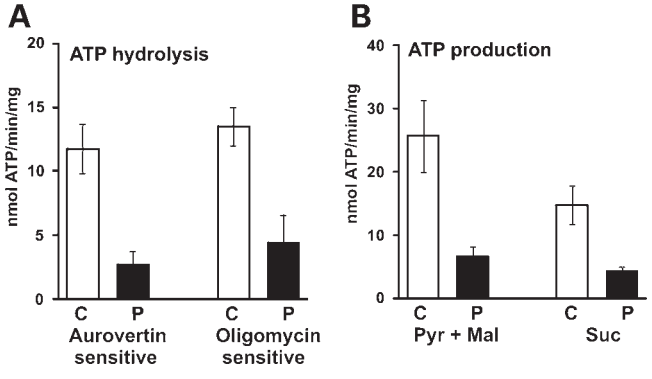

Figure 1. ATP synthase hydrolytic and synthetic activity. (**A**) Oligomycin- and aurovertin-sensitive hydrolysis was determined in the frozen/thawed fibroblasts. (**B**) ATP synthesis was measured in the digitonin-permeabilized fibroblasts supplied with respiratory substrates pyruvate (Pyr) + malate (Mal) or succinate (Suc). Values represent mean ± SD of four experiments, in control (C) and patient (P) cells.

Changes in cellular respiration and mitochondrial membrane potential

Respirometric measurements in the permeabilized patient fibroblasts supplemented with the combination of substrates glutamate, malate and succinate showed a pronounced decrease in the rate of ADP-stimulated respiration, although there was no apparent difference in FCCP-stimulated respiration compared with the control fibroblasts (Fig. 2A).

Cytofluorometric analysis of mitochondrial membrane potential ($\Delta\Psi_m$) in the permeabilized fibroblasts using tetramethylrhodamine methylester (TMRM) (Fig. 2B) revealed

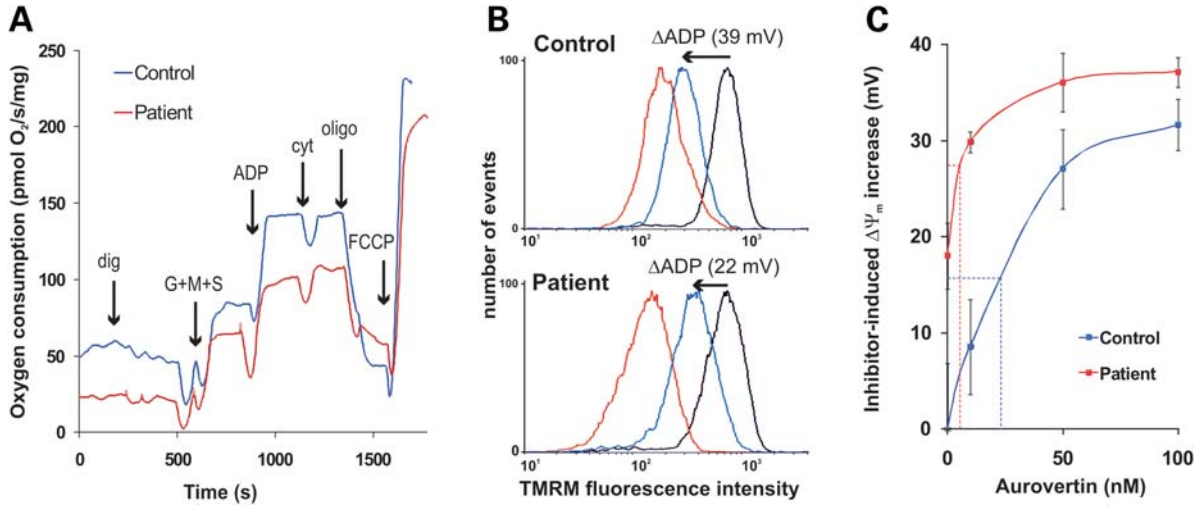

Figure 2. Respiration and mitochondrial membrane potential. (**A**) In the digitonin-permeabilized control (blue line) and patient (red line) fibroblasts, respiration with glutamate, malate and succinate (G + M + S) was measured in the presence of ADP, cytochrome *c* (cyt), oligomycin (oligo) and FCCP, as indicated. (**B**) Mitochondrial membrane potential ($\Delta\Psi_m$) was analyzed by TMRM cytofluorometry in the digitonin-permeabilized fibroblasts supplied with succinate (state 4, black line), after the addition of ADP (state 3-ADP, blue line) or FCCP (state 3-uncoupled, red line). (**C**) $\Delta\Psi_m$ at state 3-ADP was reversed back to state 4 by the inhibition of ATP synthase with aurovertin. Data show a titration with aurovertin in the control (blue line) and patient (red line) fibroblasts; the concentration of inhibitor for a 50% reversal is indicated.

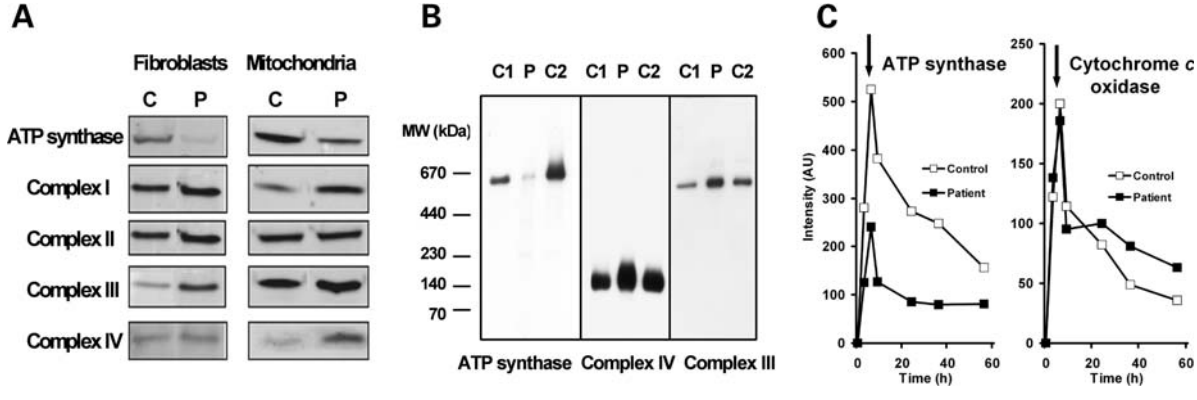

Figure 3. Specific content, size and biosynthesis of ATP synthase. (**A**) SDS–PAGE and WB analysis of ATP synthase and respiratory chain complexes in the control (C) and patient (P) fibroblasts (12 μg protein aliquots) and isolated mitochondria (3 μg protein aliquots), using antibodies to ATP synthase (F_1 α subunit), complex I (NDUFA9 subunit), complex II (SDH 70 kDa subunit), complex III (core 1 subunit) and complex IV (COX4 subunit). (**B**) BN-PAGE and WB analysis of DDM (1 g/g protein) solubilized proteins (10 μg protein aliquots) from the control (C1,C2) and patient (P) fibroblasts mitochondria, using antibodies to ATP synthase (F_1 α subunit), complex III (core1 subunit) and complex IV (COX1 subunit). The mobility of MW standards is shown on the left. (**C**) Metabolic labeling of ATP synthase and COX in cultivated fibroblasts. Fibroblasts were pulse-labeled with ^{35}S-methionine for 6 h and then chased with cold methionine (arrow). At the indicated times, the proteosynthesis was stopped by chloramphenicol and cycloheximide and mitoplasts isolated from the harvested cells were solubilized with DDM and subjected to 2D BN/SDS–PAGE. The radioactivity was quantified in assembled ATP synthase (subunits F_1 α + β) and COX (subunits COX2 + COX3 + COX4).

similar values of $\Delta\Psi_m$ at state 4 in the patient and control fibroblasts. In contrast (Fig. 2B), there was a much smaller decrease of $\Delta\Psi_m$ after an addition of ADP (i.e. at state 3-ADP) in the patient cells (20.29 ± 3.43 and 38.31 ± 6.85 mV in patient and controls, respectively), when mitochondria phosphorylate the added ADP at the expense of $\Delta\Psi_m$. In both patient and control cells, the inhibition of ATP synthase by aurovertin (or oligomycin) fully restored the $\Delta\Psi_m$ to the state 4 values. However, the titration of $\Delta\Psi_m$ at state 3-ADP with aurovertin showed a several-fold higher sensitivity of patient fibroblasts (Fig. 2C). Thus, all these functional measurements indicate that the patient cells possess an insufficient capacity of ATP synthase in relation to the capacity of respiratory chain.

Decreased content of ATP synthase complex

To determine the protein amount of ATP synthase and respiratory chain enzymes, SDS–polyacrylamide gel electrophoresis (PAGE) and western blot (WB) analysis was performed in both fibroblasts and isolated mitochondria. As shown in Fig. 3A, there was a pronounced decrease of ATP synthase

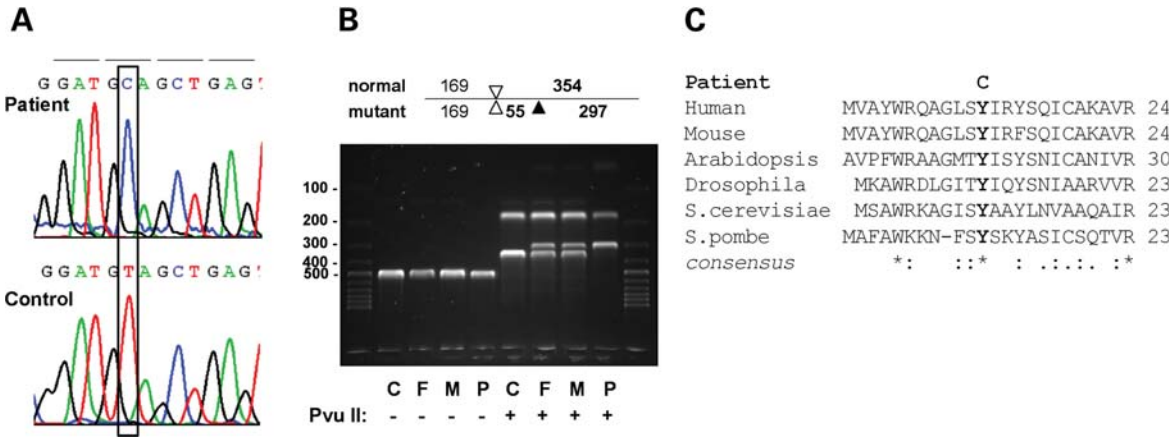

Figure 4. Presence of missense mutation c.35A>G in exon 2 of *ATP5E*, which leads to the amino acid exchange p.Tyr12Cys in the N-terminal conserved region of the subunit ε of ATP synthase. (**A**) Sequence analysis of the reverse complement cDNA of the patient (upper panel) and a control (lower panel). (**B**) RFLP analysis of genomic DNA isolated from the fibroblasts of control (C), father (F), mother (M) and patient (P). (**C**) Sequence alignment of the subunit ε from different organisms shows high conservation of the tyrosine 12 of the human protein.

F_1 α subunit content in the fibroblast homogenate corresponding to 70% reduction of the content of ATP synthase complex. The immunodetection of respiratory chain complexes with subunit-specific antibodies revealed a normal or increased content of the respiratory chain complexes I, II, III and IV corresponding to 100–150% of the control values. Analogous changes were observed in the isolated mitochondria (Fig. 3A) in accordance with the enzyme activity values (Table 1). Apparently, the observed biochemical changes are caused by an isolated decrease of the ATP synthase complex content, whereas the content of mitochondrial respiratory chain enzymes is unchanged or increased.

ATP synthase complex is fully assembled

The analysis of the ATP synthase complex in its native state was performed by blue native (BN)-PAGE and WB of dodecyl maltoside (DDM)-solubilized proteins of isolated fibroblast mitochondria. As shown in Fig. 3B, the migration of ATP synthase complex from the patient mitochondria was identical to that in the control, indicating that the complex has a normal size of about 620 kDa. The anti-F_1 α antibody showed that the ATP synthase defect in the patient cells is of quantitative character. There were two additional faint bands of lower molecular weight visible on the blot, which might be assembly or brake down intermediates; however, they did not accumulate at a high proportion. BN-PAGE analysis also confirmed an isolated decrease of the ATP synthase complex compared with the respiratory chain complexes III and IV.

Selective decrease of ATP synthase biosynthesis

The isolated decrease of ATP synthase can be caused by a decreased *de novo* synthesis of the enzyme and/or by its increased lability and thus enhanced degradation. To investigate the biosynthesis of mitochondrial oxidative phosphorylation complexes, we performed metabolic labeling with ^{35}S-methionine and followed its incorporation into the assembled oxidative phosphorylation complexes during a 6 h pulse labeling followed by a chase with unlabeled methionine. Mitoplasts were isolated from the labeled fibroblasts, and DDM-solubilized proteins were resolved by two-dimensional (2D) BN/SDS–PAGE.

The labeling of the full-sized, assembled ATP synthase holoenzyme (calculated from the labeling of two largest subunits, F_1 α and β) showed a near-linear increase during the 6 h pulse, in both control and patient cells (Fig. 3C), but the intensity of the labeling was 55% lower in patient cells. In contrast, there was no apparent difference in the intensity of the labeling of complex IV, cytochrome *c* oxidase (COX), quantified from the labeling of subunits COX2, COX3 and COX4. When the decay of the labeling of ATP synthase was quantified in the course of 50 h following the chase, an analogous time-dependent decrease was observed in the patient and control cells. Similarly, there was a comparable decay of COX labeling after the chase in the patient and control cells. These data indicate that the isolated defect of ATP synthase is caused by decreased *de novo* synthesis, whereas the half-life remains rather unchanged.

Patient cells harbor a homozygous missense mutation in the *ATP5E* gene

The genetic analysis excluded mutations in ATP synthase assembly factors and in the *TMEM70* gene (19), but sequencing of ATP synthase subunit genes at the cDNA level revealed the missense mutation c.35A>G in exon 2 of the *ATP5E* gene (Fig. 4A). This leads to the amino acid exchange p.Tyr12Cys, which corresponds to Tyr11 of the mature ε subunit which lacks the N-terminal methionine. The affected position is highly conserved among eukaryotes (Fig. 4C). This mutation introduces a restriction site for *Pvu*II into the sequence, which was used to verify the mutation by RFLP analysis at the level of genomic DNA. The mutation was homozygous and inherited from the parents who were both heterozygous carriers (Fig. 4B). The mutation was not found in 180 control chromosomes from the Austrian population.

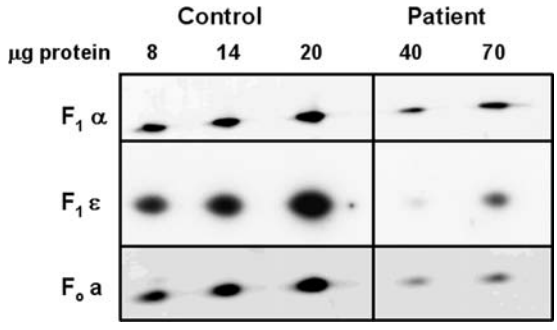

Figure 5. Mutated subunit ε is incorporated in the ATP synthase complex. DDM-solubilized mitochondrial proteins from the control and patient fibroblasts were separated by BN-PAGE in the first dimension; the gel pieces containing the full-size ATPase complex were dissected and subjected to SDS–PAGE in the second dimension. WB analysis was performed using antibodies to F_1 α, F_1 ε and F_o a subunits.

QT-PCR analysis revealed comparable levels of ε subunit mRNA (not shown), indicating that the transcription of the *ATP5E* gene is not altered in the patient cells.

ATP synthase complex contains mutated subunit ε

To examine whether the mutated ε subunit is incorporated into the ATP synthase holoenzyme in patient mitochondria, we performed 2D BN/SDS–PAGE and WB analysis using specific antibodies to F_1 subunits α and ε and F_o subunit a. As shown in Fig. 5, the anti-ε antibody reacted well with the mutated ε subunit, which could be identified in the assembled ATP synthase complex of patient mitochondria. The comparison of ε subunit signals to those of F_1 α and F_o a subunits showed similar proportions in the control and patient ATP synthase complex, indicating that although the amount of patient holoenzyme is profoundly reduced, it has a normal composition of its subunits. This means that most of the mutated ε subunit must be either degraded or present in an unassembled form or in intermediates.

Low content of ATP synthase subunit ε, but increased content of subunit c is present in patient mitochondria

We investigated whether there could be any accumulation of mutated subunit ε outside the ATP synthase holoenzyme in the patient mitochondria, and performed a detailed WB analysis of isolated fibroblasts mitochondria with the available antibodies to individual ATP synthase subunits. As shown in Fig. 6A, an analogous pronounced decrease compared with control mitochondria was found in F_1 subunits α, β and ε, as well as in F_o subunits a, d, OSCP and F6, supporting the assumption that only ATP synthase-assembled ε subunit is present in the patient cells.

However, in the patient mitochondria with the mutated ε subunit, we found an increased content of F_o subunit c that opposed the decrease of all other ATP synthase subunits. Interestingly, the content of subunit c was as low as that of all other ATP synthase subunits in the fibroblasts from the patients with the ATP synthase deficiency due to the *TMEM70* mutation (Fig. 6B). Apparently, the observed accumulation of subunit c was unique to the patients with the *ATP5E* mutation.

Accumulated subunit c is not associated with ATP synthase enzyme complex and is insoluble in DDM

When we performed 2D WB analysis of DDM-solubilized proteins from the patient mitochondria using antibodies to F_1 α and F_o c subunits, the subunit c was detected only in the full-size ATP synthase, and its content was very low, correlating with the low content of F_1 and F_o subunits. This indicated that the accumulated subunit c resisted DDM solubilization. Therefore, further experiments were performed to analyze both the DDM-soluble and DDM-insoluble material obtained after solubilization using up to 4 g DDM/g protein of mitochondria. As shown in Fig. 6C, there was a pronounced accumulation of c subunit in the DDM-insoluble fraction (pellet) of patient mitochondria, whereas almost no subunit c was found in the DDM-insoluble fraction from the control mitochondria. In the control, only 5% of the total c subunit was recovered in the pellet, whereas the pellet contained 56% of c subunit in the patient (Table 2). Thus, the subunit c content in the pellet was 17-fold higher in the patient mitochondria and the total content of subunit c was ~22% higher compared with the control. In contrast and as expected, the F_1 α subunit was mostly recovered in the soluble material (86–95%) both in the patient and control mitochondria (Fig. 6C). The respective F_o c/F_1 α ratio was similar in the patient and control DDM solubilisates, but it was 194-fold higher in DDM pellet from patient mitochondria compared with that of control (Table 2).

The pellet from the patient was analyzed also with antibodies to other F_o subunits, a and d. Their content was low and excluded any parallel accumulation with subunit c (Fig. 6C). These data clearly indicate that despite the isolated decrease of ATP synthase complex in the patient cells, the subunit c is produced and accumulates in mitochondria in the form of aggregates which cannot be solubilized with DDM, even at rather high detergent concentrations.

DISCUSSION

The present work demonstrates that in addition to nuclear encoded mutations in the assembly-biogenesis factors ATPAF2 and TMEM70, also a mutation of the subunit ε of the F_1 catalytic part can cause an isolated deficiency of ATP synthase leading to a mitochondrial disease. Mutations in all three genes lead to a very similar biochemical phenotype of selective inhibition of ATP synthase biogenesis, which apparently affects the early stage of enzyme assembly at the level of F_1. However, the mutation in the *ATP5E* gene presents with a distinct clinical phenotype as well as with a unique alteration of ATP synthase biogenesis at the level of subunit c, which contrasts with the manifestation of *TMEM70* (20) and *ATPAF2* (21) mutations.

The subunit ε of the F_1 catalytic part is one of the smallest subunits in the mitochondrial ATP synthase. Evolutionarily, it appeared probably during the formation of eukaryotic cell and is the only F_1 subunit that does not have a homolog in the

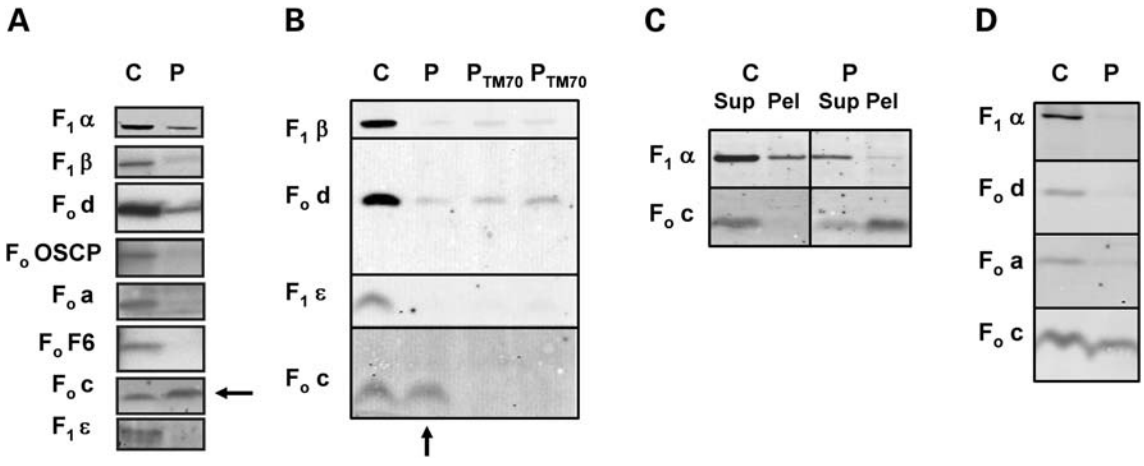

Figure 6. Patient mitochondria accumulate F_o subunit c of ATP synthase. SDS–PAGE and WB analysis of indicated ATP synthase subunits in the isolated fibroblast mitochondria (**A**) and fibroblasts (**B**) from control (C), patient with the *ATP5E* mutation (P) and two patients with a mutation in the *TMEM70* gene (P_{TM70}). (**C**) Accumulated F_o c subunits resist to DDM solubilization. Isolated mitochondria from patient and control fibroblasts were solubilized with DDM (4 g/g protein) and 29 000*g* supernatant (Sup) and pellet (Pel) were analyzed for the content of subunits F_1 α and F_o c. (**D**) Pellet from the patient accumulates F_o c but not the subunits F_o a or F_o d.

Table 2. Recovery of F_o c and F_1 α subunits in the supernatant and pellet of the patient and control fibroblast mitochondria solubilized with 4 g DDM/g protein

	Supernatant AU/mg prot	%Total	Pellet AU/mg prot	%Total	Total AU/mg prot
Patient F_1 α	1799	95.4	86	4.6	1885
Patient F_o c	1566	43.8	2009	56.2	3575
Patient F_o c/F_1 α	0.87		23.3		1.89
Control F_1 α	5872	86.4	922	13.6	6794
Control F_o c	2824	95.9	118	4.1	2942
Control F_o c/F_1 α	0.48		0.12		0.43

For quantification, 5 and 10 μg protein aliquots of 29 000 *g* supernatants and pellets were analyzed by SDS–PAGE and WB as in Fig. 6C.

bacterial and chloroplast enzyme. The mammalian ε (24) is a 51 amino acid protein that lacks the cleavable import sequence and exerts a high degree of homology with the slightly larger 62 amino acid yeast ε protein, encoded by the *ATP15* gene in *Saccharomyces cerevisiae*. The subunit ε is a part of the ATP synthase central stalk, and the function of this subunit is still unclear. The absence of ε subunit in *S. cerevisiae* (25) resulted in no detectable oligomycin-sensitive ATPase activity and in F_1 instability, but F_o subunits were still bound to F_1. The null mutation of the *ATP15* gene could be complemented by the bovine ε, indicating the structural and functional equivalence of corresponding subunits (26). Unlike in *S. cerevisiae*, the disruption of ε subunit gene in *Kluyveromyces lactis* completely eliminated the F_1-ATPase activity, indicating that the ε subunit may play an important role in the formation of the F_1 catalytic sector of eukaryotic ATP synthase (27). When the *ATP5E* gene in the mammalian HEK293 cells was knocked down, the biogenesis of ATP synthase was selectively inhibited, resulting in an isolated decrease of ATP synthase complex, insufficient ATP phosphorylating capacity, elevated levels of mitochondrial membrane potential ($\Delta\Psi_m$) and unexpected accumulation of subunit c (28).

The important finding of the present study is that the missense mutation c.35A>G (p.Tyr12Cys) in *ATP5E* results in a biochemical phenotype that is very similar to the downregulation of ε subunit biosynthesis. Interestingly, the function of ATP synthase assembled with the mutated ε subunit appears to be rather unaffected; it is able to utilize the mitochondrial membrane potential ($\Delta\Psi_m$) for ATP synthesis and also retains the sensitivity to oligomycin, which means that the F_1–F_o functional interactions are rather preserved, once the complex is formed.

The question arises at which stage of enzyme biogenesis the mutation of ε stalls the assembly process. The patient cells have similarly decreased oligomycin-sensitive and aurovertin-sensitive ATP synthase hydrolytic activities, which argues against the presence of free active F_1 catalytic part. They also do not show enhanced content of F_1 assemblies that are known to accumulate in rho^0 cells (29), upon mtDNA depletion (30), in *ATP6* mutations (31), and after the inhibition of mitochondrial protein synthesis by doxycycline (32). Also, there is no pronounced accumulation of free F_1 subunits α or β, nor their heterodimers. The initial formation of $\alpha_3\beta_3$ heterooligomer, which depends on the ATPAF1 and ATPAF2 factors, is expected to be followed by the addition of the γ, δ and ε subunits (6), but the eukaryotic F_1 assembly that involves the subunit ε is not much known. As the biochemical phenotype of patient cells indicates, a mutation in ε must be critical for the completion of F_1 or for its stability. Especially, the stability of the mutated F_1 might be a problem, and mutated F_1 could be degraded before it can establish further contacts with the F_o subunits.

As revealed by crystallographic studies (33), the ε subunit in the bovine enzyme is located in the protruding part of the central stalk and has a hairpin (helix–loop–helix) structure. It is in contact with (located between) the γ and δ subunits and is expected to play a role in the stability of the foot of the central stalk facing the c subunit oligomer. The C-terminus of ε subunit forms an extension of the β-sheet of the γ subunit, and the N-terminal region of ε subunit is in a shallow cleft of δ

subunit. Three of the five strictly conserved amino acids of the ε subunit are located at the N-terminus (Fig. 4C) and include Trp4, the only tryptophan within the mitochondrial F_1, Arg5 and Tyr11, which is replaced by Cys in our patient with the c.35A>G mutation in exon 2 of the *ATP5E* gene (amino acid numbering according to the mature ε that lacks the N-terminal methionine). Interestingly, this Tyr11 of the mature protein forms a part of the hydrophobic pocket for Trp4 and is involved in the hydrogen bonding to the δ subunit in a way that the ε subunit normally forms a stable heterodimer with the δ subunit (34,35). In the light of similarity between the Tyr11 to Cys replacement in the patient cells and the *ATP5E* knock-down in the HEK293 cells (28), it is possible to state that the disruption of these interactions at the level of stalk may be critical to the stability of the whole F_1 part of the ATP synthase. Interestingly, when the complete F_1F_o with mutated ε is formed, its stability appears to be normal.

Another important finding of our study is the accumulation of subunit c, the most hydrophobic ATP synthase subunit which forms an oligomeric ring in the F_o moiety. The accumulation of the subunit c, which appears to be present in an aggregated form, is comparable with that found in the *ATP5E* knock-down (28) and represents a substantial difference from the ATP synthase deficiency due to *TMEM70* or *ATPAF2* mutations. It also supports the view that the subunit ε plays an essential role in the incorporation and assembly of subunits c into the ATP synthase complex. Further studies are needed to test whether the p.Tyr12Cys replacement alters the F_1-c subunit oligomer formation via a changed conformation in the bottom part of the central stalk or whether the ε subunit interacts directly with the c subunits.

The accumulation of subunit c is known in neuronal ceroid lipofuscinosis (NCL), a neurodegenerative inherited lysosomal storage disease, where an altered degradation of hydrophobic proteins leads to a failure in the degradation of ATP synthase subunit c after its normal inclusion into mitochondria. Its consequent abnormal accumulation in lysosomes is apparent as autofluorescent storage bodies (lipopigment) (36). The impact of the NCL disease process differs substantially depending on the cell type. The brain neurons are most seriously affected and degenerate, whereas other cell types mostly survive without any detectable deterioration. NCL mitochondria were found to contain normal amounts of ATP synthase, and the oxidative phosphorylation was also normal (37,38). Up to now, about 160 NCL-causing mutations were found in eight NCL genes (39,40) but none of them appears to be associated with the ATP synthase biosynthesis and function.

In conclusion, the present work describes that a mutation in the third nuclear gene, *ATP5E*, leads to an isolated ATP synthase deficiency and mitochondrial disease. This is the first mutation in nuclear encoded subunit of mammalian ATP synthase that leads to an alteration of enzyme biosynthesis, and it provides with important information for the genetic diagnostics of mitochondrial diseases due to ATP synthase dysfunction. At the same time, this genetic disorder represents a unique model for further studies on the functional role of the ε subunit, one of the least understood subunits of the mitochondrial enzyme, in the biogenesis of the ATP synthase in eukaryotic cells.

MATERIALS AND METHODS

Case report

The girl was born after 37 weeks of gestation with the birth weight of 2440 g (small for gestational age). Pregnancy was uneventful; she is the first and only offspring of non-consanguineous, healthy Austrian parents. She was symptomatic from the first day of her life with poor sucking and respiratory distress. Laboratory investigations showed hyperlactacidemia (3.8 mmol/l) and 3-methylglutaconic aciduria. A metabolic disease was supposed; the girl recovered slowly and could be discharged home at the age of 2 months. The clinical course over the first years of life was marked by recurrent metabolic crises, mostly triggered by (upper airway) infections with elevated plasma lactate and partly hyperammonemia (up to 500 μmol/l). The girl started walking at the age of 18 months, the course of the disease stabilized at the age of 5–6 years. She entered and successfully completed a regular school and works now as an employee in an office. The diagnosis was finally established at the age of 17 years. The ATP synthase deficiency was diagnosed in cultured skin fibroblasts during a workup aimed at explaining her persistent 3-methylglutaconic aciduria and lactic acidosis. Electrophoretic analysis confirmed the isolated defect of the ATP synthase. The patient shows moderate ataxia, horizontal nystagmus, exercise intolerance and weakness, a strongly shortened walking distance and a severe peripheral neuropathy with the loss of tendon reflexes in the legs and mild hypertrophy of the left ventricle. The motor nerve conduction velocity of the peroneal nerve was severely reduced with a significantly diminished amplitude proofing mixed axonal and demyelizating neuropathy. Electromyography showed increased amplitudes and prolonged polyphasic potentials during the innervation as a symptom of reorganization. MRI showed slightly increased signal intensities in the caudate and lentiformis nuclei at the age of 14 years; MRI of the brain was normal 3 years later and the signal intensities disappeared.

Ethics

This study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Committees of Medical Ethics at all collaborating institutions. Informed consent was obtained from the parents.

Cell cultures and isolation of mitochondria

Fibroblast cultures were established from skin biopsies and cells were cultivated in DMEM medium (Gibco BRL) with 10% fetal calf serum (Sigma, USA) at 37°C in 5% CO_2 in air. Cells were grown to ~90% confluence and harvested using 0.05% trypsin and 0.02% EDTA. Detached cells were diluted with ice-cold culture medium, sedimented by centrifugation and washed twice in cold phosphate-buffered saline (PBS; 8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4). Mitochondria were isolated from the fibroblasts by the method of Bentlage *et al*. (41), utilizing the hypotonic shock cell disruption. To avoid proteolytic degradation, the isolation medium (250 mM sucrose, 40 mM KCl, 20 mM

Tris–HCl, 2 mM EGTA, pH 7.6) was supplemented with the protease inhibitor cocktail (Sigma P8340). The isolated mitochondria were stored at −70°C.

Enzyme activity measurements

The following respiratory chain enzyme activities were determined accordingly: citrate synthase (42), complex I and I + III (43), complex II and II + III (44), complex III (43), complex IV (45), with modifications described in Berger *et al*. (46). The ATPase hydrolytic activity was measured in ATP-regenerating system (47) using frozen–thawed fibroblasts or isolated mitochondria. The sensitivity to aurovertin and oligomycin was determined at 2 μM inhibitor concentration. All spectrophotometric measurements were performed at 37°C.

The rate of ATP synthesis was measured as described before (13) in fibroblasts permeabilized with digitonin (0.1 g/g protein; Fluka, USA) and supplemented with 1 mM ADP and respiratory substrates 10 mM pyruvate + 2.5 mM malate or 10 mM succinate. The reaction was started by the addition of fibroblasts; then, in time intervals of 5–15 min, samples were quenched with dimethylsulfoxide (1/1, v/v), and ATP content was measured by a luciferase assay (48). The ATP production was expressed in nmol ATP/min/mg protein.

The protein content was measured by the method of Bradford (49), using bovine serum albumin as a standard. Samples were sonicated for 20 s prior to the protein determination.

High-resolution oxygraphy

Oxygen consumption by cultured fibroblasts was determined at 30°C as described before (50) using Oxygraph-2k (Oroboros, Austria). Freshly harvested fibroblasts were suspended in a KCl medium (80 mM KCl, 10 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM potassium phosphate, pH 7.4) and cells were permeabilized with digitonin (0.05 g/g protein). For measurements, 10 mM glutamate, 2.5 mM malate, 10 mM succinate, 1.5 mM ADP, 25 μM cytochrome *c*, 1 μM oligomycin and 0.5 μM FCCP were used. The oxygen consumption was expressed in pmol oxygen/s/mg protein.

Flow cytometry analysis of mitochondrial membrane potential ΔΨm

The cells were resuspended in the KCl medium used for oxygraphy at the protein concentration of 1 mg/ml. The aliquots of the cells (0.3 mg protein) were diluted in 1.5 ml KCl medium containing 10 mM succinate, permeabilized with 0.1 g digitonin/g protein and incubated with 20 nM TMRM (Molecular Probes, USA) for 15 min. For measurements, 1.5 mM ADP and 1 μM FCCP were used. Cytofluorometric analysis was performed on PAS-III flow cytometer (Partec, Germany) equipped with the 488 nm Ar–Kr laser, and TMRM fluorescence was analyzed in the FL2 channel (band pass filter 580 ± 30 nm). Data were acquired with FloMax software (Partec, Germany) and analyzed with Summit Offline V3.1 software (Cytomation, USA). The changes in ΔΨm were calculated in millivolts (51).

Electrophoresis and WB analysis

SDS–PAGE (52) was performed on 10% polyacrylamide slab minigels (MiniProtean II system; Bio-Rad). The samples were boiled for 3 min in a sample lysis buffer [2% (v/v) mercaptoethanol, 4% (w/v) SDS, 50 mM Tris–HCl, pH 7.0, 10% (v/v) glycerol], and the protein aliquots were loaded as indicated. Blue native-PAGE (BN-PAGE) (53) was performed on 6–15% gradient polyacrylamide slab minigels. Fibroblast mitochondria or mitoplasts were solubilized with 1–4 g/g protein of DDM for 20 min at 0°C and centrifuged for 20 min at 29 000*g*. The protein concentration in the supernatant was estimated and Serva Blue G was added at a ratio 0.25 g/g DDM. For 2D analysis, strips of gel from BN-PAGE were incubated for 1 h in 1% (v/v) mercaptoethanol, 1% (w/v) SDS and subjected to SDS–PAGE in the second dimension.

The gels were blotted onto a PVDF membrane (Immobilon P, Millipore) by the semidry electrotransfer for 1 h at 0.8 mA/cm^2, and the membrane was blocked in PBS, 0.1% Tween-20, 5% (w/v) fat-free dried milk. The membranes were incubated for 3 h with primary antibodies diluted in PBS with 0.3% (v/v) Tween-20 (PBST). The following antibodies were used: monoclonal antibodies from Mitosciences (USA) to SDH70 (MS204), Core 1 (MS303), COX4 (MS407), NDUFA9 (MS111), ATP synthase subunits α (MS502), β (MS503), d (MS504), OSCP (MS505), F6 (MS508) and porin (MSA03); Abnova (USA) antibody to ATP synthase subunit ε (H00000514-M01); polyclonal antibodies to ATP synthase subunits a (54) and c (55). Incubation with Alexa Fluor 680-labeled secondary antibodies (Molecular Probes) in PBST was performed for 1 h using either goat anti-mouse IgG (A21058) or goat anti-rabbit IgG (A21109). The fluorescence was detected using Odyssey Imager (LI-COR) and the signal was quantified using Aida 3.21 Image Analyzer software (Raytest).

DNA analysis

Genomic DNA was isolated from fibroblasts or blood with a standard technology (NucleoSpin, Machery-Nagel). Total RNA was extracted from fibroblasts (TRI reagent, MRC, Inc.), treated with DNase I (Ambion) for 15 min and reverse-transcribed (Superscript III, Invitrogen). The *ATP5E* gene was amplified from cDNA with the following forward ATP5E-F GACATTGCCGGCGTCTTG and reverse ATP5E-R TCCC ATGGAATGAGATCCTTT primers (5′ to 3′). Sequence analysis (CEQ-8000, Beckman Coulter) was performed according to the manufacturer. The presence of the c.35A>G mutation was assessed from the genomic DNA by PCR–RFLP analysis with the following forward ATP5E-intron1-F AGATCATCCATCCAAACACC and reverse ATP5E-intron2-R GCCAGGGGACTTCTATAACC primers employing a mutation-induced restriction site for *Pvu*II in the sequence.

Metabolic labeling

Fibroblasts were grown in 75 cm^2 flasks until 80% confluency. Cells were washed twice with PBS and then incubated for 3 h in methionine-free medium (Sigma D0422) with 10% dialyzed

FCS (Gibco BRL 26400-044) for the depletion of intracellular methionine. The met$^-$ medium was removed and labeling was started by the addition of 6 ml of met$^-$ medium with 10% FCS and 100 μCi ^{35}S-methionine (MP Radiochemicals No. 51004) into each flask. For chase, 10 mM methionine was added. Labeling was performed for the indicated time intervals and then stopped by 2.5 mM chloramphenicol and 2.5 mM cycloheximide. After 10 min, the cells were washed twice with PBS containing both inhibitors and harvested by trypsinization. Mitoplasts prepared by digitonin treatment [0.8 g/g protein (56)] were solubilized with DDM and analyzed by 2D BN/SDS–PAGE. Radioactivity of proteins was quantified in dried gels using BAS-5000 system (Fuji). The labeling of ATP synthase complex was calculated from the radioactivity of subunits α and β, and the labeling of COX was calculated from the radioactivity of subunits COX2, COX3 and COX4 identified in Coomassie Blue R-250-stained gels.

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Conflict of Interest statement. None declared.

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ARTICLE 5

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Knockdown of F_1 epsilon subunit decreases mitochondrial content of ATP synthase and leads to accumulation of subunit c

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ABSTRACT

The subunit ε of mitochondrial ATP synthase is the only F_1 subunit without a homolog in bacteria and chloroplasts and represents the least characterized F_1 subunit of the mammalian enzyme. Silencing of the *ATP5E* gene in HEK293 cells resulted in downregulation of the activity and content of the mitochondrial ATP synthase complex and of ADP-stimulated respiration to approximately 40% of the control. The decreased content of the ε subunit was paralleled by a decrease in the F_1 subunits α and β and in the F_o subunits a and d while the content of the subunit c was not affected. The subunit c was present in the full-size ATP synthase complex and in subcomplexes of 200–400 kDa that neither contained the F_1 subunits, nor the F_o subunits. The results indicate that the ε subunit is essential for the assembly of F_1 and plays an important role in the incorporation of the hydrophobic subunit c into the F_1-c oligomer rotor of the mitochondrial ATP synthase complex.

1. Introduction

The mammalian ATP synthase (F_oF_1 ATPase) is a heterooligomeric complex of ~650 kDa localized in the inner mitochondrial membrane. It consists of at least 16 different types of subunits [1,2]. Six of them (α, β, γ, δ, ε and inhibitor protein IF_1) form the F_1-catalytic domain on the matrix side of the membrane. The remaining ten subunits (a, b, c, d, e, f, g, OSCP, A6L, F6), two of which (a and A6L) are encoded by mitochondrial DNA (mtDNA) [3], comprise the membrane-embedded F_o portion, functioning as a proton channel, and two stalks connecting the F_1 and F_o domains [4–7]. Two additional proteins MLQ and AGP are possibly involved in the dimerization of ATP synthase [8], and the enzyme function can be modulated by the coupling factor B [9].

The three largest subunits of the F_1 catalytic part of ATP synthase — α, β and γ — exert a varying degree of homology among ATP synthases from mitochondria, chloroplast and bacteria, while the mammalian subunit δ corresponds to the ε subunit in the bacterial enzyme [1]. The only F_1 subunit that does not have a homolog in bacteria and chloroplasts is the ε subunit [1], which is the smallest and functionally the least characterized mitochondrial F_1 subunit. The mammalian ε subunit [10] encoded by the *ATP5E* gene is a 51AA protein of 5.8 kDa that lacks a cleavable import sequence. It exerts a high degree of homology with the slightly larger yeast ε of 6.6 kDa, which is encoded by the *ATP15* gene, and consists of 62 AA in *S. cerevisiae* [11]. As revealed by complementation experiments, the yeast and mammalian ε are structurally and functionally equivalent [12].

The F_1 subunits γ, δ and ε together with the subunit c oligomer form the rotor of ATP synthase [13]. The subunit ε was shown to form heterodimers with the subunit δ [14,15] and presumably also makes contacts with F_o. As revealed by crystallographic studies [5,16], the mitochondrial ε subunit is located in the protruding part of the central stalk and it has a hairpin (helix–loop–helix) structure. It maintains contact with the γ and δ subunits and is expected to be involved in the stability of the foot of the central stalk facing the c subunit oligomer. The C-terminus of ε subunit forms an extension of the β-sheet of γ subunit and the N-terminal region of ε subunit is located in a shallow cleft of δ subunit [5].

The involvement of ε subunit in the ATP synthase biogenesis and function was repeatedly studied in yeast by means of disruption of the *ATP15* gene. The absence of ε subunit in *S. cerevisiae* resulted in no detectable oligomycin-sensitive ATPase activity, decreased content of γ, δ and F_o subunits in immunoprecipitated ATP synthase and F_1 instability. High proton leak, which was shown to be sensitive to oligomycin, indicated a conformationally changed F_o [17]. Also, disruption of the *ATP15* gene in *K. lactis* resulted in a complete elimination of F_1-ATPase activity, suggesting that the ε subunit may have an important role in the formation of the F_1 catalytic sector of eukaryotic ATP synthase [18]. In contrast, if the null mutations of F_1 subunits α, β, γ, δ and ε were made in *S. cerevisiae*, mutations in all but the ε subunit gene were unable to grow on a nonfermentable carbon source indicating that ε is not an essential component of the ATP synthase [11].

Abbreviations: DDM, dodecyl maltoside; F_1, catalytic part of ATP synthase; F_o, membrane-embedded part of ATP synthase

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With the aim to investigate the functional role of ε subunit in the biogenesis and formation of mammalian ATP synthase complex, we have downregulated the expression of *ATP5E* gene in HEK293 cells by means of RNA interference (RNAi). We have found that the inhibition of ε subunit biosynthesis has a pronounced effect on the mitochondrial content and activity of ATP synthase and leads to a relative accumulation of subunit c. Our results demonstrate the essential role of subunit ε in the assembly of F_1 and the incorporation of hydrophobic subunit c into the F_1-c oligomer rotor structure of mitochondrial ATP synthase in higher eukaryotes.

2. Materials and methods

2.1. Cell culture

Human embryonic kidney 293 cells (HEK293 from ATCC) were grown at 37 °C in a 5% (v/v) CO_2 atmosphere in high-glucose Dulbecco's modified Eagle's medium (PAA) supplemented with 10% (v/v) fetal calf serum (PAA). Cell transfections were carried out with a Nucleofector™ device (Amaxa) using the HEK293 cell-specific transfection kit.

2.2. RNAi

For the silencing of subunit ε of ATP synthase we used two miR-30-based shRNAs (shRNAmirs) shE1 (TGCTGTTGACAGTGAGCGAACAATGTCAATAAATTGAAATTAGTGAAGCCACAGATGTAATTTCAATTTATTGACATTGTCTGCCTACTGCCTCGGA) and shE2 (TGCTGTTGACAGTGAGCGAACATGTTATGGCAGATTGAAATAGTGAAGCCACAGATGTATTTCAATCTGCCATAACATGTGTGCCTACTGCCTCGGA) targeted to the coding sequence of human *ATP5E* gene, which were cloned to plasmid pGIPZ™ (V2LHS-77373 and V2LHS-773734, Open Biosystems). Plasmid DNA was isolated by an endotoxin-free kit (Qiagen) and HEK293 cells were transfected with the shE1 or shE2 shRNA constructs or with the non-silencing, empty vector (negative control, NS cells). At 48 h after the transfection the cells were split into culture medium containing 1.5 μg/ml puromycin (Sigma-Aldrich) and antibiotic-resistant colonies were selected over a period of three weeks.

ATP5E mRNA and *18S* RNA levels were determined in the transfected cells by QT RT-PCR. The total RNA was isolated with TRIzol reagent (Invitrogen) and cDNA was synthesized with SuperScript III reverse transcriptase using random primers (Invitrogen). PCR was performed on the LightCycler 480 instrument (Roche Diagnostics) with a SYBR Green Master kit (Qiagen) using *ATP5E* (F: 5′-GATGCACTGAAGACAGAATTCAAAG-3′, R: 5′-GCTGCCAGAAGTCTTCTCAGC-3′) and *18S* (F: 5′-ATCAGGGTTCGATTC CGGAG-3′, R: 5′-TTGGATGTGGTAGCCGTTTCT-3′) primers.

2.3. Isolation of mitochondria

Cells (~90% confluent) were harvested with 0.05% trypsin and 0.02% EDTA and washed twice in phosphate-buffered saline (PBS, 8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na_2HPO_4, 0.20 g/l KH_2PO_4). Mitochondria were isolated by a method utilizing hypotonic shock cell disruption [19]. To avoid proteolytic degradation, isolation medium (250 mM sucrose, 40 mM KCl, 20 mM Tris–HCl, 2 mM EGTA, pH 7.6) was supplemented with protease inhibitor cocktail (Sigma P8340). The protein content was measured by the Bio-Rad Protein Essay (Bio-Rad Laboratories), using BSA as a standard. The isolated mitochondria were stored at −70 °C.

2.4. Electrophoresis

Blue-Native polyacrylamide gel electrophoresis (BN-PAGE) [20] was performed on a 6–15% polyacrylamide gradient minigels (Mini Protean, Bio-Rad). Mitochondria were solubilized with dodecyl maltoside (DDM, 2 g/g protein) for 20 min on ice in 1.75 M aminocaproic acid, 2 mM EDTA and 75 mM Bis–tris (pH 7.0). Samples were centrifuged for 20 min at 26000 × *g*, Serva Blue G (0.1 g/g detergent) was added to supernatants and the electrophoresis was run at 45 V for 30 min and then at 90 V.

SDS-Tricine polyacrylamide gel electrophoresis (SDS-PAGE) [21] was performed on 10% (w/v) polyacrylamide slab minigels. The samples were incubated for 20 min at 40 °C in 2% (v/v) mercaptoethanol, 4% (w/v) SDS, 10 mM Tris–HCl, 10% (v/v) glycerol. For two-dimensional (2D) analysis, the stripes of the first dimension BN-PAGE gel were incubated for 1 h in 1% (w/v) SDS and 1% (v/v) mercaptoethanol and then subjected to SDS-PAGE in the second dimension [21].

2.5. Western blot analysis

Gels were blotted on to PVDF membrane (Millipore) by semi-dry electrotransfer (1 h at 0.8 mA/cm^2). Blocked membranes (5% (w/v) non-fat dry milk in PBS) were incubated in PBS, 0.01% (v/v) Tween 20 with the following primary antibodies — polyclonal antibodies against F_o-a (1:500 [22]) and F_o-c (1:1000 [23]), monoclonal antibodies against F_1-α (1:1000, MS502, MitoSciences), F_1-β (1:2000, MS503, MitoSciences), F_1-ε (1:5000, Abnova), F_o-d (1:100; Molecular Probes), SDH70 (1:10000, MS204, Mitosciences), Core 2 (1:1000, MS304, Mitosciences) and porin (1:1000, MSA03, Mitosciences) and with fluorescent secondary antibodies (goat anti-mouse IgG, 1:3000, Alexa Fluor 680 A-21058 or goat anti-rabbit IgG, 1:3000, Alexa Fluor 680 A-21109, Molecular Probes). The fluorescence was detected on an ODYSSEY system (LI-COR) and the signal was quantified using Aida 3.21 Image Analyser software.

2.6. ATPase assay

The ATP synthase hydrolytic activity was measured in ATP-regenerating system as described by [24]. Digitonin (0.05 g/g protein) permeabilized cells were incubated in a medium containing 40 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 10 mM KCl, 2 mM phosphoenolpyruvate, 0.2 mM NADH, 1 μM rotenone, 3 μM FCCP, 0.1% (w/v) BSA, 5 U of pyruvate kinase, 5 U of lactate dehydrogenase for 2 min. The reaction was started by addition of 1 mM ATP. The sensitivity to aurovertin or oligomycin was determined by parallel measurements in the presence of 2 μM inhibitor.

2.7. Respiration measurements

Respiration was measured at 30 °C by an Oxygraph-2k (Oroboros). Freshly harvested cells were suspended in a KCl medium (80 mM KCl, 10 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM potassium phosphate, pH 7.4) and permeabilized with digitonin (0.1 g/g of protein). Respiration was measured using 10 mM glutamate, 3 mM malate, 1.5 mM ADP, 1 μM oligomycin, 1 μM FCCP and 1 μM antimycin A. Oxygen consumption was expressed in pmol oxygen s^{-1} mg $protein^{-1}$.

2.8. Mitochondrial membrane potential $\Delta\Psi_m$ measurements

$\Delta\Psi_m$ was measured with TPP^+-selective electrode in 1 ml of KCl medium as described in [25]. Cells (2.5 mg protein/ml) were permeabilized with digitonin (0.05 g/g protein) and the following substrates and inhibitors were used: 10 mM succinate, 10 mM glutamate, 3 mM malate, 1.5 mM ADP, 1 μM oligomycin and 1 μM FCCP. The membrane potential was plotted as pTPP, *i.e.* natural logarithm of TPP^+ concentration (μM).

3. Results

3.1. Downregulation of ATP5E gene decreases the content and activity of ATP synthase

Transfections of HEK293 cells with miR-30-based shRNAs (shE1, shE2) targeted to the *ATP5E* gene encoding the ε subunit of ATP synthase were followed by puromycin selection and resulted in three shE2 stable lines that showed a variable decrease of *ATP5E* mRNA levels relative to 18S RNA. These shEa, shEb and shEc exhibited an *ATP5E* mRNA level of 16%, 47% and 29%, respectively, compared with the parental HEK293 cells transfected with empty vector (NS cells). The cell lines exerted normal viability under standard cultivation conditions. There was no significant difference in cell growth rate between the silenced and control cell lines.

The cell lines were analyzed for the content and activity of mitochondrial ATP synthase as well as the function of mitochondrial respiratory chain. Quantification of the cellular content of respiratory chain enzymes by SDS-PAGE and WB showed in all silenced cell lines normal content of complexes II and III, but decreased content of complex V — ATP synthase (Fig. 1A), indicating that the specific knockdown of *ATP5E* gene expression affected selectively the biogenesis of ATP synthase complex. Based on the immunodetection with the antibody to F_1 subunit β, the content of ATP synthase showed a decrease of 60–70%. The same result was obtained with the antibody to the α subunit (not shown). This was confirmed by analysis of ATP synthase at native conditions in dodecyl maltoside-solubilized proteins from isolated mitochondria using BN-PAGE and WB (Fig. 1B). In comparison with the controls (the original HEK293 and NS cells), the *ATP5E*-silenced cell lines contained reduced amounts of assembled ATP synthase complex, which, however, retained the same mobility as the ATP synthase complex from control cells corresponding to about 650 kDa. Control cells contained a small amount of F_1 subcomplex of ~370 kDa, which was not detected in silenced cell lines. The quantification of WB data from BN-PAGE revealed also a 60–70% reduction of ATP synthase complex in the *ATP5E*-silenced cell lines.

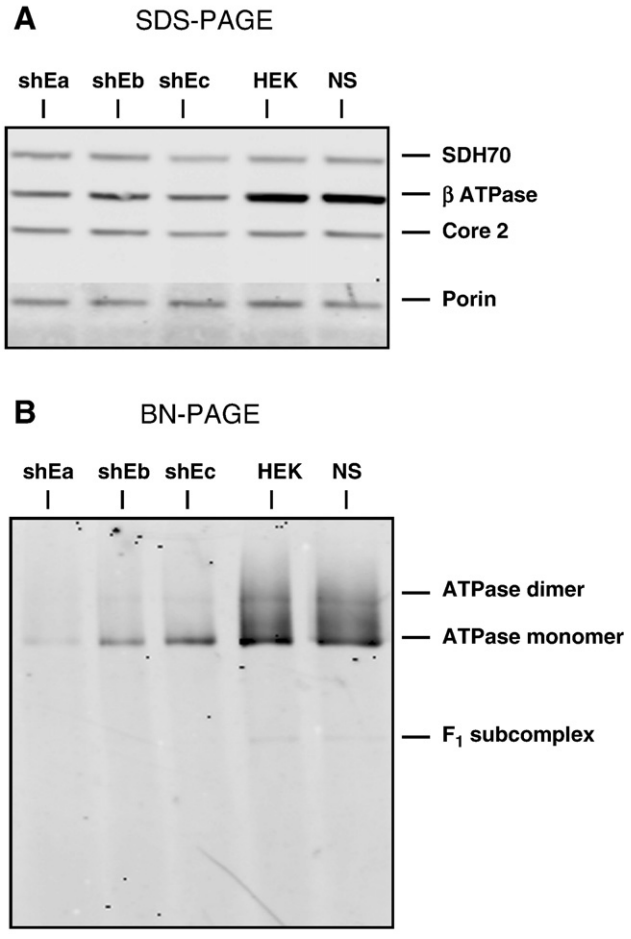

Fig. 1. Selective reduction of ATP synthase in *ATP5E*-silenced cell. (A) Isolated mitochondria (10 μg protein aliquots) from control (HEK293 and HEK293 transfected with empty vector (NS)) and *ATP5E*-silenced (shE2a, shE2b, shE2c) cells were analyzed by SDS-PAGE and WB with antibodies to ATP synthase (β) and to respiratory chain complexes II (SDH70) and III (Core 2). (B) DDM-solubilized (2 g/g protein) mitochondrial proteins (15 μg protein aliquots) from control (HEK293, NS) and *ATP5E*-silenced (shE2a, shE2b, shE2c) cells were analyzed by BN-PAGE and WB using antibody to ATP synthase β subunit.

Furthermore, the *ATP5E*-silenced cell lines had a low ATP synthase hydrolytic activity compared with the control HEK293 and NS cells. Oligomycin-sensitive ATP hydrolysis showed a 54–64% decrease of activity and aurovertin-sensitive ATP hydrolysis was 64–68% decreased in comparison with the control cells. The activity measurements data corresponded well with the electrophoretic analysis. The same results obtained with F_1-interacting aurovertin and F_o-interacting oligomycin indicated further that all remaining ATP hydrolytic activity was due to complete ATP synthase complexes with unaltered F_1–F_o interaction and not due to a presence of free and active F_1-ATPase molecules.

3.2. Downregulation of ATP5E gene decreases mitochondrial ATP production but does not uncouple oxidative phosphorylation

The functional effects of *ATP5E* silencing on mitochondrial energy conversion were analyzed by mitochondrial respiration in digitonin-permeabilized cells. As shown in Fig. 2A, mitochondria in the *ATP5E*-silenced cells were tightly coupled at state 4, but ADP-stimulated respiration was significantly lower although these cells had comparable respiratory capacity after uncoupling with FCCP (state 3 uncoupled) when compared with the control NS (Fig. 2A) or HEK293 (not shown) cells. The ADP-stimulated respiration in the *ATP5E*-silenced cells was fully sensitive to oligomycin. Direct measurements of mitochondrial membrane potential $\Delta\Psi_m$ with TPP^+-selective electrode (Fig. 2B) revealed comparable state 4 values of $\Delta\Psi_m$ in both control and *ATP5E*-silenced cells. In fact, an even higher state 4 value was found in the *ATP5E*-silenced cells (ΔpTPP, i.e. the difference of membrane potential with respect to the pTPP value with FCCP, was 0.47 and 0.64 in the control and silenced cells, respectively). Addition of ADP led to a much smaller decrease of $\Delta\Psi_m$ in the silenced cells (ΔpTPP decrease of 0.28 and 0.05 in the control and silenced cells, respectively), but the decrease was fully reversed by oligomycin, much the same as in control cells. Membrane potential measurements thus further supported the conclusion that the *ATP5E*-silenced cells are well coupled but the low content of ATP synthase complex limits the function of mitochondrial oxidative phosphorylation.

3.3. Silencing of the ATP5E gene leads to relative accumulation of F_o subunit c

An alteration of ATP synthase assembly due to low availability of ε subunit may lead to an accumulation of incomplete assemblies consisting of some enzyme subunits, e.g. F_1 ATPase subcomplexes, assuming that the ε subunit is added at the late stage of F_1 formation, i.e. after the γ and/or δ subunits. Having determined the cellular content of individual ATP synthase subunits, we found that *ATP5E* silencing reduced the content of the F_1 subunits α and ε as well as of the F_o subunits a and d (Fig. 3A) to a similar extent. The only subunit that was not reduced was the F_o subunit c. Normal content of subunit c was maintained in all cell lines with silenced *ATP5E*, demonstrating

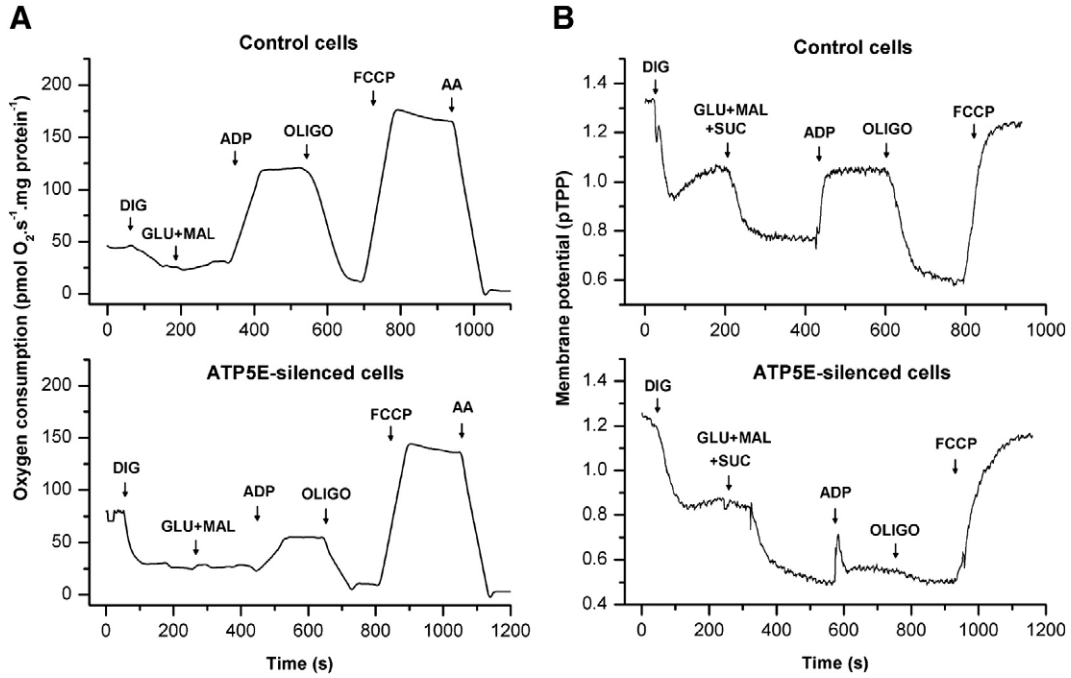

Fig. 2. ADP-stimulated respiration and ADP-induced decrease of mitochondrial membrane potential $\Delta\Psi_m$ in *ATP5E*-silenced cells. (A) Respiration and (B) $\Delta\Psi_m$ were measured in shE2a and NS cells permeabilized with digitonin (DIG) using 10 mM glutamate (GLU), 3 mM malate (MAL), 10 mM succinate (SUC), 1.5 mM ADP, 1 μM oligomycin (OLIGO), 1 μM FCCP and 1 μM antimycin A (AA). Respiration was expressed as oxygen consumption in pmol O2 s^{-1} mg $protein^{-1}$, mitochondrial membrane potential $\Delta\Psi_m$ measured with TPP^+-selective electrode was plotted as pTPP, *i.e.* natural logarithm of TPP^+ concentration (μM).

that if the ATP synthase assembly process was inhibited, the "excess" subunit c was not degraded and cleared out as other ATP synthase subunits.

To characterize further the accumulated subunit c, mitochondrial proteins were solubilized with DDM and analyzed for the content of ATP synthase subunits. As shown in Fig. 3B, the subunit c of *ATP5E*-silenced mitochondria was recovered in both soluble and insoluble fractions. In comparison with the control NS cells, both fractions from *ATP5E*-silenced cells showed a much higher content of subunit c relative to subunits α or d. Thus the solubilized proteins from *ATP5E*-silenced mitochondria were 2–2.5-fold enriched in subunit c and the DDM-insoluble pellet was enriched 4–10-fold.

When the solubilized mitochondrial proteins were subjected to 2D electrophoresis, BN/SDS-PAGE and WB analysis (Fig. 4), in control mitochondria all subunit c as well as subunit a were present in assembled F_oF_I ATP synthase complex and neither subunit c nor subunit a could be detected around 370 kDa where the F_1 subcomplex migrates. In mitochondria from the *ATP5E*-silenced cells, the subunit c signal was also present in the F_oF_1 complex, in a smaller amount, in a good correspondence with the reduced content of ATP synthase. In addition, significant signal of subunit c was found in the BN-PAGE region of about 200–400 kDa. In this region, however, no F_1 subunits α and β or F_o subunits a and d could be detected (Fig. 4).

4. Discussion

The present study demonstrates that the ε subunit is essential for the biosynthesis of F_1 catalytic part of mammalian ATP synthase complex and that a decreased amount of available subunit ε due to *ATP5E* silencing limits the cellular content of assembled and functional ATP synthase. As revealed by respiration and mitochondrial membrane potential $\Delta\Psi_m$ measurements, *ATP5E* silencing consequently decreases the activity of mitochondrial oxidative phosphorylation, not affecting the tight coupling of mitochondria. These data clearly indicate that the ε subunit plays an important role in the assembly and/or stability of F_1 moiety of mammalian ATP synthase.

The biogenesis of eukaryotic ATP synthase is a highly organized process depending on mutual action of different ancillary factors. At least 13 ATP synthase-specific factors exist in yeast. They are involved in transcription and translation of mtDNA-encoded subunits and in the assembly of the ATP synthase complex [26–29]. Much less is known about the mammalian enzyme where only 4 specific factors have been found so far. ATP11 and ATP12 are essential for the assembly of F_1 subunits α and β, similarly as their yeast homologues [30]. There is also a mammalian homologue of ATP23, yeast metalloprotease and chaperone of subunit 6 [27,31], but its function is not known. Recently, TMEM70 was identified as a novel factor of ATP synthase biogenesis in higher eukaryotes [32]. Its deficiency results in diminished amount of the full-size ATP synthase complex with detectable traces of the free F_1-part in some patients' tissues [33].

The biosynthesis and assembly of the F_1 catalytic part begins with the formation of $\alpha_3\beta_3$ oligomer catalyzed by ATP11 and ATP12 assembly factors [26] to which are then added subunits γ, δ and ε. It is not clear when and how exactly the subunit ε is inserted, but an ε null mutant of *S. cerevisiae* [17] indicated the presence of F_1 subcomplexes lacking also γ and δ subunits. Their expected size would be below that of $\alpha_3\beta_3\gamma\delta\varepsilon$ complex (ca. 370 kDa), and if they accumulate, they should be resolved by electrophoresis at native conditions. In our experiments with mammalian HEK293 cells, apparently neither ε-less F_1 molecules nor any smaller α/β-containing subassemblies could be detected after *ATP5E* silencing, indicating that either the lack of ε prevents their formation or such incomplete assemblies are very unstable and short-lived. This is in accordance with the observed low stability of F_1 in the ε null mutant in *S. cerevisiae* [17].

Another important finding of our study is that of unchanged content of subunit c in mitochondria upon *ATP5E* silencing. It demonstrates that in contrast to other ATP synthase subunits, the "excess" subunit c is not degraded. A major part of accumulated subunit c was resistant to solubilization with mild detergent DDM and likely represents insoluble and strongly hydrophobic subunit c aggregates. However, even the DDM-soluble fraction of *ATP5E*-silenced mitochondria was enriched in subunit c, this was resolved

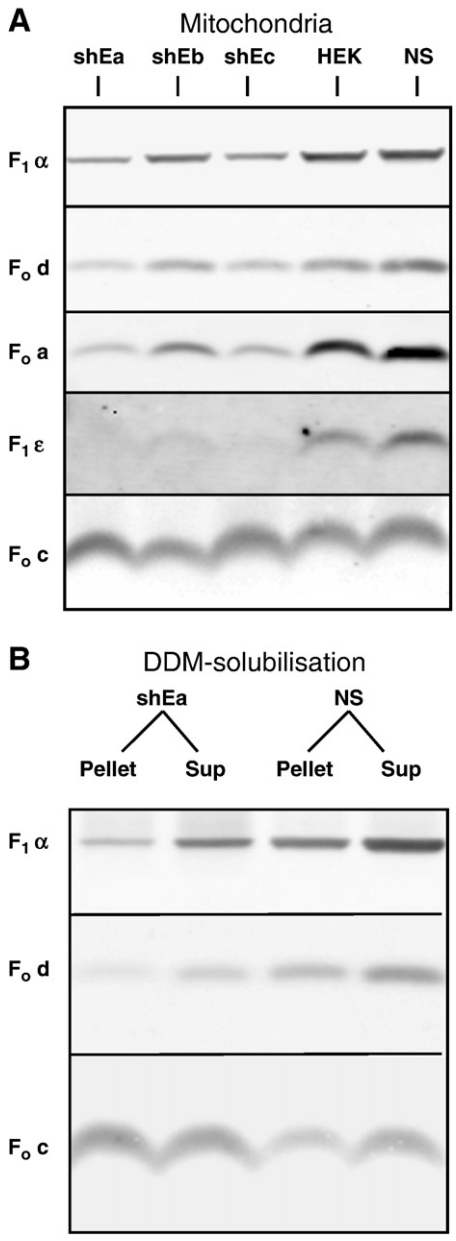

Fig. 3. Mitochondrial content of F_1 and F_o subunits in *ATP5E*-silenced cells. (A) Isolated mitochondria (10 μg protein aliquots) from silenced (shE2a, shE2b, shE2c) and control (HEK293, NS) cells and (B) 10 μg protein aliquots of 26000 ×*g* supernatant (Sup) and pellet (Pellet) from DDM-solubilized (2 g/g protein) mitochondria from silenced (shE2a) and control (NS) cells were analyzed by SDS-PAGE and WB. For detection antibodies to F_1 subunits α, and ε and F_o subunits a, d and c were used as indicated.

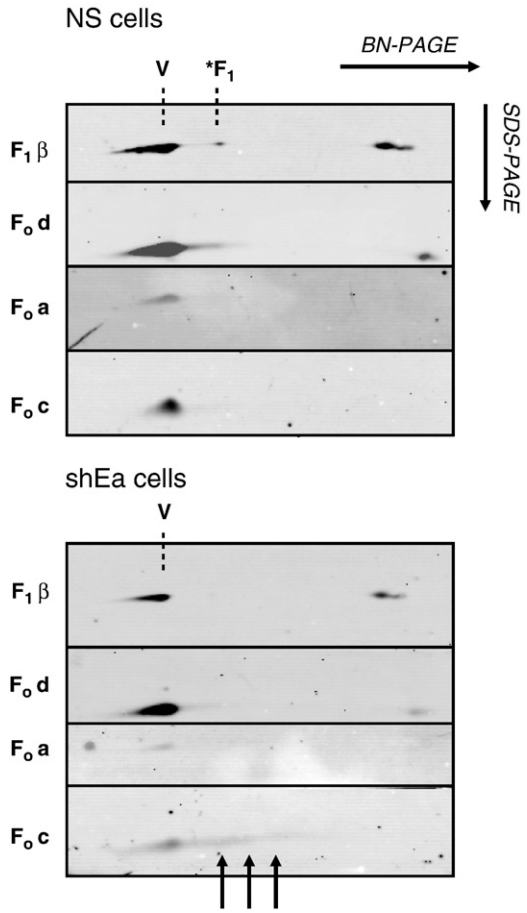

Fig. 4. Two-dimensional electrophoretic analysis of ATP synthase subunits in *ATP5E*-silenced cells. DDM-solubilized (2 g/g protein) proteins of mitochondria from shEa and NS cells were subjected to 2D electrophoresis and WB analysis was performed with indicated antibodies to ATP synthase subunits. V and $*F_1$ indicate position of ATP synthase monomer and F_1 subcomplex, arrows indicate accumulated subunit c.

by BN/SDS-PAGE in the second dimension corresponding to native complexes of about 200–400 kDa, which contained no F_1 subunits. Their origin is unclear at present, and they could represent breakdown products of subunit c oligomer attached to an unstable ε-lacking F_1 intermediate. However, their size is much larger than that of an oligomer of 10–12 copies of subunit c. Accumulated subunit c aggregates were also free of other F_o subunits, notably the subunit a, which is closely apposed to the c oligomer forming together the proton channel in the F_o structure. Apparently *ATP5E* silencing did not lead to enhanced proton conductivity of the inner mitochondrial membrane although a high proton leak was associated with ungated F_o structures in yeast ε null mutants [17]. Contrary to these findings, mitochondria of *ATP5E*-silenced cells showed an even tighter coupling than controls, in our work.

The accumulation of F_1 and larger assembly intermediates containing subunit c complexes of F_1 with subunit c oligomer were observed in various types of mammalian cultured cells or tissue samples with altered biogenesis of ATP synthase. They most likely represent dead-end products of a stalled assembly process resulting from a lack of mtDNA-encoded subunit a [34–36] or mutations in this subunit [36–38]. Upon *ATP5E* silencing in a human cell line, however, such intermediates are not present (Fig. 4). Interestingly, yeast F_1 mutants have been recently shown to inhibit translation of *ATP6* and *ATP8* mRNAs, but also in this case no F_1 or F_1-subunit c intermediates could be found in the *ΔATP15* strain [39].

Further studies are needed to resolve the properties and mechanism of subunit c accumulation, which is specifically induced by the absence of subunit ε and suggests a direct interaction and a regulatory role of ε in the assembly of ATP synthase rotor structure. Interestingly, no similar storage of subunit c could be found in mitochondria with ATP synthase deficiency of nuclear genetic origin due to mutations in *ATP12* [40] or *TMEM70* genes [32,41].

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