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Genetické a funkční příčiny mitochondriálních chorob vyvolaných defekty ATP syntázy

Genetic and functional characterisation of mitochondrial diseases caused by ATP synthase defects

Disertační práce

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ABSTRACT

Disorders of ATP synthase, the key enzyme of mitochondrial energy provision belong to the most severe metabolic diseases presenting mostly as early-onset mitochondrial encephalo-cardio-myopathies. Mutations in four nuclear genes can result in isolated deficiency of ATP synthase, all sharing a similar biochemical phenotype – pronounced decrease in the content of fully assembled and functional ATP synthase complex. The thesis summarises studies on two distinct causes of ATP synthase deficiency. First is TMEM70 protein, a novel ancillary factor of ATP synthase, which represents most frequent determinant of severe inborn deficiency of ATP synthase. TMEM70 is a 21 kDa protein of the inner mitochondrial membrane, facilitating the biogenesis of mitochondrial ATP synthase, possibly through TMEM70 protein region exposed to the mitochondrial matrix, but the proper regulatory mechanism remains to be elucidated. In TMEM70-lacking patient fibroblasts the low content of ATP synthase induces compensatory adaptive upregulation of mitochondrial respiratory chain complexes III and IV, interestingly by a posttranscriptional mechanisms.

The second type of ATP synthase deficiency studied was mtDNA *m.9205delTA* mutation affecting maturation of MT-ATP8/MT-ATP6/MT-CO3 mRNA and thus biosynthesis of Atp6 (subunit *a*) and Cox3 structural subunits. With the help of transmitochondrial cybrids with varying mutation load it was possible to elucidate gene–protein relationship of the pathogenic mechanism with mutation threshold close to homoplasmy. Characterisation of resulting enzyme deficiencies revealed pronounced decrease of cytochrome *c* oxidase biosynthesis contrasting with increased amount of structurally and functionally altered ATP synthase, unable to produce ATP.

Key words: Mitochondrial diseases, OXPHOS system, ATP synthase deficiency, ATP synthase biogenesis, TMEM70, mtDNA mutation, *MT-ATP6*, heteroplasmy, threshold effect.

ABSTRAKT

Poruchy ATP syntázy, klíčového enzymu mitochondriální tvorby energie, patří mezi velmi časté příčiny metabolických onemocnění, které se projevují nejčastěji jako mitochondriální encefalo-kardio-myopatie s časným nástupem nemoci. U pacientů s izolovaným defektem ATP syntázy byly nalezeny mutace ve čtyřech jaderných genech, se shodným biochemickým projevem – výrazně sníženým množstvím plně asemblovaného a funkčního komplexu ATP syntázy. V této práci jsou shrnuty výsledky studií proteinu TMEM70, nového pomocného faktoru v biogenezi ATP syntázy, jenž představuje nejčastější genetickou příčinu závažných vrozených poruch ATP syntázy. TMEM70 o velikosti 21 kDa je proteinem vnitřní mitochondriální membrány, který napomáhá tvorbě ATP syntázového komplexu. Funkce proteinu TMEM70 je nejspíše zprostředkována pomocí C-konce, exponovaného do mitochondriální matrix, ale přesný mechanismus stále není znám. Ve fibroblastech pacientů, které neobsahují protein TMEM70, je nízká aktivita ATP syntázy kompenzována zvýšením obsahu komplexů dýchacího řetězce III a IV pomocí posttranslačního mechanizmu.

Dalším typem defektu ATP syntázy, který byl v rámci této práce studován, je mtDNA mutace *m.9205delTA*, která ovlivňuje maturaci MT-ATP8/MT-ATP6/MT-CO3 mRNA a tím i biosyntézu strukturních podjednotek Atp6 (podjednotka *a*) a Cox3. Pomocí několika linií transmitochondriálních cybridů s různým množstvím mutace bylo odhaleno, že patogenní mechanismus se odehrává na úrovni gen–protein, a že tato mutace má vysokou prahovou hodnotu blížící se homoplazmii. Na enzymové úrovni byl zjištěn výrazný pokles tvorby cytochrom *c* oxidázy, který je v kontrastu se zvýšeným množstvím strukturně a funkčně pozměněné ATP syntázy, která není schopna tvořit ATP.

Klíčová slova: Mitochondriální onemocnění, OXPHOS systém, nedostatečnost ATP syntázy, biogeneze ATP syntázy, TMEM70, mutace mtDNA, MT-ATP6, heteroplazmie, prahový efekt.

Contents

Abbreviations

AA	amino acid
AAC	adenine nucleotide carrier; ADP/ATP carrier
Acetyl-CoA	acetyl-coenzyme A
ADP	adenosine di-phosphate
ATP	adenosine tri-phosphate
BAT	brown adipose tissue
BNE	blue native electrophoresis
CHD	choline dehydrogenase
CMT	Charcot-Marie-Tooth hereditary neuropathy
CoQ, Q	coenzyme Q
COX	cytochrome *c* oxidase
ETF-QO	electron-transferring flavoprotein-ubiquinone oxidoreductase
FAD	flavin adenine dinucleotid
$FADH_2$	reduced form of flavin adenine dinucleotid
Fe-S	iron-sulfur cluster
FMN	flavin mononucleotide
G3P	glycerol-3-phosphate
GPDH	glycerol-3-phosphate dehydrogenase
IMM	inner mitochondrial membrane
IMS	mitochondrial intermembrane space
LA	lactic acidosis
LRPPRC	leucine-rich pentatricopeptide repeat-containing protein
LS, LLS	Leigh or Leigh-like syndrome
mGPDH	mitochondrial glycerol-3-phosphate dehydrogenase
MILS	maternally inherited Leigh syndrome
MITRAC	mitochondrial translation regulation assembly intermediate of COX
mtDNA	mitochondrial DNA
mtEFTu	mitochondrial elongation factor Tu
mtPAP	mitochondrial poly(A) polymerase
mtSSB	mitochondrial single-stranded-DNA-binding protein
nDNA	nuclear DNA
NADH	reduced form of nicotinamide adenine dinucleotide
NARP	neurogenic muscle weakness, ataxia, and retinitis pigmentosa syndrome
OMM	outer mitochondrial membrane
OXPHOS	oxidative phosphorylation system
Pi	inorganic phosphate
PiC	inorganic phosphate carrier
POLG	polymerase γ
POLRMT	mitochondrial RNA polymerase
RITOLS	RNA incorporated throughout the lagging strand mechanism
ROS	reactive oxygen species
SCs	respiratory chain enzymes supercomplexes
SDH	succinate dehydrogenase
SDM	strand-displacement mechanism
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis

ssDNA	single-stranded DNA
TCA	tricarboxylic acid cycle
TEFM	transcription elongation factor of mitochondria
TFAM	mitochondrial transcription factor A
TIM	translocase of the inner mitochondrial membrane
TMEM70	transmembrane protein 70
TOM	translocase of the outer mitochondrial membrane
3-MGA	3-methylglutaconic aciduria

1. Introduction

1.1 Mitochondrion

Mitochondria are organelles of eukaryotic cells that originated from the endosymbiosis of α-protheobacteria with original, nucleus-containing eukaryotic cells (Gray M.W. et al., 1999). Most of the bacterial genome had been moved to the nucleus, but a small piece stay preserved in the mitochondria, and thus mitochondrial proteins are uniquely encoded by both nuclear (nDNA) and mitochondrial DNA (mtDNA).

Mitochondria are usually thin oval shape 0.5 – 1 μm long in diameter. Thanks to the two mitochondrial membranes and the fact that they can undergo a fusion/fission process, mitochondria create dynamic mitochondrial network within the cell. They function primarily as a cellular power station, since they produce more than 90 % of cellular ATP via oxidative phosphorylation system (OXPHOS), but they accommodate also numerous metabolic pathways. Tricarboxylic acid cycle (TCA cycle) and β oxidation of free fatty acids are the key processes of nutrient catabolism. Anabolic pathways take place in mitochondria too, including heme and phospholipids synthesis, as well as synthesis of mtDNA encoded subunits including transcription and translation of mtDNA. In addition, mitochondria are involved also in regulation of apoptosis, calcium homeostasis, reactive oxygen species (ROS) production or controlling redox status of the cell.

The structure of a single mitochondrion is shown in Figure 1. It is composed of mitochondrial outer membrane (OMM) and mitochondrial inner membrane (IMM), intermembrane space (IMS), and mitochondrial matrix. The outer mitochondrial membrane is smooth and partially permeable for molecules up to 5 kDa. Metabolite flow between cytosol and IMS is facilitated and regulated by voltage dependent anion-selective channel, VDAC protein. Since mitochondrial proteins encoded by nDNA are synthesized in cytosol, specific transporting system is required for the import of the proteins into appropriate mitochondrial compartment. Transport across the OMM is ensured by the machinery of several proteins, associated in the complex called translocase of the outer membrane, TOM complex. The intermembrane space, a space between the membranes, contains cytochrome *c*, one of the key components of OXPHOS system and also apoptotic signalling molecule. Contrary to the outer membrane, the inner mitochondrial membrane is impermeable. Proteins are transported through the translocase of the inner membrane, TIM complex.

Transport of metabolites and ions is facilitated by many specific channels and carriers. The inner membrane is thickly curved into the mitochondrial cristae, significantly increasing its surface area. Since the structure of cristae is dependent on the content of functional OXPHOS enzymes, especially ATP synthase, the remodelling of the cristae structure is a marker of mitochondrial quality. The inner membrane encloses the mitochondrial matrix, enzymatically most active part of the mitochondria.

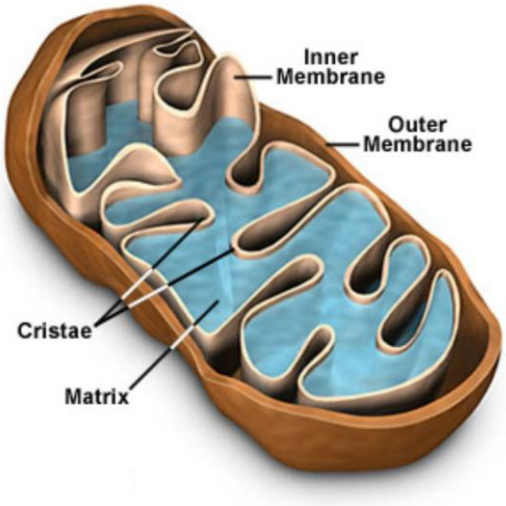


Figure 1: Structure of the single mitochondrion. *Mitochondrion is bounded by the outer mitochondrial membrane. Interior of the mitochondrion, the mitochondrial matrix, is enclosed by the inner mitochondrial membrane, which is curved and creates mitochondrial cristae. The space between the membranes is so called intermembrane space. Adapted from http://micro.magnet.fsu.edu/cells/mitochondria/mitochondria.html.*

1.2 Oxidative phosphorylation system

The main role of mitochondria is energy provision in the form of ATP. The synthesis of ATP takes place in the inner mitochondrial membrane, where NADH or $FADH_2$, the end products of the catabolism are oxidised by the respiratory chain complexes. Electrons from the substrates are transferred along respiratory chain complexes to oxygen, that is consumed and it can be detected as mitochondrial respiration. In the respiratory chain the electron-proton carriers (FMN, FAD, coenzyme Q) are alternated with the electron carriers (Fe-S clusters, cytochromes *b*, *c* and c_1) and the substrate oxidation is accompanied by the pumping of the protons from matrix to the intermembrane space. Generated proton gradient across the membrane (ΔpH) provides the energy for ATP synthase to phosphorylate ADP to ATP. Thus the oxidation of the substrates is coupled with the ADP phosphorylation and this process is called oxidative phosphorylation. Basically two major nutrients and thus

two major pathways generate substrates for the OXPHOS. The first major nutrients, sugars are degraded to pyruvate in the cytosol by the process of glycolysis. After the pyruvate is transported to the mitochondrial matrix, it is oxidative decarboxylated to acetyl-coenzyme A (acetyl-CoA). The second major nutrients, lipids are cleaved in the cytosol to glycerol and free fatty acids. Fatty acids are then transported into the matrix where they undergo β-oxidation, resulting in the production of acetyl-CoA, NADH and $FADH_2$. Both sugars and lipids metabolisms are linked via glycerol, that is converted to glycerol-3-phosphate (G3P). G3P is then oxidised by mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) to dihydroxyaceton phosphate, which can either enter the glycolysis or be reconverted to G3P via NADH-dependent cytosolic GPDH. In the matrix, acetyl-CoA from both sugars and lipids enters another important metabolic pathway of mitochondria, TCA cycle producing reduced substrates for the respiratory chain, NADH and $FADH_2$.

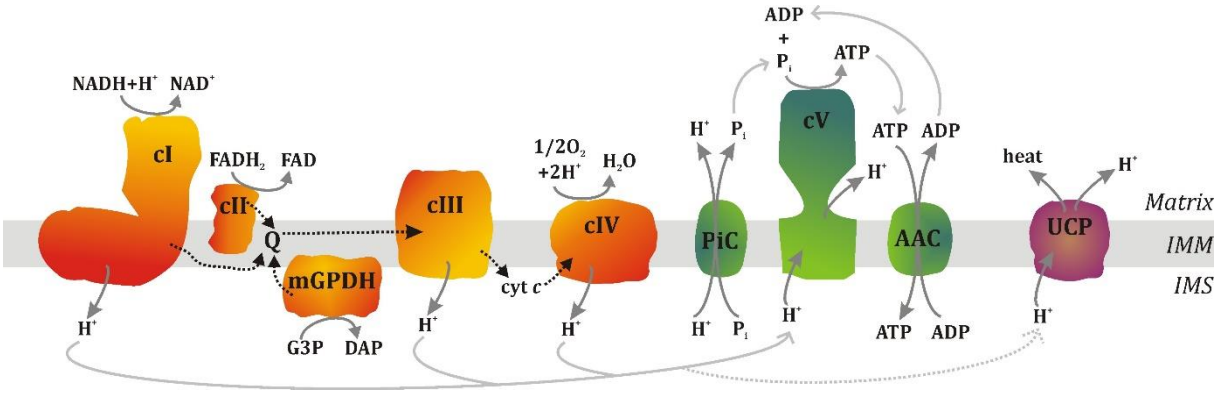


Figure 2: Mitochondrial OXPHOS system. *OXPHOS system is localised in the inner membrane. Substrates in the form of NADH or $FADH_2$ are oxidised on the matrix side of the membrane by respiratory chain complexes I or II (cI, cII). At the IMS side, glycerol-3-phosphate (G3P) is oxidised by mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) to dihydroxyaceton phosphate (DAP). The electrons then flow through complex III (cIII) to cytochrome c oxidase (cIV), where molecular oxygen is reduced to H_20. The transfer of electrons between individual complexes is ensured by mobile carriers coenzyme Q (Q) and cytochrome c (cyt c). Proton (H^+) gradient generated by respiratory chain is utilised by ATP synthase (cV) for the production of ATP. Phosphate carrier (PiC) and ADP/ATP carrier (AAC) provide substrates for the synthesis of ATP. Alternatively, the proton gradient is dissipated by uncoupling protein (UCP) in the form of heat.*

Several enzyme complexes with different functions are involved in the OXPHOS system (Fig. 2). The main components are four complexes of the respiratory chain (cI–cIV) and ATP synthase (complex V, cV). Mobile carriers coenzyme Q (ubiquinone ↔ ubiquinol) and cytochrome *c* (Fe^{3+} ↔ Fe^{2+}) are responsible for the transfer of electrons between

respiratory chain complexes. Mitochondrial glycerol-3-posphate dehydrogenase (mGPDH) is associated with the respiratory chain as another source of electrons for coenzyme Q (CoQ), especially in brown adipose tissue (BAT). Since the inner membrane is impermeable, transport of the substrates for ATP synthase is mediated by two carriers. Inorganic phosphate is transported into mitochondrial matrix by phosphate carrier (PiC) together with H^+. ADP/ATP carrier (AAC) facilitates the import of ADP to the matrix accompanied by export of ATP to the intermembrane space. In brown adipose tissue the proton gradient generated by respiratory chain can be also used by the uncoupling protein 1 instead of ATP synthase to produce heat.

1.2.1 Complex I

Mitochondrial complex I (NADH:ubiquinone oxidoreductase, NADH dehydrogenase) is the largest component of the respiratory chain with molecular mass about 1 MDa. In mammals it is composed by 45 subunits, 7 of which are encoded by mtDNA. The catalytic core of the complex is composed by 14 subunits that are conserved throughout evolution (Lazarou M. et al., 2009). These core subunits are organised in the L-shaped boot, with the peripheral arm consisting of 7 hydrophilic subunits oriented into the matrix, and the hydrophobic membrane arm composed by 7 hydrophobic, mtDNA encoded subunits (Hirst J., 2013). Flavin cofactor FMN and seven iron-sulphur (Fe-S) clusters are localised in the peripheral arm, while the CoQ is bound at the domains interface. The remaining complex I subunits, called supernumerary subunits (as they are missing in some evolutionary older forms of cI), vary widely between species and their roles are rather unknown. It is suggested they are involved in the assembly and stability of the complex, or in the protection from oxidative damage and ROS production. The supernumerary subunit GRIM-19 was described as a regulatory protein of apoptosis, suggesting the involvement of these subunits in other cellular processes (Hirst J., 2013). Based on the current knowledge, the assembly of mammalian complex I is a dynamic process, in which the complex oscillates between several intermediates and fully assembled complex I. Due to its large size and regulation by two genomes many helper proteins are expected to be required in the assembly of complex I. Several of them have already been described in mammals, NDUFAF1, NDUFAF2, NDUFAF3, NDUFAF4, NDUFAF6, ACAD9, AIF, C20orf7, C3orf1, Ecsit, FoxRed1, Ind1 and TMEM126B (Vahsen N. et al., 2004, Dunning C.J. et al., 2007, Vogel R.O.

et al., 2007a, Vogel R.O. et al., 2007b, Saada A. et al., 2008, Lazarou M. et al., 2009, Saada A. et al., 2009, Sheftel A.D. et al., 2009, Nouws J. et al., 2010, McKenzie M. et al., 2011, Zurita Rendon O. and Shoubridge E.A., 2012, Formosa L.E. et al., 2015). Mitochondrial ferredoxin Fdx2, involved in the biosynthesis of Fe-S clusters, is a putative unspecific assembly factor of complex I as well (Sheftel A.D. et al., 2010, Spiegel R. et al., 2014).

The function of complex I enables the oxidation of NADH linked substrates. The hydrogens from NADH are received by flavin cofactor FMN in the hydrophilic arm of the complex. Subsequently the electrons are passed to CoQ via series of Fe-S clusters, and the protons are pumped into the intermembrane space (Hirst J., 2013). Complex I is one of the key players in the mitochondrial ROS production (Cadenas E. and Davies K.J., 2000). Under the conditions when the electron flow from NADH to CoQ is inhibited, or when the reverse electron flow occurs, complex I leaks electron and these electrons reduce O_2 to superoxide or H_2O_2.

1.2.2 Complex II

Mitochondrial complex II (succinate:ubiquinone oxidoreductase, succinate dehydrogenase, SDH) consists of only four nDNA encoded subunits. Thus SDH complex of approximately 140 kDa is the smallest one of the respiratory chain complexes and the only one fully encoded by nuclear genome. Similar to complex I, complex II is composed of two domains, the hydrophilic head protruding to the matrix, and the hydrophobic tail anchoring complex II to the membrane (Rutter J. et al., 2010). The soluble part contains the two large complex II subunits, SDHA with covalently bound FAD cofactor, and SDHB containing three Fe-S clusters. The membrane domain is composed of SDHC and SDHD subunits, and of heme bound at the subunit interface. There are two CoQ binding sites – Q_P lying on the matrix side of the IMM, and Q_D placed closer to the IMS side of the membrane. Contrary to other OXPHOS complexes, assembly of SDH is not as complex, however, there should be specific ancillary factors for the proper assembly of the Fe-S clusters and FMN. Three complex II specific assembly factors (SDHAF1, SDHAF2 and SDHAF4) and three rather unspecific factors (Tcm62, Flx1 and Fdx2) were described (Rutter J. et al., 2010, Sheftel A.D. et al., 2010, Spiegel R. et al., 2014, Van Vranken J.G. et al., 2014).

Succinate dehydrogenase represents an important link between TCA and respiratory chain, since it functions in the both pathways. Succinate is oxidised to fumarate via flavin

cofactor FAD and electrons from $FADH_2$ are transferred to CoQ by three Fe-S clusters (Rutter J. et al., 2010). In contrast to other respiratory chain complexes, during the succinate oxidation the protons are not pumped to the IMS. Recently complex II was described to generate ROS under low succinate concentrations (Quinlan C.L. et al., 2012).

1.2.3 Complex III

Mitochondrial complex III (ubiquinol:cytochrome *c* oxidoreductase, bc_1 complex) is composed of 11 different subunits, of which only cytochrome *b* is mtDNA encoded. The functional form of complex III is a dimer with molecular mass of 480 kDa, embedded in the inner membrane. Only three subunits are involved in the electron transfer: cytochrome *b*, cytochrome c_1 and Fe-S cluster containing Rieske protein. The function of the remaining subunits is unclear, they are proposed to have supportive role (Fernandez-Vizarra E. et al., 2009). There are two binding sites for CoQ, Q_o at the IMS side of the membrane, and Q_i at the matrix side. The assembly of complex III is proposed to start with the formation of several independent subcomplexes (Fernandez-Vizarra E. et al., 2009). The process then continues with the association of these subcomplexes to the so called core subcomplex and by the addition of the remaining subunits. Several assembly factors for complex III were described in humans. First and best characterised is BCS1L protein (Fernandez-Vizarra E. et al., 2007), others recently found include TTC19, LYRM7/MZM1L, UQCC1, UQCC2 and UQCC3 (Ghezzi D. et al., 2011, Sanchez E. et al., 2013, Tucker E.J. et al., 2013, Wanschers B.F. et al., 2014). Fdx2 is thought to be involved in the complex III biogenesis, same as in the complex I and complex II assembly (Spiegel R. et al., 2014).

The function of complex III is ensured by the Q cycle, in which electrons from ubiquinol (generated by complexes I and II) are transferred to cytochrome *c* and as a result the protons are pumped into the IMS (Trumpower B.L., 1990). The two electrons from ubiquinol pass via two different ways. At Q_o site, the first electron is accepted by Fe-S cluster of Rieske protein, from which it is transferred via cytochrome c_1 to mobile cytochrome *c*. The second electron is accepted by cytochrome *b*, through which it is delivered to the Q_i site, where ubiquinone is reduced to ubiquinol, which is further oxidised in the Q_o site. Same as complexes I and II, also complex III is an important producer of ROS, specifically the Q_o site of the complex (Chen Q. et al., 2003).

1.2.4 Complex IV

Complex IV (cytochrome *c* oxidase, COX) is the terminal enzyme of respiratory chain, embedded in the membrane but partially extending to both the IMS and matrix. This ~200 kDa complex consists of 13 subunits in humans. The three largest mtDNA encoded subunits Cox1, Cox2, and Cox3 form the catalytic core of the enzyme. There are four redox centres; hem *a*, hem a_3, and two copper ions Cu_A and Cu_B. Subunit Cox1 is carrying the binuclear centre composed of hem a_3 and Cu_B and hem *a*. Cu_A and cytochrome *c* binding site are localised on Cox2 subunit. The ten small subunits (Cox4, Cox5a, Cox5b, Cox6a, Cox6b, Cox6c, Cox7a, Cox7b, Cox7c, and Cox8) encoded in the nuclear genome are involved in the assembly and regulation of the enzyme (Kadenbach B. et al., 1991). COX assembly proceeds stepwise through several proposed intermediates (S1–S4). Cox1 represents the first intermediate S1 which progresses to Cox1-Cox4-Cox5a subassembly and Cox2 then assumingly joins this intermediate S2. The process continues with the formation of intermediate S3 by the addition of Cox3 and most of the remaining subunits. The COX holoenzyme (S4) is completed by the addition of Cox7a/b and Cox6a to S3 as the last ones (Fornuskova D. et al., 2010). Over 20 specific factors were described in yeast complex IV assembly, mostly acting in the incorporation of hemes and copper ions to Cox1 and Cox2 subunits (Soto I.C. et al., 2012). Although many homologs of the yeast factors were found in humans, their function in human COX assembly was confirmed only in some cases. Of them, Cox10 and Cox15 are necessary for the biosynthesis and assembly of both heme *a* and heme a_3 (Stiburek L. et al., 2006). Assembly of the copper centres is assisted by Cox17, Cox19, Cox20, hCoa6, Pet191, Sco1 and Sco2 proteins (Leary S.C. et al., 2004, Oswald C. et al., 2009, Huigsloot M. et al., 2011, Leary S.C. et al., 2013, Bourens M. et al., 2014, Ghosh A. et al., 2014). Next COX-specific assembly factor with unclear function is Surf1 protein (Tiranti V. et al., 1998, Yao J. and Shoubridge E.A., 1999, Taanman J.W. and Williams S.L., 2001, Stiburek L. et al., 2005, Kovarova N. et al., 2012). Recently Pet100 protein was described to be involved in the COX assembly, perhaps in the early stages of the process (Olahova M. et al., 2014). Other yeast proteins serving as copper or heme *a* chaperones, whose homologs were found in humans are Cox11, Cox18, Cox23, Cmc1, Cmc2, Fdx2 and Adr (Soto I.C. et al., 2012). There are also several factors, required for the translation of mtDNA encoded subunits, ordinarily assigned as COX assembly factors. Cox14, hCoa3 and TACO1 are involved in the translation of Cox1

(Weraarpachai W. et al., 2009, Szklarczyk R. et al., 2012, Clemente P. et al., 2013), LRPPRC is involved in the stability of all three mtDNA encoded subunits Cox1, Cox2 and Cox3 (Mootha V.K. et al., 2003).

Cytochrome *c* oxidase catalyses the final step of electron transfer represented by the reduction of molecular oxygen to water. Electrons from cytochrome *c*, reduced by complex III, enter COX through the Cu_A centre, subsequently the electrons are transmitted to heme *a* and from here they are passed to the binuclear centre. The entry of the first two electrons is accompanied by the breaking of the atomic bond of the molecular oxygen, which is reduced by the next two electrons and reacts with the protons from the matrix to form a molecule of water. The reduction of oxygen is coupled with the pumping of protons from the matrix to the intermembrane space.

1.2.5 Other mitochondrial dehydrogenases

In mitochondria there are at least four additional flavin dehydrogenases which can serve as electron donors for CoQ pool (Huang S. and Lin Q., 2003, Nichols D.G. and Ferguson S.J., 2013). Electron-transferring flavoprotein-ubiquinone oxidoreductase (ETF-QO), localised on the matrix side of the inner membrane, serves as a short electron transfer pathway for electrons from several flavin-containing dehydrogenases, involved in the fatty acid oxidation or amino acid (AA) catabolism (Watmough N.J. and Frerman F.E., 2010). Second dehydrogenase localised on the matrix side of the IMM is choline dehydrogenase (CHD), that catalyses dehydrogenation of choline to betaine aldehyde. However, the putative role of CHD in donating electrons to CoQ needs further studies to validate this hypothesis (Salvi F. and Gadda G., 2013). Contrary to ETF-QO and CHD, dihydroorotate dehydrogenase involved in the pyrimidine biosynthesis is localised on the outer side of the inner mitochondrial membrane (Nichols D.G. and Ferguson S.J., 2013).

From the bioenergetics point of view the most interesting is mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH). Although the mGPDH research started several decades ago, the structure and function of the enzyme is not yet fully understood (Mracek T. et al., 2013). mGPDH was found in many tissues, but in a highly variable amount, being highest in mammalian brown adipose tissue and in insect fly muscles. Rat mGPDH is composed of the single subunit of 74 kDa, encoded by nDNA and associated to the oligomeric structures (Mracek T. et al., 2014). Based on the crystal structures of bacterial homologs, mGPDH is

suggested to be strongly associated with the inner membrane as a peripheral protein, at the IMS side of the membrane. In contrast with the rest of respiratory chain complexes, mGPDH is proposed to contain only one prosthetic group, FAD. Mitochondrial GPDH localised at the outer side of IMM together with cytosolic GPDH forms a glycerol-3-phosphate cycle, enabling oxidation of cytosolic NADH and serving as a link between oxidative phosphorylation and glucose and lipid metabolisms (Mracek T. et al., 2013). Oxidation of glycerol-3-phosphate in the intermembrane space to dihydroxyacetone phosphate is accompanied by the reduction of FAD to $FADH_2$, and electrons from $FADH_2$ are transferred to coenzyme Q. Mitochondrial GPDH is one of the key producers of ROS in mitochondria (Drahota Z. et al., 2002) and it was recently shown, that ROS is generated in mGPDH at the CoQ site (Mracek T. et al., 2009, Mracek T. et al., 2014).

1.3 Mitochondrial ATP synthase

1.3.1 Structure and assembly of F_1F_o ATP synthase

Proton translocating mitochondrial ATP synthase (F_1F_o-ATP synthase), the fifth enzyme complex (complex V) of OXPHOS system, is located in the inner mitochondrial membrane. ATP synthase complex of ~600 kDa is composed of 16 different subunits (*α*, *β*, *γ*, *δ*, *ε*, *a*, *b*, *c*, *d*, *e*, *f*, *g*, *A6L*, *F_6*, *OSCP* and *IF1*) organised into membrane-extrinsic F_1 catalytic part and membrane-embedded F_o part that are connected by two stalks (Walker J.E., 2013). Matrix oriented F_1 is formed by the $\alpha_3\beta_3$ hexamer and by central stalk subunits *γ*, *δ* and *ε*. The stalk subunits attach the F_1 to subunit *c*-oligomer (*c*-ring), occupying about half of the F_o. The F_o sector is further composed of subunits *a*, *e*, *f*, *g*, *A6L* and *b*. Subunits *b* and *A6L* extend to the matrix and together with subunits *d*, *F_6* and *OSCP* (oligomycin sensitivity conferring protein) form the peripheral stalk connecting the F_o part to F_1. Small regulatory subunit *IF1* binds to F_1 at low pH and prevents the enzyme to switch to a hydrolytic mode and hydrolyse ATP. Other proteins described to be associated with ATP synthase are membrane proteins *DAPIT* (Diabetes-Associated Protein in Insulin-sensitive Tissue, AGP) and 6.8 kDa proteolipid (*MLQ*) (Chen R. et al., 2007, Meyer B. et al., 2007, Ohsakaya S. et al., 2011). In mammals, only two ATP synthase subunits - *a* and *A6L* are encoded by mitochondrial DNA.

The assembly of ATP synthase is very complex process proceeding via several structural modules, but still only partly understood (Fig. 3). It starts with the independent formation

of $\alpha_3\beta_3$ hexamer and the ring of subunit *c*. Once the F_1 part is complete after addition of the subunits *γ*, *δ* and *ε*, it interacts with the newly formed *c*-ring. The ATP synthase assembly then follows by the addition of F_o and peripheral stalk subunits. The last added seem to be mtDNA encoded subunits *a* (Atp6) and *A6L* (Atp8), since almost complete ATP synthase complex was described in the cells lacking mtDNA (Wittig I. et al., 2010). The ATP synthase biogenesis is assisted by numerous, enzyme-specific factors that markedly differ between lower and higher eukaryotes. In *S. cerevisiae*, where the *c* subunit is also encoded by mtDNA (Atp9), several yeast-specific factors (Nca1-3, Nam1, Aep1-3, Atp10, Atp22 and Atp25) are involved in the F_o biogenesis, namely in mRNA stability, translation and processing of mtDNA encoded subunits Atp6 and Atp9 (Tzagoloff A. et al., 2004, Ackerman S.H. and Tzagoloff A., 2005, Zeng X. et al., 2007a, Zeng X. et al., 2008) or their assembly (Atp10, Atp22); however, none of them exists in mammals reflecting differences in structure of mitochondrial genes and expression of mtDNA-encoded subunits. Additional factor Atp23 (Osman C. et al., 2007, Zeng X. et al., 2007b) with metalloprotease/chaperone activity participates in processing of Atp6 and its association with *c*-oligomer, but there is only a partial homolog of Atp23 in mammals and its function remains unknown.

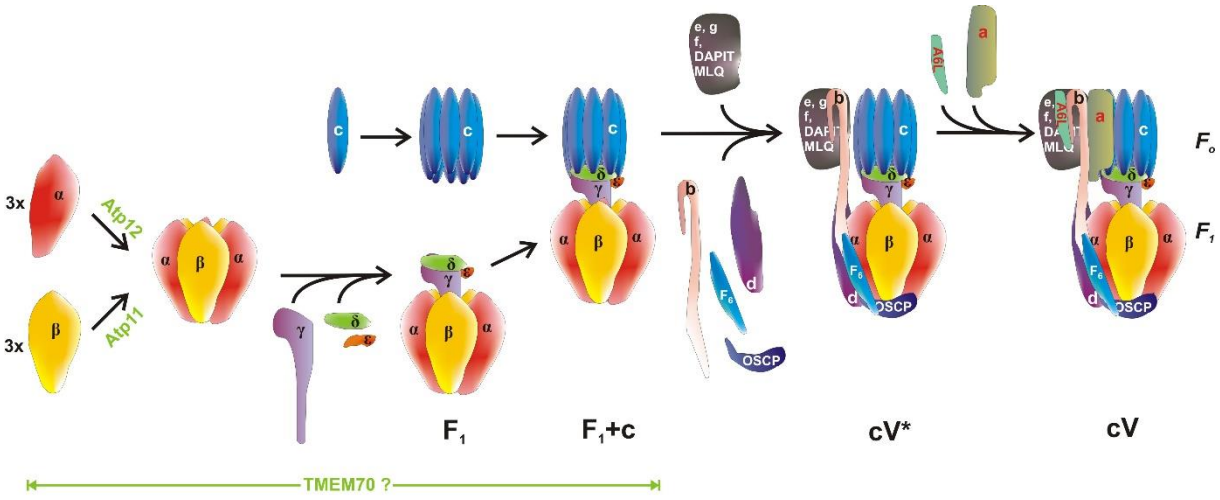


Figure 3. Mammalian ATP synthase assembly. *ATP synthase assembly starts with the independent formation of F_1 part (composed of subunits α, β, γ, δ and ε) and subunit c-oligomer, that are in the next step connected together to form F_1+c intermediate. Assembly then follows with the subsequent addition of the remaining subunits, the mtDNA encoded subunits a and A6L (in red) are added last. Assembly factors Atp11p and Atp12p interact with subunits β and α, respectively, during the formation of $\alpha_3\beta_3$ hexamer. TMEM70 protein is proposed to play a role in the early steps of mammalian ATP synthase assembly. cV* – almost complete ATP synthase complex lacking subunits a and A6L. cV – complete ATP synthase complex. Adapted from (Hejzlarova K. et al., 2014).*

Assembly of the yeast F_1 part depends on three additional factors, Atp11, Atp12 and Fmc1 (Ackerman S.H. and Tzagoloff A., 1990, Lefebvre-Legendre L. et al., 2001, Ackerman S.H. and Tzagoloff A., 2005). Only the first two have their homologs in humans (Wang Z.G. et al., 2001) interacting with the subunits *β* and *α*, respectively, during the $\alpha_3\beta_3$ hexamer formation (Fig. 3). Recently new ATP synthase ancillary factor was described (Cizkova A. et al., 2008, Houstek J. et al., 2009). Transmembrane protein 70 (TMEM70), specific for higher eukaryotes, is suggested to be involved in the early stages of ATP synthase assembly (Houstek J. et al., 1999).

1.3.2 Function of F_1F_o ATP synthase

ATP synthase operates as a unique molecular motor utilising proton gradient generated by the respiratory chain to synthesise ATP from ADP and P_i. During some conditions it operates in a reverse mode and uses ATP hydrolysis for translocation of protons out from the matrix.

In the synthetic mode, protons in the intermembrane space bind to subunit *a* and are passed to the *c*-ring. Key residues of this proton channel are best characterised for the yeast (*S. cerevisiae*) and bacterial enzymes (*E. coli*), while the studies on mammalian ATP synthase are less complex. The binding of protons and their transfer to subunit *c* is conveyed via yeast arginine 186, histidine 195 and glutamate 233 of subunit *a*, homologues to bacterial arginine 210, glutamate 219 and histidine 245 respectively. The protonation/deprotonation of subunit *c* takes place on yeast glutamate 59 or bacterial aspartate 61 (Devenish R.J. et al., 2000). Corresponding residues of human ATP synthase are arginine 159, histidine 168 and glutamate 203 of subunit *a*, and glutamate 119 of subunit *c*. As the protons pass at the interface of subunit *a* and *c*-oligomer, the proton gradient powers rotation of *c*-ring leading to the rotation of the subunits *γ*, *δ* and *ε* (Fig. 4). As *γ* rotates inside the $\alpha_3\beta_3$ hexamer it provides energy for ATP synthesis by a rotary catalysis explained by a "binding change mechanism" originally proposed by P. Boyer (Boyer P.D., 1975). Movements of *γ* induce sequential conformational changes of subunits *β*, and the three catalytic sites of F_1 cooperatively undergo three different states, open, loose and tight. At loose conformation ADP and Pi are bound, then *β* is converted to tight, closed state when adjacent ADP and P_i react and ATP is formed. Finally the catalytic site opens and ATP can be released. Since one complete 360° rotation of *γ* subunit is divided into three steps, one step should comprise 120°

rotation. In 2001 it was shown that the rotation is divided rather into four steps, two 120° steps, and one 90° and 30° substeps each (Yasuda R. et al., 2001), later corrected to 80° and 40° (Ueno H. et al., 2005, Masaike T. et al., 2008). Frequency of rotation reaches several hundreds per second and the efficacy of the process stems from the number of protons translocated per one rotation of the *c*-oligomer as for each copy of *c* subunit one proton is utilised. The most efficient is thus mammalian enzyme containing 8 subunits, while 10–15 are found in yeast and bacterial enzymes (Walker J.E., 2013).

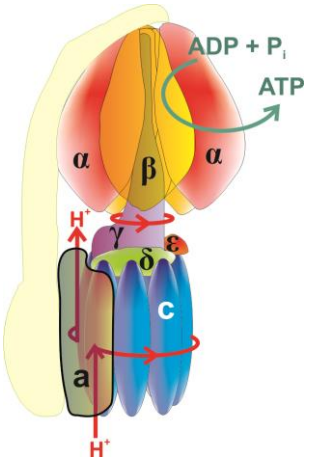


Figure 4. Production of ATP by mitochondrial ATP synthase. *Protons at the IMS side of the membrane are bound to subunit a and passed to subunit c. Binding of protons powers rotation of c-ring, that causes rotation of subunit γ. As γ rotates inside the hexamer it causes conformational changes of subunit β, required for interaction of ADP and P_i to create ATP.*

1.4 Supramolecular organisation of the OXPHOS system

Within last years, important progress has been achieved in understanding structural organisation of mitochondrial OXPHOS system composed of the membrane embedded respiratory chain enzymes, free mobile carriers (coenzyme Q, cytochrome *c*) and ATP synthase. For several decades two major theories have been discussed and tested about organisation of respiratory chain complexes in the inner mitochondrial membrane. According to a widely accepted random collision model of Hackenbrock, the individual complexes were localised in the inner membrane phospholipid matrix as a single moieties, free to diffuse laterally and independently of one another in the membrane plane (Gupte S. et al., 1984, Hackenbrock C.R. et al., 1986). In contrast, in the other model, respiratory chain complexes would be arranged as an ordered macromolecular assembly (Boumans H. et al., 1998) and numerous observations have been reported indicating that multicomplex units can be isolated (see (Rich P.R., 1984)).

Thanks to the advances in the methodology for isolation of the membrane complexes, namely the use of milder conditions for the protein solubilisation by non-ionic detergents and separation by native electrophoresis, in 2000 supramolecular organisation of OXPHOS complexes was described in yeast and mammalian mitochondria (Schagger H. and Pfeiffer K., 2000). In bovine heart mitochondria, complexes I, III and IV were found to associate into several different structures called supercomplexes (SCs). Thenceforth, supercomplexes were studied by several groups under different conditions, and nowadays SCs are generally accepted as one of the native forms of respiratory chain complexes. It was shown, that formation of SCs as well as their stability and function depend on the lipid composition of the membrane and, importantly, SCs have been found as individual functional units (Bianchi C. et al., 2004), containing cytochrome *c* and CoQ and capable to respire with different substrates (Acin-Perez R. et al., 2008). Since isolated complexes maintain their respiratory activity, SCs are not essential for the respiration, but they may increase efficiency of the electron flow by substrate channelling. Another important role of supercomplexes could be the defence against electron leak and ROS production, as both complex I and complex III dependent ROS production is increased under conditions where the formation of SCs is blocked. The presence of complex II in SCs was suggested by Acín-Pérez at al. (Acin-Perez R. et al., 2008) but remains questionable as recent study focused on the native forms of complex II argues rather against direct and stable association with other respiratory chain complexes (Kovarova N. et al., 2013). Complex II was found in higher structures partially overlapping with complex IV containing SCs and with dimeric ATP synthase, but only when using much milder conditions for solubilisation of the proteins than conditions under which the SCs are normally detected, suggesting the possible interaction with complex IV SCs is very weak and/or oligomers of complex II only comigrated with SCs during separation on native gels. In contrast, the interaction of complex II with ATP synthase was confirmed by coimmunoprecipitation of these two complexes. Interestingly, both complex II and ATP synthase were previously described as possible components of mitoK_{ATP} channel (Ardehali H. et al., 2004).

Under standard conditions of Blue native electrophoresis, ATP synthase was usually detected as monomer preserving its hydrolytic activity (Arnold I. et al., 1999). When the physiological state of ATP synthase in the membrane was studied using electron microscopy and clear native electrophoresis, ATP synthase complex was found predominantly

in homooligomeric structures, dimers and multiple oligomers, in a number of species including mammals (Arnold I. et al., 1998, Schagger H. and Pfeiffer K., 2000, Krause F. et al., 2005, Strauss M. et al., 2008). In cells where dimerisation/oligomerisation of ATP synthase is blocked an altered mitochondrial morphology was found, suggesting dimers and oligomers of ATP synthase are involved in the cristae formation of the inner mitochondrial membrane (Paumard P. et al., 2002, Habersetzer J. et al., 2013). It was shown, that ATP synthase dimers are formed via interaction of F_o parts of two ATP synthase monomers (Wittig I. et al., 2008). The angles between the ATP synthase monomers in the dimeric structure have been proposed to determine the curvature of the membrane. Basically two types of dimers can be resolved, with lower angle about 30–40° (Dudkina N.V. et al., 2006, Couoh-Cardel S.J. et al., 2010) and with higher angle above 70° that vary between species (Dudkina N.V. et al., 2006, Baker L.A. et al., 2012). The lower angle dimers represents rather unspecific associations of hydrophobic F_o raised in the conditions used for the solubilisation, or perhaps the angle can be reduced via binding of *IF1* to the F_1 heads (Minauro-Sanmiguel F. et al., 2005). In mammalian cells the dimers with angles about 80–90° are present (Davies K.M. et al., 2011, Baker L.A. et al., 2012). The role of ATP synthase dimers in the membrane cristae formation was recently got a boost by finding that the dimers are present in the rows along the membrane curvature (Strauss M. et al., 2008, Davies K.M. et al., 2011, Davies K.M. et al., 2012). Recently the F_o sector subunits *e* and *g* were confirmed to be necessary for the dimer formation and oligomerisation of mammalian ATP synthase (Habersetzer J. et al., 2013).

Besides homooligomers and mitoK_{ATP} mentioned above, ATP synthase is expected to be a part of mitochondrial ATP phosphorylating assembly called ATP synthasome (Ko Y.H. et al., 2003, Chen C. et al., 2004). This supercomplex is composed of ATP synthase and two carriers – ADP/ATP transporter (AAC) which exports matrix ATP and imports cytosolic ADP, and phosphate carrier PiC, facilitating import of phosphate anion from cytosol. The interaction of ATP synthase with its substrate providers can increase the efficiency of proton translocation linked to the production of ATP. Same as in case of respiratory chain supercomplexes, also detection of ATP synthasome depends on the conditions used for the solubilisation. Originally it was described in rat liver mitochondria only for the monomeric ATP synthase (Ko Y.H. et al., 2003). When bovine heart mitochondria and different detergents were used, the association of oligomeric ATP synthase and AAC was found (Wittig I. and Schagger H., 2008).

1.5 Mitochondrial genetics

During the evolution, most of mitochondrial genes were lost or displaced in the nucleus resulting in the most compact mtDNA in higher eukaryotes. In mitochondria genes for tRNAs and rRNAs are preserved, as well as few structural genes. The number of encoded polypeptides varies between species, however only a minor portion of mitochondrial proteins are encoded by mtDNA, all representing the structural subunits of OXPHOS system. Thus the expression and repair of all mitochondrial genes is controlled completely by nuclear DNA. However, functional mitochondrial genome is required for normal assembly and function of OXPHOS complexes.

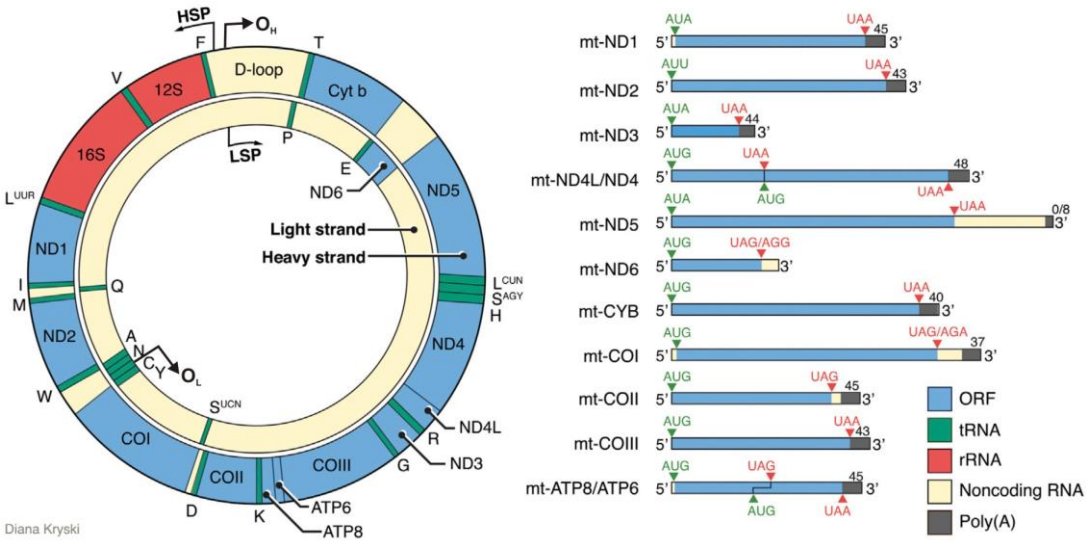


Figure 5. Structure of the mammalian mitochondrial genome. *Mitochondrial DNA is composed by two circular strand – Heavy strand and Light strand. It encodes 2 rRNAs (red boxes), 22 tRNAs (green boxes) and 11 mRNAs with 13 open reading frames (ORFs, blue boxes). D-loop, a long noncoding region on the Heavy strand, contains origin for its replication (O_H) and transcription (HSP). Similarly the Light strand contains origin for its replication (O_L) and transcription (LSP). The structure of the mature mRNAs with polyadenylation tails (Poly(A)) is shown in the right panel. Adapted from (Hallberg B.M. and Larsson N.G., 2014).*

Mammalian mitochondrial DNA is a 16 569 bp long double-stranded circular molecule with a modified genetic code. In contrast to nuclear genome, mtDNA is strictly maternally inherited, exists in multiple copies per cell and is highly prone to mutagenesis. MtDNA encodes 2 rRNAs, 22 tRNAs and 13 polypeptides, subunits of OXPHOS complexes (Taanman J.W., 1999). Its small size reflects low number of genes as well as absence introns (Figure 5). The two strands of mtDNA are named according sedimentation properties due to the guanine nucleotides content. The majority of mRNAs and tRNAs are synthesized by transcription of the

outer H (heavy) guanine rich strand. The inner L (light) guanine poor strand encodes only six tRNAs and one protein subunit (Figure 5). On the H strand a wide noncoding region of mtDNA called D-loop is involved in the initiation of replication and transcription. Mammalian mtDNA is packed into compact structures called nucleoids. Nucleoids have size about 100 nm and are thought to contain only single copy of mtDNA (Kukat C. et al., 2011). For the nucleoid packaging is essential mitochondrial transcription factor A (TFAM) (Bogenhagen D.F. et al., 2008). Many other proteins are thought to be associated with nucleoid structures. These are not only proteins involved in mitochondrial nucleic acids metabolism, such as mtSSB, Twinkle helicase, POLG, POLRMT, mtETFu, mitoribosomal proteins, LRPPRC, TACO1, CRIF1, but also proteins associated with mitochondrial dynamics and protein homeostasis, for example OPA1, YME1L, β-actin, Prohibitins 1 and 2, and Hsp70 (Hensen F. et al., 2014).

1.5.1 Replication of mtDNA

The mechanism of mtDNA replication is more complicated than originally proposed (Kasamatsu H. and Vinograd J., 1973) and still not fully understood. Currently there are at least three possible modes for this process in human; strand-displacement mechanism (SDM), RITOLS (RNA Incorporated ThroughOut the Lagging Strand) and strand-coupled mechanism (Holt I.J. et al., 2000, Bogenhagen D.F. and Clayton D.A., 2003, Yasukawa T. et al., 2006). The strand-coupled model was found only in the cells recovering from mtDNA depletion. In both SDM and RITOLS the replication starts with the formation of triple-stranded structure in the H strand origin (O_H), localised at D-loop (Fig. 5). The process then continues with the synthesis of the new H strand, with parental strand maintained in a single-stranded form (ssDNA). The synthesis of the L strand proceeds in the opposite direction from the O_L origin, with the different initiation of the L strand replication in SDM and RITOLS. Irrespective of the proper mechanism, three nuclear encoded proteins are necessary for replication of mtDNA (McKinney E.A. and Oliveira M.T., 2013). Unwinding of double-stranded DNA into single-stranded DNA is ensured by the replicative helicase Twinkle. The resulting ssDNA is then used by DNA polymerase γ (POLG) as a template for the synthesis of new mtDNA. As ssDNA is more prone to the degradation, it is protected from nucleolysis by association with mitochondrial single-stranded-DNA-binding protein (mtSSB). For the L strand replication an RNA primer formed by the transcription at the L strand promoter is required. This RNA transcript is created by the mitochondrial RNA polymerase (POLRMT).

1.5.2 Synthesis of mtDNA encoded proteins – mitochondrial transcription and translation

Mitochondrial DNA transcription by POLRMT results in the production of two long transcripts, one transcript from each strand. In the D-loop region there are transcription promoters for both H and L strands (Hallberg B.M. and Larsson N.G., 2014). In the initiation of transcription mitochondrial transcription factor A (TFAM) and mitochondrial transcription factor B2 (TFB2M) are involved. Recently transcription elongation factor of mitochondria (TEFM) has been identified to be required for synthesising promoter-distal transcripts from both strands (Minczuk M. et al., 2011). Once the transcripts are synthesised, they undergo a mitochondrial RNA processing. The first step of the processing is excision of tRNAs by RNAse P and tRNAase Z (ELAC2) (Szczesny R.J. et al., 2013). Since almost all mitochondrial rRNAs and protein coding sequences are separated by tRNAs (Fig. 5), excision of tRNAs results in the formation of pre-mRNAs, pre-rRNAs and pre-tRNAs. Polycistronic transcripts originating from the transcription of the mRNAs not flanked by tRNAs are further cleaved, but the mechanism remains unclear. However, mature MT-ND4L/MT-ND4 and MT-ATP8/MT-ATP6 transcripts are bicistronic, containing two partially overlapping open reading frames (Fig. 5). The maturation of pretranscripts includes adenylation of mRNAs, methylation of rRNAs, addition of the CCA sequence to the 3´end of tRNAs and other tRNAs modification (Szczesny R.J. et al., 2013). A number of proteins have been described to be involved in these processes (Hallberg B.M. and Larsson N.G., 2014), playing a role as the regulators of expression of mitochondrial encoded proteins, as will be discussed later.

Mitochondrial translation occurs on mitochondrial ribosomes (mitoribosomes) immediately after the transcription. Mammalian mitoribosome (55S) is composed of small mitoribosomal subunit (SSU, 28S) and large mitoribosomal subunit (LSU, 39S). SSU consists of 12S rRNA and more than 28 mitoribosomal proteins, LSU consists of 16S rRNA and about 50 mitoribosomal proteins (Hallberg B.M. and Larsson N.G., 2014). Both 12S and 16S rRNAs are encoded by mitochondrial DNA, while all mitoribosomal proteins are products of the nuclear genes. Since mitochondrial genetic code is somewhat different from the universal code, mitochondria have their own tRNAs. Synthesis of all mtDNA encoded proteins starts with the formylated methionine. Mature mitochondrial mRNAs contain start codon at or near to the 5´end of each mRNA, in addition the two bicistronic transcripts have an internal start codon.

As in nucleus, mitochondrial translation can be divided into several phases – initiation, elongation, termination, peptide release and mitoribosome dissociation, each requiring many ancillary factors. Two mitochondrial initiation factors mtIF2 and mtIF3 were found to be involved in the 55S dissociation, binding of mRNA and fMet-tRNA, and in 55S initiation complex reassembly (Christian B.E. and Spremulli L.L., 2012). In the elongation phase, which is the most conserved part of mitochondrial translation, three factors were described. Mitochondrial elongation factor Tu (mtEFTu) is necessary for the binding of aminoacyl-tRNA into the A-site of the mitoribosome, while mitochondrial elongation factor Ts (mtEFTs) promotes the exchange of mtEFTu-GDP for mtEFTu-GTP to refresh mtEFTu for the next elongation steps. Mitochondrial elongation factor G1 (mtEFG1) catalyses the removing of deacetylated tRNA from the P-site and transfer of the peptidyl-tRNA from the A-site to P-site. The final phase of the protein synthesis is the release of the nascent peptide from mitoribosome and the ribosome recycling. Mitochondrial mRNAs have four stop codons, which are recognised by mitochondrial release factors. There are four release factors found in mammalian mitochondria, mtRF1, mtRF1a, ICT1 and C12orf65 (Hallberg B.M. and Larsson N.G., 2014). The specific role of these factors is not fully understood. mtRF1, mtRF1a and ICT1 are suggested to recognise the stop codons, while the GGQ motif promoting termination by triggering hydrolysis of the peptidyl-tRNA bond was found in mtRF1a, ICT1 and C12orf65 sequences (Christian B.E. and Spremulli L.L., 2012, Hallberg B.M. and Larsson N.G., 2014). For the subsequent protein synthesis cycle the mitoribosome needs to be recycled through the disassembly of the two subunits and release of the deacetylated tRNA and mRNA. In mammalian mitochondria, this process is promoted by ribosomal recycling factors mtRRF1 and mtRRF2, which is known as elongation factor G2 (mtEFG2).

1.6 Biogenesis of OXPHOS system

As discussed in chapter 1.2., OXPHOS system is composed of several multisubunit complexes. Their biosynthesis is further complicated by the fact, that four of these complexes have dual genetic origin. It means that a part of the complex subunits is encoded by mitochondrial DNA, while the rest is encoded in the nucleus. Taken together, OXPHOS biogenesis is very complex process requiring cooperation of many factors. It is controlled not only at the level of the assembly of the individual complexes, that is quite complicated in itself,

but also by the coordination of the both genome expression, levels of expression etc. Many of the processes were extensively studied in the lower eukaryotes, especially in *Saccharomyces cerevisiae*. However, it is not easy to extrapolate the knowledge from studies in yeast to mammals, because of several differences between lower and higher eukaryotes. *S. cerevisiae* mtDNA encodes more OXPHOS subunits, mtDNA structure and mRNA expression is more complex and some proteins are posttranslationally modified. Furthermore, many yeast specific factors essential for mitochondrial biogenesis lack their homologs in mammals, or the respective proteins play different roles in the different species. In the recent years, the studies on mitochondrial biogenesis became focused more on the mammalian models. Although performed in the distinctly shorter time, thanks to the huge progress in the methodology and impact of human mitochondrial diseases, these studies rapidly improve our knowledge of biogenesis of mitochondrial proteins in mammals.

1.6.1 Regulation of expression of mitochondrial proteins

Different mammalian tissues have different energy demands and thus the levels of OXPHOS complexes vary within an organism. Strict coordination of expression of mitochondrial proteins is necessary for keeping the proper levels of proteins involved in the OXPHOS system to facilitate tissue specificity, but also to enhance the ability to respond to actual conditions. The activity of OXPHOS system is controlled via regulation of both nuclear and mitochondrial genes expression. As all components of mtDNA replication and expression are nuclear encoded, the biosynthesis of all mitochondrial proteins is under the control of the nuclear genes. The synthesis of mtDNA encoded proteins can be regulated at various levels. The basal indicator is mtDNA copy number, which varies between different tissues according to tissue-specific energy demands and in response to some physiological conditions. Thus mtDNA expression can be indirectly influenced by the expression of proteins involved in mtDNA replication. One of the pivotal regulators of mtDNA copy number in mammals is Twinkle helicase (Szczesny R.J. et al., 2013). Similarly, mtDNA transcription can be controlled by regulation of activity of the respective mtDNA transcription factors, mainly the POLRMT and TEFM (Rorbach J. and Minczuk M., 2012). Recently mitochondrial topoisomerase I was described as a negative regulator of mitochondrial transcription in mammals, but it is not clear whether it participates directly in the inhibition of transcription or if it decreases the stability of mitochondrial transcripts (Sobek S. et al., 2013).

As mitochondrial genes are usually transcribed with equal efficacy, regulation of the individual mRNAs levels is the main way how to regulate the biosynthesis/translation of individual proteins encoded by mtDNA. Human mitochondrial mRNAs transcribed form the heavy strand are polyadenylated at their 3´ends. Two proteins have been described as the main regulators of this process. Mitochondrial poly(A) polymerase (mtPAP) is essential for the polyadenylation (Tomecki R. et al., 2004, Nagaike T. et al., 2005), while the 2´phosphodiesterase PDE12 is responsible for the removing of poly(A) tails of mitochondrial transcripts (Rorbach J. et al., 2011). Polyadenylation is required for the formation of functional STOP codon of the mRNAs with U or UA at the 3´end (Anderson S. et al., 1981). Moreover, it was suggested that poly(A) tails are necessary for the heavy strand transcripts stability (Temperley R.J. et al., 2003). However, when the polyadenylation is decreased by silencing or depleting of mtPAP (Tomecki R. et al., 2004, Nagaike T. et al., 2005), or when poly(A) tails are removed by the cytosolic poly(A)-specific exoribonuclease (PARN) targeted to the mitochondria (Wydro M. et al., 2010), the stability of individual transcripts is affected in a different way. Similarly the deadenylation of the transcripts by PDE12 altered the steady-state levels of only some mitochondrial mRNAs (Rorbach J. et al., 2011). Both PARN and PDE12 stimulated deadenylation resulted in the inhibition of protein synthesis, but in these models the removal of functional STOP codons leading to the translation inhibition cannot be excluded. Nevertheless, the interference of the poly(A) tails by mitochondria targeted PABP1 did not shorten the transcripts but caused an inhibition of mitochondrial translation (Wydro M. et al., 2010). In the fibroblasts from patients with mtPAP mutation, the translation of several OXPHOS subunits was altered, but there was no strict correlation between decreased stability/levels of the transcripts and their respective protein products (Wilson W.C. et al., 2014).

Taken together, in human mitochondria the deadenylation is not sufficient for direct mRNA degradation, and the stability of the transcripts is controlled by another, yet unidentified factor(s). Hot candidates are RNA-binding proteins from PPR (pentatricopeptide repeat) protein family. Except POLRMT, LRPPRC (leucine-rich pentatricopeptide repeat-containing) protein is the most known PPR protein that was described to be associated with the human pathology based on the cytochrome *c* oxidase deficiency (Mootha V.K. et al., 2003), where the loss of COX activity was found to be caused by decreased translation of COX mtDNA-encoded subunits (Sasarman F. et al., 2014). Using mouse model, Xu et al. suggests that

LRPPRC is specific for the stability of Cox1 mRNA (Xu F. et al., 2012). However, the function of LRPPRC seems to be more universal, since it was found together with SLIRP protein to form a complex that is involved in the regulation of stability of all mature mitochondrial mRNAs (Sasarman F. et al., 2010). Furthermore, loss of LRPPRC was also associated with disrupted assembly of ATP synthase, caused probably by the lack of mtDNA-encoded subunit *a*, since specific ATP synthase subassemblies were found (Mourier A. et al., 2014). Another PPR protein, PTCD2 (pentatricopeptide repeat domain 2) is suggested to stabilise MT-CYB mRNA (Xu F. et al., 2008). Apart from proteins regulating expression of mtDNA in general, also specific factors are found for individual protein subunits and their genes, such as Cox14, hCoa3 and TACO1, mentioned earlier as "assembly" factors of cytochrome *c* oxidase, although they are rather translational factors required for the translation of Cox1 subunit (Weraarpachai W. et al., 2009, Szklarczyk R. et al., 2012, Clemente P. et al., 2013). However, no such a specific factor has been found in case of mtDNA-encoded subunits of ATP synthase.

Since the vast majority of mitochondrial proteins, including OXPHOS subunits, are encoded in the nucleus, for the OXPHOS biogenesis the regulation of expression of respective nDNA genes is crucial. There are several key nuclear factors and their coactivators, regulating the transcription of nuclear genes coding for mitochondrial proteins in general. They include nuclear respiratory factors NRF1 and NRF2 and estrogen-related receptor ERRα (Scarpulla R.C., 2006). These nuclear factors are activated via several other proteins, glucocorticoids and thyroid hormones. The central master activators are proteins from the peroxisome proliferator-activated receptor γ coactivator 1 family (PGC1), namely PGC1α, PGC1β and PRC (Austin S. and St-Pierre J., 2012). Apart from the general regulators described above, expression of mitochondrial proteins could be regulated in a specific way. For example in brown adipose tissue the amount of subunit *c* is limiting for the expression of the rest ATP synthase subunits (Kramarova T.V. et al., 2008).

1.6.2 Complex interactions among components of mitochondrial protein synthesis and assembly

Assembly of the multisubunit complexes with dual genetic origin in the inner mitochondrial membrane is the crucial step in the biogenesis of OXPHOS apparatus. Subunits encoded by nDNA and synthesized in cytosol need to be transported into mitochondria and assembled in to the membrane together with the subunits encoded by mtDNA

and synthesized in the mitochondrial matrix. Basic knowledge about these processes also came from the yeast studies. In yeast the synthesis of mtDNA encoded subunits takes place on the membrane-bound ribosomes, suggesting the cotranslational import of the proteins into the membrane. Mitochondrial translational products are then inserted into the membrane via several proteins, of them the best characterised is the Oxa1 (Herrmann J.M. and Neupert W., 2003). The association of mitoribosomes with the inner mitochondrial membrane was found also in mammals, and Oxa1L, human homolog of the yeast Oxa1, is suggested to bind to the mitoribosomes as well (Haque M.E. et al., 2010). Oxa1L is required for the proper biogenesis of respiratory chain complex I and ATP synthase, since its knockdown in human HEK293 cells leads to the impaired assembly of these two complexes (Stiburek L. et al., 2007). CRIF1 is the next mitochondrial protein, which was described to be associated with large mitoribosomal subunit (Kim S.J. et al., 2012). Mouse CRIF1 deficiency results in the decrease of the subunits of complexes I, III, IV and V, and fully assembled complexes were completely lacking. Since the only unaffected was complex II, composed of solely nuclear-encoded subunits, CRIF1 protein is probably essential for the synthesis and insertion of all mtDNA encoded subunits.

In yeast it was found, that accumulation of the early assembly intermediates of cytochrome *c* oxidase containing assembly factors Coa1, Coa3 and Cox14 lead to the reduction of Cox1 translation, representing the link between COX assembly and Cox1 mRNA translation. Recently so called MITRAC complexes were described in humans, containing not only COX subunits and specific factors for Cox1 assembly and translation, but also Tim21 protein (Mick D.U. et al., 2012). Tim21 is a part of TIM23 complex, involved in the transport and insertion of proteins from cytosol across/to the inner mitochondrial membrane. Taken together, MITRAC complex represents the link between the transport of mitochondrial proteins from cytosol, the assembly of the inner membrane complexes and the regulation of translation of mitochondrial mRNAs. The interaction of Tim21 with complex I assembly intermediates and stable association with MITRAC15 suggests, that these two proteins are involved in the assembly of at least complex I and complex IV and can have more general function in the OXPHOS biogenesis. MITRAC12, a yeast Coa3 ortholog, was found in the MITRAC complex with opposite effect to the Cox1 translation than yeast Coa3, suggesting the link between assembly and translation of mitochondrial complexes is evolutionary conserved, but comprising different factors with diverse functions.

1.7 Mitochondrial diseases

As mitochondria are the main "producer" of energy in the living cells, defects in mitochondrial metabolism are frequent cause of metabolic diseases. Mitochondrial dysfunctions affect mostly tissues with high energy demands such as heart, brain, liver and skeletal muscle. OXPHOS system defects are associated with broad range of clinical phenotypes ranging from early onset severe and devastating encephalo-cardio-myopathies to late onset and milder forms of mitochondrial diseases, but also with polygenic neurodegenerative diseases (Alzheimer disease, Parkinson disease), complex diseases (obesity, metabolic syndrome) or aging. Due to a dual genetic origin of OXPHOS system, mitochondrial diseases can be caused by mutations in both nuclear and mitochondrial genes. Mutations in the maternally-transmitted mtDNA are already extensively characterised. Several hundreds of them have been described to date (MITOMAP inventory, www.mitomap.org, (Ruiz-Pesini E. et al., 2007)) and their detection and screening has become rather routine task (DiMauro S., 2007). However, according to current estimates, mtDNA mutations are responsible for only 25 % of mitochondrial diseases. The remainder originates from mutations in nuclear genes, and their identification and characterisation represents major challenge of current mitochondrial research. Within the last couple of years pathogenic mutations resulting in a mitochondrial disease have been uncovered in ~ 110 nuclear genes (Vafai S.B. and Mootha V.K., 2012) including numerous novel factors of mitochondrial biogenesis (e.g. TMEM70 (Cizkova A. et al., 2008, Houstek J. et al., 2009), C12orf65 (Antonicka H. et al., 2010, Weraarpachai W. et al., 2012) C20orf7 (Sugiana C. et al., 2008), TACO1 (Weraarpachai W. et al., 2009), RMND1 (Janer A. et al., 2012), SDHAF1 (Ghezzi D. et al., 2009), SDHAF2 (Hao H.X. et al., 2009)). Nevertheless, many of the disease-causing genes from more than thousand nuclear genes contributing to the mitochondrial proteome (www.mitocarta.com (Pagliarini D.J. et al., 2008)) still remain to be identified.

1.7.1 Mitochondrial DNA mutations

Mutations in mitochondrial DNA are often associated with severe metabolic defects in newborns, and to a lesser extend also with neurodegenerative syndromes, deafness, optic neuropathy and other diseases (MITOMAP). Human pathologies are caused by mutations in structural genes mutations as well as tRNA and rRNA genes. Mutations in tRNA and rRNA typically result in combined OXPHOS defects, as they affect synthesis of mtDNA encoded

proteins in general, while mutations in structural genes are associated with isolated defects of the respective enzyme whose subunit is mutated. About 280 mtDNA mutations in structural genes and about 300 mutations in tRNA or rRNA were described, involving point mutations and simple deletions or insertions. In most cases mtDNA mutations are missense and cause replacement of respective amino acid. Less often premature Stop codon is created or shift in the frame can occur due to nonsense or frameshift mutations. In addition to maternally inherited point mutations also more than a hundred of mainly somatic deletions or insertions of large mtDNA regions were described.

Clinical and biochemical phenotypes of the patients with mtDNA mutations can differ substantially in the cases harbouring the same mutation. This can reflect different nuclear genetic background of the patients, but most often the reason is mtDNA heteroplasmy, coexistence of wild type and mutated DNA within a single cell which typically contains several thousands of copies of mtDNA. The degree of mutation load determines the pathogenic phenotype. Importantly, the relationship between heteroplasmy and disease progression is often not linear and exerts a threshold, when the disease starts to manifest. This is usually achieved when number of mutated copies reaches 80–90 %.

Homoplasmy is on the other hand the state when all mtDNA copies are identical. While the most pathogenic mtDNA mutations are usually heteroplasmic, homoplasmy is usually found in case of mutations with very mild phenotypes not affecting the key life functions.

1.7.2 Mitochondrial DNA mutations associated with ATP synthase defects

Isolated ATP synthase deficiency is frequently caused by mutations in mtDNA encoded subunit *a* (*MT-ATP6*) while *A6L*, the other subunit of ATP synthase coded for by *MT-ATP8* gene is seldom affected. The majority of them are missense mutations in mtDNA *MT-ATP6* gene (summarised in Table 1) associated with severe brain, heart and muscle disorders with early-onset, but also with deafness, multiple sclerosis, autism, optic neuropathy and diabetes in combination with other mtDNA mutations. Contrary to *MT-ATP6*, in *MT-ATP8* gene only six mutations were found in heart and brain diseases, usually found in combination with other possibly pathogenic mutations. Finally, three cardio-myopathic mutations in *MT-ATP6/MT-ATP8* overlapping region affecting both genes or only *MT-ATP8* were described. These single mutations are summarised in Table 1.

Genetics of MT-ATP6 mutations

The most common *MT-ATP6* mutations are in nt position 8993, *m.8993T>G* (p.L156R) or *m8993T>C* (p.L156P), manifesting usually as milder Neurogenic muscle weakness, Ataxia, and Retinitis Pigmentosa syndrome (NARP) or more severe Maternally Inherited Leigh Syndrome (MILS) (Holt I.J. et al., 1990, Puddu P. et al., 1993, Houstek J. et al., 1995, Uziel G. et al., 1997, Vazquez-Memije M.E. et al., 1998, Rubio-Gozalbo M.E. et al., 2000, Carelli V. et al., 2002, Sciacco M. et al., 2003, Enns G.M. et al., 2006, Morava E. et al., 2006, Cortes-Hernandez P. et al., 2007, Craig K. et al., 2007, Mkaouar-Rebai E. et al., 2009, Vazquez-Memije M.E. et al., 2009, Kara B. et al., 2012). In case of T>G transition the severity of symptoms seems to be heteroplasmy-dependent, other mtDNA variations and additional factors play a role as well. T>C transition is not as usual but the symptoms are rather milder and late-onset. Similar features were described for the next common transitions at nt 9176, *m.9176T>G* (p.L217R) and *m.9176T>C* (p.L217P) associated with Leigh disease or Familial Bilateral Striatal Necrosis (Thyagarajan D. et al., 1995, Carrozzo R. et al., 2001, Akagi M. et al., 2002, Hung P.C. and Wang H.S., 2007, Vazquez-Memije M.E. et al., 2009, Ronchi D. et al., 2011). T>C transition was found in the patients with Charcot-Marie-Tooth hereditary neuropathy (CMT), the disease with a very mild course (Synofzik M. et al., 2012), and with the late-onset Hereditary spastic paraplegia (Verny C. et al., 2011). Yet another mutation usually associated with Leigh syndrome, *m.9185T>C* transition was recently described in CMT patients (Moslemi A.R. et al., 2005, Castagna A.E. et al., 2007, Saneto R.P. and Singh K.K., 2010, Pitceathly R.D. et al., 2012). Other rare *MT-ATP6* mutations (*m.8597T>C, m.8839G>C*, *m.8851T>C*, *m.8969G>A, m.8989G>C, m.9035T>C, m.9191T>C,*) present as Leigh or Leigh-like syndrome, NARP or cardio-myopathy (De Meirleir L. et al., 1995, Moslemi A.R. et al., 2005, Sikorska M. et al., 2009, Tsai J.D. et al., 2012, Blanco-Grau A. et al., 2013, Duno M. et al., 2013, Honzik T. et al., 2013, Burrage L.C. et al., 2014).

Distinct phenotypes of different *MT-ATP6* mutations are related to the mutation load, but with variable relationships between heteroplasmy and phenotypic presentation. In many cases lower or higher threshold for the disease progression was found. However, in some cases patients with very low heteroplasmy were described, suggesting the threshold effect is not always linked with mtDNA mutations. Although several studies were performed on cybrid cell lines with different heteroplasmy to resolve this problem, the results are not consistent and the question about the threshold in *MT-ATP6* mutations remain unanswered.

The asymptomatic family members often have mutation load lower than the affected patients, however the implicated threshold mutation level for the disease manifestation varies. The best example of phenotypic dependence on mutation load represents *m.8993T>G* transition – the severity of the disease increases with mutation load and a milder NARP manifests at lower heteroplasmy (around 70 %) than early-onset devastating MILS (around 90 %). In some cases the severity of symptoms in 8993 patients was found to be heteroplasmy-dependent but without distinct threshold level for the disease manifestation (Puddu P. et al., 1993, Carelli V. et al., 2002, Baracca A. et al., 2007) or even with a linear correlation between mutation load and biochemical parameters (Sgarbi G. et al., 2006).

Table 1: Single *MT-ATP8/6* and *MT-ATP6* mutations associated with isolated ATP synthase disorders. *MT-ATP6* mutations associated with human pathologies in combination with other mtDNA mutation are not included.

Gene	Mutation	Clinical phenotype	References
MT-ATP8/6	*m.8528T>C* missense (p.W55R; p.M1T)	Infantile cardio-myopathy	(Ware S.M. et al., 2009)
	m.8529G>A nonsense; silent (p.W55X; p.M1M)	Apical HCMP	(Jonckheere A.I. et al., 2008)
MT-ATP6	*m.8597T>C* missense (p.I24T)	LS	(Tsai J.D. et al., 2012)
	m.8668T>C missense (p.W48R)	LHON	(Kumar M. et al., 2010)
	m.8719G>A nonsense (p.G65X)	Suspected mitochondrial disease	(Tang S. et al., 2013)
	m.8836A>G missense (p.M104V)	LHON	(Abu-Amero K.K. and Bosley T.M., 2006)
	m.8839G>C missense (p.A105P)	NARP	(Blanco-Grau A. et al., 2013)
	m.8851T>C missense (p.W109R)	BSN, LS, ataxia	(De Meirleir L. et al., 1995, Honzik T. et al., 2013)
	m.8950G>A missense (p.V142I)	LHON	(Abu-Amero K.K. and Bosley T.M., 2005)
	m.8969G>A missense (p.S148N)	MLASA	(Burrage L.C. et al., 2014)

MT-ATP6	*m*.8989G>C missense (p.A155P)	NARP	(Duno M. et al., 2013)
	m.8993T>G missense (p.L156R)	NARP, LS	(Holt I.J. et al., 1990, Puddu P. et al., 1993, Houstek J. et al., 1995, Uziel G. et al., 1997, Carelli V. et al., 2002, Enns G.M. et al., 2006, Morava E. et al., 2006, Cortes-Hernandez P. et al., 2007, Mkaouar-Rebai E. et al., 2009, Vazquez-Memije M.E. et al., 2009)
	m.8993T>C missense (p.L156P)	NARP, LS, ataxia	(Rubio-Gozalbo M.E. et al., 2000, Sciacco M. et al., 2003, Morava E. et al., 2006, Cortes-Hernandez P. et al., 2007, Craig K. et al., 2007, Vazquez-Memije M.E. et al., 2009, Kara B. et al., 2012)
	m.9035T>C missense (p.L170P)	Ataxia	(Sikorska M. et al., 2009)
	m.9101T>C missense (p.L222P)	LHON	(Lamminen T. et al., 1995)
	m.9176T>G missense (p.L217R)	LS	(Carrozzo R. et al., 2001, Akagi M. et al., 2002)
	m.9176T>C missense (p.L217P)	LS, CMT, BSN, Hereditary spastic paraplegia	(Thyagarajan D. et al., 1995, Hung P.C. and Wang H.S., 2007, Vazquez-Memije M.E. et al., 2009, Ronchi D. et al., 2011, Verny C. et al., 2011, Synofzik M. et al., 2012)
	m.9185T>C missense (p.L220P)	LS, CMT, Motor neuron syndrome, ataxia	(Moslemi A.R. et al., 2005, Castagna A.E. et al., 2007, Saneto R.P. and Singh K.K., 2010, Pitceathly R.D. et al., 2012, Brum M. et al., 2014)
	m.9191T>C missense (p.L222P)	LS	(Moslemi A.R. et al., 2005)
	m.9205delTA frameshift; splicing (p.X227M)	Encephalo-myopathy, LA	(Seneca S. et al., 1996, Jesina P. et al., 2004)

BSN, Bilateral Striatal Necrosis; CMT, Charcot-Marie-Tooth hereditary neuropathy; HCMP, Hypertrophic Cardio-MyoPathy; LA, Lactic Acidosis; LHON, Leber Hereditary Optic Neuropathy; LS, Leigh Syndrome; LVNC, Left Ventricular Non-Compaction cardio-myopathy-assoc.; MIDD, Maternally Inherited Diabetes and Deafness; MLASA, Mitochondrial myopathy, lactic acidosis, and sideroblastic anemia; NARP, Neurogenic muscle weakness, Ataxia, and Retinitis Pigmentosa.

Biochemistry of MT-ATP6 mutations

Subunit *a* is necessary for the proton translocation and thus for ATP synthesis, and is thought to stabilise ATP synthase complex. Mutations in *MT-ATP6* usually affect the efficiency of proton translocation leading to the reduced production of ATP (Pallotti F. et al., 2004, Sgarbi G. et al., 2006, Baracca A. et al., 2007, Sikorska M. et al., 2009, Vazquez-Memije M.E. et al., 2009, Blanco-Grau A. et al., 2013). In some cases the underlying mechanism may be increased ROS production (Baracca A. et al., 2007, Sikorska M. et al., 2009, Verny C. et al., 2011).

As shown by native electrophoresis, the stability of ATP synthase complex is affected by *MT-ATP6* mutations as well. In fibroblasts from patients with either mutated or missing subunit *a* the content of full size ATP synthase was strongly decreased and instead, incomplete forms of enzyme, F_1 containing subcomplexes accumulated (Houstek J. et al., 1995, Nijtmans L.G. et al., 1995, Carrozzo R. et al., 2006, Smet J. et al., 2009). In ρ^0 cells devoid of mtDNA these subcomplexes originate from almost complete ATP synthase complex lacking subunits *a* and *A6L*, which falls apart during BNE (Wittig I. et al., 2010).

In 1996, Seneca et al. found a new type of mtDNA mutation that affects *MT-ATP6* and *MT-CO3* genes by microdeletion of two bases TA in mtDNA at positions 9205–9206 (*m.9205delTA*). This mutation removes the STOP codon of *MT-ATP6* gene and alters the splicing site for processing of the polycistronic MT-ATP8/MT-ATP6/MT-CO3 transcript. Mutation *m.9205delTA* can be expected to alter the levels of MT-ATP6 and MT-CO3 transcripts and thus the synthesis of subunit *a* (Atp6) of ATP synthase and Cox3 subunit of cytochrome *c* oxidase, which could limit the biogenesis of these two respiratory chain complexes. The first case with *m.9205delTA* mutation presented with a relatively mild phenotype – seizures with several episodes of transient lactic acidosis (Seneca S. et al., 1996). Analysis of patient fibroblasts with reported homoplasmic mutation revealed no changes in MT-ATP6 and MT-CO3 mRNAs processing, significant increase in deadenylation of MT-ATP8/MT-ATP6 bicistron, and relatively insignificant biochemical changes (Temperley R.J. et al., 2003, Chrzanowska-Lightowlers Z.M. et al., 2004). The second case of *m.9205delTA* mutation was a child with severe encephalopathy and hyperlactacidemia (Jesina P. et al., 2004). In accordance with the fatal clinical course, the patient fibroblasts showed a pronounced alteration of ATP synthase structure and a low activity and protein content of COX resulting in a ~70 % decrease in mitochondrial ATP synthesis. There was a marked and

specific decrease in MT-ATP8/MT-ATP6/MT-CO3 primary transcript processing. Subunit *a* content and its de novo synthesis were reduced 10-fold when compared to the other ATP synthase subunits. Both cases were reported to be homoplasmic.

1.7.3 Nuclear DNA mutations leading to the mitochondrial dysfunction

Nuclear DNA mutations are frequent cause of mitochondrial metabolism disorders. In 1995, Bourgeron et al. (Bourgeron T. et al., 1995) reported the first defect in a nuclear gene resulting in a mitochondrial respiratory chain deficiency – mutation in *SDHA* gene affecting complex II, succinate dehydrogenase. As most of OXPHOS disorders are transmitted as autosomal recessive traits, the number of recognised nuclear disease-causing genes was growing rapidly and by 2001, nuclear genetic defects had been reported in all electron transporting complexes of mitochondrial respiratory chain (Shoubridge E.A., 2001). Since then, increasing interest in nuclear genes constituting mitochondrial proteome further stimulated progress in identification of affected nuclear genes encoding either structural proteins or biogenetic and regulatory factors of OXPHOS machinery – so called “direct and indirect hits” (Dimauro S., 2011). As described in OMIM database, most of the OXPHOS structural genes mutations were found in complex I, the largest respiratory chain complex, where mutations affecting at least 16 subunits from total 45 were found (OMIM 252010, OMIM 256000). In addition, mutations of 8 complex I assembly factors were described. Mutations in all 4 complex II subunits and in 1 specific assembly factor were described to be associated with human pathologies (OMIM 252011, OMIM 185470, OMIM 602413 and OMIM 602690). In case of complex III, mutations in 4 structural genes and in 5 genes coding for complex III assembly factors are known (OMIM 124000). Only 4 mutated structural genes of cytochrome *c* oxidase contrast with high number of mutations in COX assembly factors and other COX-related proteins, where 13 of them have been found to be mutated in patients with different clinical symptoms (OMIM 220110, OMIM 309801, OMIM 612714, OMIM 615119, OMIM 300887, OMIM 616039).

Another important group represent mutations of factors involved in the mtDNA stability, replication and expression. These are for example Polymerase γ POLG mutations, Twinkle helicase or succinate-CoA ligase (alfa subunit SUCLG1, beta subunit SUCLA2) mutations. In recent years a growing number of patients with mutated mitochondrial tRNA synthetases (YARS2, SARS2, DARS2, RARS2) have been reported. Direct linkage between

OXPHOS deficiency and nuclear gene mutations can be found also in case of mutated proteins involved in the iron-sulfur cluster assembly (BOLA3, ISCU) or coenzyme Q biosynthesis (CoQ2, CoQ4, CoQ9). Yet another cause of mitochondrial diseases is represented by mutations in the genes for proteins with chaperone function (HSPD1), proteins involved in the fusion/fission processes (MFN2, OPA1) or in mitochondrial metabolism (pyruvate dehydrogenase subunit E1-alfa).

1.7.4 Nuclear DNA mutations associated with ATP synthase defects

At present, inborn and isolated disorders of ATP synthase are associated with four nuclear genes, two of which code for the structural subunits α and ε while the other two encode biogenetic factors Atp12 and TMEM70 that are not a part of the enzyme structure. All these mutations share a similar biochemical phenotype with pronounced decrease in the content of fully assembled and functional ATP synthase. However, their incidences, mechanism of molecular pathogenesis, clinical manifestation, as well as the course of the disease progression, differ substantially (Table 2). Up to now, only two patients with mutations in *ATP5A1* and one patient each with mutations in *ATP5E* and *ATPAF2* genes were reported pointing to the rareness of these disorders. In contrast, the number of patients with mutations in *TMEM70* gene is steadily increasing. Apparently *TMEM70* gene is highly prone to mutagenesis and this type of rare mitochondrial disease has relatively frequent incidence.

Mutations in ancillary factors of ATP synthase – ATPAF2 and TMEM70

The first indication that ATP synthase dysfunction could be linked to a nuclear gene came from the study of Holme et al. (Holme E. et al., 1992) who were unable to find any mtDNA mutation in a child with cardio-myopathy, lactic acidosis (LA), persisting 3-methylglutaconic aciduria (3-MGA) and severely decreased activity of ATP synthase. Seven years later and in another patient, Houštěk et al. (Houstek J. et al., 1999) demonstrated by the use of mitochondrial cybrids that mitochondrial disease presenting as early onset neonatal and fatal LA, cardio-myopathy and hepatomegaly due to a 70 % isolated decrease of ATP synthase complex was of nuclear origin. Number of similar patients have been later described (Sperl W. et al., 2006) by the joint effort of several European mitochondrial centres that focus specifically on putative disorders of ATP synthase. In all of them DNA sequencing excluded mutations in genes for structural subunits.

In 2004 De Meirleir et al. (De Meirleir L. et al., 2004) described one patient with severe

neonatal encephalopathy who harboured missense mutation in Atp12 protein, an assembly factor essential for incorporation of *α* subunit into F_1-ATPase structure. A homozygous *c.280T>A* mutation in *ATPAF2* gene coding for Atp12 was found in a girl with dysmorphic features, cortical-subcortical brain atrophy followed by basal ganglia atrophy and metabolic acidosis, who died at the age of 14 months. In liver mitochondria, severe reduction in native complex V without accumulation of F_1 containing subcomplexes was discovered. Significantly reduced content of individual ATP synthase subunits suggested that F_1 assembly could be disturbed at the early stage and the unassembled subunits were rapidly degraded. The TGG>AGG transition caused replacement of evolutionary conserved neutral tryptophan in position 94 to a basic arginine (p.W94R), probably affecting the Atp12 interaction with *α* subunit. Both parents and unaffected sibling were heterozygous carriers of the mutation. With the help of a yeast model, it was later shown that this mutation affects the solubility of Atp12 protein, with the mutated form showing tendency to aggregate (Meulemans A. et al., 2010).

In 2008, we used the homozygosity mapping and sequencing of candidate genes in other known patients with ATP synthase deficiency, severe neonatal lactic acidosis and encephalo-cardio-myopathy and identified TMEM70 as another disease-causing gene. The splicing site *c.317–2A>G* mutation at the end of the second intron of *TMEM70* gene preventing the synthesis of TMEM70 protein was found in 24 cases including the first patient reported in 1999 (Cizkova A. et al., 2008).

TMEM70 was first described in 2006 by Calvo et al. (Calvo S. et al., 2006) as a gene coding for potentially mitochondrial protein. Since TMEM70 patients have very low levels of fully assembled ATP synthase and accumulate some F_1 containing subassemblies, TMEM70 was recognised as a new biogenetic factor of ATP synthase (Cizkova A. et al., 2008). However, the proper mechanism of TMEM70 function remained to be elucidated.

Analysis of mutations in affected patients represents an important step towards better understanding of TMEM70 involvement in the biogenetic mechanism. Within the last years, numerous other *TMEM70* mutations have been described with a broad spectrum of phenotypes (Table 2). *TMEM70* mutations associated with isolated ATP synthase deficiency are either homozygous or compound heterozygous, parents and healthy siblings of the patients are usually heterozygous carriers of the respective mutation(s).

Table 2: Nuclear DNA mutations associated with isolated deficiency of ATP synthase.

Gene	Mutation	Clinical phenotype	References
ATP5A1	*c.985C>T* missense (p.R329C) (*c.–49+418C>T* substitution*)*	Severe neonatal encephalopathy	(Jonckheere A.I. et al., 2013)
ATP5E	*c.35A>G* missense (p.Y12C)	Neonatal respiratory distress, LA, 3-MGA, severe peripheral neuropathy, exercise intolerance	(Mayr J.A. et al., 2010)
ATPAF2	*c.280T>A* missense (p.W94R)	3-MGA, LA, neonatal encephalopathy, dysmorphism	(De Meirleir L. et al., 2004)
TMEM70	*c.317–2A>G* splicing	IUGR, LA, HA, EOH, FD, HCMP, 3-MGA, cataract, encephalopathy, FTT, PMR, persistent PAH	(Cizkova A. et al., 2008, Wortmann S.B. et al., 2009, Honzik T. et al., 2010, Tort F. et al., 2011, Torraco A. et al., 2012, Stojanovic V. and Doronjski A., 2013, Catteruccia M. et al., 2014, Magner M. et al., 2014, Diodato D. et al., 2015)
	c.317–2A>G/ c.118_119insGT frameshift (p.S40CfsX11)	LA, 3-MGA, HA, HCMP, FD, PMR,	(Cizkova A. et al., 2008, Honzik T. et al., 2010, Cameron J.M. et al., 2011, Magner M. et al., 2014)
	c.317–2A>G/c.494G>A missense (p.G165D)	LA, 3-MGA, HA, HCMP, Reye-like syndrome, exercise intolerance	(Scaglia F. et al., 2002, Shchelochkov O.A. et al., 2010)
	c.336T>A nonsense (p.Y112X)	IUGR, LA, HA, HCMP, FD, PMR	(Spiegel R. et al., 2011, Magner M. et al., 2014)
	c.316+1G>T splicing	IUGR, Encephalopathy, HCMP, EOH, LA, FD	(Spiegel R. et al., 2011, Magner M. et al., 2014)
	c.238C>T nonsense (p.R80X)	IUGR, EOH, LA, 3-MGA, HA, HCMP, multiorgan failure, dysmorphism,	(Spiegel R. et al., 2011, Magner M. et al., 2014, Diodato D. et al., 2015)

TMEM70	*c.578_579delCA* frameshift (p.N198X)	IUGR, EOH, LA, 3-MGA, FD, cataract, Encephalopathy, HCMP, PMR	(Spiegel R. et al., 2011, Magner M. et al., 2014)
	c.211–450_317–568del (2290bp deletion) frameshift	IUGR, HCMP, LA, 3-MGA, PMR	(Tort F. et al., 2011)
	g.2436–3789 in-frame deletion (1353bp)	IUGR, LA, HA, HCMP, PMR, ptosis	(Jonckheere A.I. et al., 2011)
	c.317–2A>G/c.628A>C missense (p.T210P)	HCMP, LA, 3-MGA, HA, resistent or persistent PAH, WPW	(Torraco A. et al., 2012, Catteruccia M. et al., 2014, Magner M. et al., 2014)
	c.535C>T missense (p.Y179H)	IUGR, LA, EOH, FD, HCMP, bilateral cataract, PMR, HA	(Atay Z. et al., 2013, Magner M. et al., 2014)
	c.317–2A>G/ c.349_352del frameshift (p.I117A, p.224X?)	IUGR, LA, PMR, HCMP, EOH, dysmorphism, HA	(Diodato D. et al., 2015)
	c.317–2A>G/c.783A>G frameshift (p.X261Wext17)	IUGR, LA, PMR, HCMP, dysmorphism	(Diodato D. et al., 2015)
	c.701A>C missense (p.H234P)	IUGR, LA, 3-MGA, PMR, HCMP, HA, EOH, dysmorphism, leuko-encephalopathy, persistent PAH	(Catteruccia M. et al., 2014, Diodato D. et al., 2015)
	c.317–2A>G/c.251delC	Hypoglycemic seizures, epilepsy	(Magner M. et al., 2014)
	c.317–2A>G/c.470T>A	n.a.	(Magner M. et al., 2014)
	c.359delC	n.a.	(Magner M. et al., 2014)

n.a., not available; 3-MGA, 3-methylglutaconic aciduria; EOH, Early-Onset Hypotonia; FD, facial dysmorphism; FTT, Failure To Thrive; HA, hyperammonemia; HCMP, Hypertrophic Cardio-myopathy; IUGR, Intrauterine growth retardation; LA, Lactic Acidosis; PAH, pulmonary arterial hypertension; PMR, Psychomotor Retardation; WPW, Wolf-Parkinson-White pre-excitation syndrome.

The most common and originally described is the homozygous *c.317–2A>G* mutation that removes the splicing site prior to the third exon and results in the generation of multiple incomplete and labile transcripts preventing synthesis of the protein. It has already been

described in 48 patients (Cizkova A. et al., 2008, Wortmann S.B. et al., 2009, Honzik T. et al., 2010, Tort F. et al., 2011, Torraco A. et al., 2012, Stojanovic V. and Doronjski A., 2013, Catteruccia M. et al., 2014, Magner M. et al., 2014, Diodato D. et al., 2015) and other cases are known but have not been formally reported. Absence of TMEM70 protein in homozygous patients typically presents as early-onset and severe LA, 3-MGA, hyperammonemia, hypertrophic cardio-myopathy, dysmorphism, hypotonia, ataxia, failure to thrive and psychomotor retardation. Out of 24 cases reported in 2008, about half died within the first few years and mostly in the first months of life. On the other hand some patients can survive significantly longer, with three of them currently reaching 13, 14 and 18 years. As pointed out by a detailed retrospective clinical study (Honzik T. et al., 2010), if the patient survived the critical postnatal period of the first weeks and months of life, the metabolic problems and cardiac disorders may improve. Till now, only one patient presented with rather late disease manifestation at the age of 3 years as a mild form of 3-MGA without hyperammonemia during the metabolic crisis (Stojanovic V. and Doronjski A., 2013).

Another two homozygous mutations were reported resulting in putative aberrant TMEM70 protein lacking 34 AA in the region 71–105. The consequence of these mutations could be inefficient import of TMEM70 into mitochondria or defective membrane assembly. Two siblings, harbouring *c.316+1G>T* splicing site mutation, presented with typical features described above but without 3-MGA and they died after 10 days and 5 months respectively. (Spiegel R. et al., 2011). Contrary to *c.316+1G>T* patients, the patient carrying homozygous *g.2436–3789* in-frame deletion with psychomotor retardation survived much longer; he was reported at the age of 6 years (Jonckheere A.I. et al., 2011).

Four additional homozygous nonsense or frameshift mutations creating premature stop codon and leading to the synthesis of potentially truncated TMEM70 have been reported. Two siblings with symptoms similar to other TMEM70 patients plus bilateral cataract harboured frameshift *c.578_579delCA* deletion resulting in a putative 197 AA long protein lacking almost two thirds of the C-terminus. Surprisingly their survival is remarkably different; one is 24 years old while the second died at 3.5 years (Spiegel R. et al., 2011). Three *TMEM70* mutations predicted to result in very short TMEM70 protein (112 AA at maximum) were described in four patients. Missense *c.336T>A* mutation (Spiegel R. et al., 2011) and *c.211–450_317–568del* frameshift mutation (Tort F. et al., 2011) were found in one patient each. Both patients presented with hypertrophic cardio-myopathy, metabolic acidosis, mental

retardation and facial dysmorphism at the age of 1 and 7 years, respectively. The last nonsense mutation *c.238C>T* had severe phenotype with encephalopathy, cardio-myopathy, hypotonia and metabolic acidosis. One patient died 7 days old (Spiegel R. et al., 2011), while the second was reported at 1 year of age (Diodato D. et al., 2015). The malfunction of the three short variants of TMEM70 protein is not surprising as even the longest one (112 AA) only reaches till the first half of the first transmembrane domain.

The common *c.317–2A>G* mutation can also be found as compound heterozygous in combination with other *TMEM70* mutations. Two patients were described carrying *c.317–2A>G* and *c.118_119insGT* frameshift mutation resulting in premature stop codon and truncated TMEM70 protein p.S40CfsX11 (Cizkova A. et al., 2008, Cameron J.M. et al., 2011). Both patients show typical TMEM70 symptoms and they are alive. Another three patients have a combination of *c.317–2A>G* with a missense mutations, such as *c.494G>A* changing neutral glycine 165 to acidic aspartate at the C-terminus (Shchelochkov O.A. et al., 2010) or *c.628A>G* changing highly conserved threonin 210 to prolin (Torraco A. et al., 2012, Catteruccia M. et al., 2014). The clinical outcome of the patient reported by Shchelochkov et al. presented as Reye-like syndrome was mild, while both patients reported by Torraco et al. are strongly affected by metabolic acidosis and cardio-myopathy. Two other compound heterozygous mutations affecting the C-terminus of TMEM70 protein were found in combination with the common mutation (Diodato D. et al., 2015). Frameshift microdeletion *c.349_352delC* is predicted to change isoleucin 117 to alanin and result in a truncated protein shorter of 36 AA, while *c.783A>G* missense mutation is predicted to change the stop codon to tryptophan and result in an aberrant protein with 17 extra AA at the C-terminus. Although the patients suffer from severe encephalo-cardio-myopathy, the onset of the disease was quite late, 8 month and 2 years, respectively.

Only two homozygous missense mutations were described so far. The first is *c.535C>T* mutation, which changes the highly conserved tyrosine to a histidine at position 179 at the beginning of the C-terminus (Atay Z. et al., 2013, Magner M. et al., 2014). The second missense mutation *c.701A>C* is changing conserved histidine 234 to proline (Catteruccia M. et al., 2014, Diodato D. et al., 2015). Both patients presented with typical phenotypes such as cardio-myopathy, hypotonia and metabolic crisis, and with less frequent arterial hypertension.

From 48 patients whose clinical outcomes were discussed in the study of Magner et al. (Magner M. et al., 2014), ten patients were not previously reported. Seven of them were

homozygous for the common *c.317–2A>G* mutation (included in the above mentioned total count), one was homozygous for the novel *c.359delC* microdeletion, and the remaining two patients were compound heterozygotes, harbouring two novel mutations in combination with the common mutation: microdeletion *c.251delC* and *c.470T>A* mutation. Interestingly, none of the patients with neonatal onset surviving to the age five years as well as none of the patients with later onset died, so far.

Although the changes in the quantity and structure of TMEM70 protein were not specifically addressed in most of the case reports, from the described mutations it appears that majority of the genetic defects lead to the absence of this factor or the synthesis of incomplete truncated forms, lacking either a part of import sequence (AA 1–81) or a major part of the second transmembrane domain and/or C-terminal sequence. A combination of splicing site and missense mutation could lead to the synthesis of sole TMEM70 protein with amino acid replacement if the splice variant mRNA was unstable and degraded. Interestingly both such missense mutations, p.G165D and p.T210P affect the C-terminal region of the protein, same as both homozygous missense mutations p.Y179H and p.H234P.

Mutations in structural subunits of ATP synthase α and ε

It was not unexpected that isolated ATP synthase deficiency, similarly as isolated disorders of other OXPHOS complexes can also be caused by a "direct hit". In 2008 our search for mutated gene in 25 patients with ATP synthase deficiency revealed TMEM70 mutation in all but one patient (Cizkova A. et al., 2008) who also differed by rather mild and distinct phenotype (Sperl W. et al., 2006). Subsequent sequencing of ATP synthase genes detected a mutation in *ε* subunit of F_1 part as the first mutation in nuclear-encoded subunit of the enzyme (Mayr J.A. et al., 2010). Three years later mutation in another structural subunit of F_1 was discovered, in this case affecting *ATP5A1* gene, coding for *α* subunit (Jonckheere A.I. et al., 2013).

A nuclear mutation affecting *ε* subunit of ATP synthase was found in 22 years old patient. Clinical phenotype started with early-onset lactic acidosis, 3-MGA, but no cardiac involvement, followed by mild mental retardation, exercise intolerance and peripheral neuropathy. Sequencing of *ATP5E* gene coding for subunit *ε* uncovered homozygous missense mutation *c.35A>G* replacing highly conserved tyrosine 12 with cysteine (Mayr J.A. et al., 2010). In patient fibroblasts an insufficient capacity of ATP synthase was indicated. Surprisingly, the

mutated subunit ε depressed biosynthesis of ATP synthase but it was incorporated in the enzyme complex without altering its functions. The reduction of assembled 600 kDa complex V was not accompanied by the presence of F_1 subcomplexes. In the patient's fibroblasts all enzyme subunits were correspondingly reduced with the exception of subunit *c*, which was not degraded. When *ATP5E* was knocked down in HEK293 cells (Havlickova V. et al., 2010) a similar phenotype was found – isolated decrease of fully functional ATP synthase complex corresponding to the low levels of subunit *ε* and accumulation of subunit *c*. Changes in ATP synthase structure and function due to the mutation and downregulation of *ε* subunit therefore indicated an essential role of *ε* subunit in the biosynthesis and assembly of the F_1 part of ATP synthase. Moreover, *ε* subunit seems to be involved in the incorporation of subunit *c* into the rotor structure of the mammalian enzyme.

Most recently, the first mutation was found in *ATP5A1* structural gene for subunit *α* in two siblings of nonconsanguineous parents presenting with severe neonatal encephalopathy (Jonckheere A.I. et al., 2013). Both patients died at the first week of life and extensive damage of brain structures was accompanied by multiorgan lesions indicative of mitochondrial disease. Fibroblasts of both patients showed decreased oxygen consumption with unusually high activation by an uncoupler and isolated defect in ATP synthase hydrolytic activity. Strong reduction in specific content of fully assembled complex V without F_1 subcomplexes accumulation was accompanied by the reduced levels of individual ATP synthase subunits. In both siblings exome sequencing detected heterozygous *c.985C>T* mutation in *ATP5A1* gene that changed conserved basic arginine to neutral cysteine at position 329. The arginine 329 is predicted to be involved in the interaction between *α* and *β* subunits, thus Arg>Cys replacement can destabilise the $\alpha_3\beta_3$ hexamer. Observed missense mutation in subunit *α* indicates defect in the early stage of F_1-ATPase assembly when $\alpha_3\beta_3$ hexamer is formed. *ATP5A1* mutation revealed remarkable genetics. Healthy father was heterozygous carrier for the mutation, while mother expressed only the wild type sequence. Extensive analyses of the sequences and mRNA expression of *ATP5A1* alleles of both parents suggested that the maternal allele was inherited by both affected children but its expression was silenced. Therefore the pathogenic phenotype was dominated by the paternal mutated allele. The reason for altered expression of the maternal allele remains unknown. The only polymorphism found was *c.-49+418C>T* variant in the first intron, circa 5.4 kb from the core promoter of *ATP5A1* gene but there is no indication that it could affect the gene regulation.

2. Aims of the study

Genetic defects of mitochondrial ATP synthase represent a frequent cause of severe mitochondrial diseases affecting paediatric patients. These disorders are associated with the dysfunction of proteins involved in the mitochondrial biogenetic apparatus, as well as with the defects of the structural components of the ATP synthase enzyme complex.

Our knowledge of the underlying biochemistry is still limited and thus it represented main focus of this thesis. Major objectives were: (i) To elucidate molecular pathogenic mechanisms responsible for several types of inborn defects of mitochondrial ATP synthase detected in paediatric patients, in collaboration with 1st Medical Faculty, Charles University in Prague, Paracelsus University in Salzburg and Vrije Universiteit in Brussel; (ii) To further characterise the basic mechanisms and components of ATP synthase biogenesis, *de-novo* synthesis and enzyme assembly with the help of various cellular models of ATP synthase dysfunction.

Specific aims of the thesis were:

- To search for the genetic cause of severe metabolic disorders of fatal neonatal cardio-myopathy due to isolated ATP synthase deficiency of nuclear-genetic origin.

- To test whether such ATP synthase defect can be "compensated" for by upregulation of other respiratory chain enzymes.

- To characterise structure and function of TMEM70 protein, the novel ancillary factor of mammalian ATP synthase, with respect of membrane topology and possible interaction with other proteins.

- To uncover factors responsible for the different manifestation of unique mtDNA *m.9205delTA* mutation, which affects the expression of subunit *a* (Atp6) of ATP synthase and subunit Cox3 of cytochrome *c* oxidase.

3. Summary of the results

This thesis is based on 6 publications, all of which have been already published. Four publications are focused on the discovery and characterisation of TMEM70 protein, the novel factor involved in the ATP synthase biogenesis, whose dysfunction turned to be the most frequent cause of isolated ATP synthase deficiency of nuclear origin. The fifth publication is a review which summarises our knowledge about nuclear genetic defects of mitochondrial ATP synthase. In the last and the most recent publication we focused on the pathogenic mechanism of mtDNA *9205delTA* mutation was elucidated.

1. **TMEM70 mutations cause isolated ATP synthase deficiency and neonatal mitochondrial encephalo-cardio-myopathy.** Alena Čížková, Viktor Stránecký, Johannes A. Mayr, Markéta Tesařová, Vendula Havlíčková, Jan Paul, Robert Ivánek, Andreas W. Kuss, Hana Hansíková, Vilma Kaplanová, Marek Vrbacký, Hana Hartmannová, Lenka Nosková, Tomáš Honzík, Zdeněk Drahota, Martin Magner, Kateřina Hejzlarová, Wolfgang Sperl, Jiří Zeman, Josef Houštěk and Stanislav Kmoch. *Nature Genetics*, 2008, 40:1288–1290. **IF = 30.259**

The first publication describes an important discovery uncovering the genetic basis of isolated deficiency of ATP synthase. Within last 15 years the number of patients suffering from severe metabolic disorders caused by dysfunction of mitochondrial ATP synthase was steadily increasing. In many cases nuclear genetic origin was confirmed, but the affected gene(s) responsible for the enzyme defect remained unknown.

Using whole-genome homozygosity mapping, gene expression analysis and DNA sequencing in the large group of 25 patients with isolated ATP synthase deficiency we found two mutations in *TMEM70* gene. The most prevalent was homozygous *c.317–2A>G* splicing mutation at the end of the second intron of *TMEM70*, altering the splicing site of intron 2 and resulting in the loss of TMEM70 transcript; one patient was compound heterozygote harbouring *c.317–2A>G* and *c.118_119insGT* frameshift mutation resulting in a truncated TMEM70 protein. TMEM70 patients` fibroblasts showed decreased ADP-stimulated respiration, low ATP synthase activities and significantly reduced levels of fully assembled complex V with increased content of F_1 subcomplexes. All structural and functional changes in patient fibroblasts were complemented after transfection with wild type *TMEM70*.

As the defect affected assembly of the enzyme, TMEM70 was recognised as a new biogenetic factor of mitochondrial ATP synthase.

Based on the DNA sequence analysis, TMEM70 protein containing conserved DUF1301 domain was found in genomes of multicellular eukaryotes and plants, but not in *S. cerevisiae* and fungi. TMEM70 thus represents the first ATP synthase assembly factor specific for higher eukaryotes.

2. **Expression and processing of the TMEM70 protein.** Kateřina Hejzlarová, Markéta Tesařová, Alena Vrbacká-Čížková, Marek Vrbacký, Hana Hartmannová, Vilma Kaplanová, Lenka Nosková, Hana Kratochvílová, Jana Buzková, Vendula Havlíčková, Jiří Zeman, Stanislav Kmoch and Josef Houštěk. *Biochimica et Biophysica Acta-Bioenergetics*, 2011, 1807(1):144–149. **IF = 4.843**

The second study was focused on the characterisation of TMEM70 protein, a novel biogenetic factor of mitochondrial ATP synthase, with special attention to its biosynthesis, localisation within mitochondria and the structural properties.

TMEM70 gene codes for 260 AA long protein with predicted N-terminal signal sequence of 81 AA and two transmembrane domains. When we analysed C-terminal tagged form of TMEM70 (TMEM70-FLAG and TMEM70-GFP) we found, that 29 kDa precursor of TMEM70 protein is processed to the mature protein of 21 kDa. Import studies confirmed that transport of the newly synthesized TMEM70 protein into mitochondria is followed by the cleavage of approximately 9 kDa signal sequence. Mitochondrial localisation of TMEM70 was further confirmed by the colocalisation of TMEM70-FLAG signal (using *anti*FLAG antibody) with the signal of mitochondrial dye MitoTracker Red. When we subfractionated mitochondria from cells expressing TMEM70-FLAG, the protein behaved similarly to Cox1, an inner mitochondrial membrane protein. 2D (BNE/SDS-PAGE) analyses of control fibroblasts revealed two forms of TMEM70 protein. Finally, we found TMEM70 protein completely absent in the fibroblasts of patients with *c.317–2A>G TMEM70* mutation, where the low amount of ATP synthase was detected.

Taken together, TMEM70 is a 21 kDa protein of the inner mitochondrial membrane, able to associate into higher structures, possibly dimers. It is involved in the biogenesis of ATP synthase, but is not absolutely essential as small, but still insufficient amount of ATP synthase is formed.

3. **Compensatory upregulation of respiratory chain complexes III and IV in isolated deficiency of ATP synthase due to TMEM70 mutation.** Vendula Havlíčková Karbanová, Alena Čížková Vrbacká, Kateřina Hejzlarová, Hana Nůsková, Viktor Stránecký, Andrea Potocká, Stanislav Kmoch and Josef Houštěk. *Biochimica et Biophysica Acta-Bioenergetics*, 2012, 1817:1037–1043. **IF = 4.624**

Low levels of functional ATP synthase, due to *TMEM70* mutations result in the decreased production of ATP, elevated mitochondrial membrane potential and increased ROS production. To test whether and how the cells of TMEM70 patients may respond to such a metabolic disbalance, we performed a quantitative analysis of respiratory chain complexes and mitochondrial proteases involved in the turnover of mitochondrial OXPHOS complexes.

We analysed fibroblast cell lines obtained from 10 patients with the common homozygous *c.317–2A>G TMEM70* mutation. Individual subunits content as well as the amount of fully assembled OXPHOS complexes were examined to search for the putative compensatory changes in the mitochondrial energy provision system. As expected, individual ATP synthase subunits and fully assembled ATP synthase complexes were drastically reduced by 82–89 % of control. In contrast, complex III was significantly increased by 22–53 % and complex IV by 50–162 % when compared to control cells. The content of Lon protease, paraplegin and prohibitins 1 and 2 were not significantly changed. Although a generalised upregulation of transcriptional activity was observed, it was not accompanied with 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 was also not changed in the patient cells.

The results indicate that the posttranscriptional events are of key importance for the adaptive regulation of mitochondrial biogenesis, which allows for compensatory increase of respiratory chain complexes III and IV in response to deficiency of ATP synthase.

4. Mitochondrial membrane assembly of TMEM70 protein. Hana Kratochvílová*, Kateřina Hejzlarová*, Marek Vrbacký, Tomáš Mráček, Vendula Karbanová, Markéta Tesařová, Adriána Gombitová, Dušan Cmarko, Ilka Wittig, Jiří Zeman and Josef Houštěk. *Mitochondrion*, 2014, 15: 1–9. **IF = 3.524 (2013)**

* Equal contribution

Although the number of patients with different *TMEM70* mutations steadily increases, the proper mechanism of its function remains unclear. In this study we proceeded with the characterisation of TMEM70 protein structure, which could shed more light onto the functional properties of this factor. Further we searched for the possible interaction of TMEM70 protein with ATP synthase or other mitochondrial proteins.

Since most of the deleterious *TMEM70* mutations affect the C-terminus of the protein, and based on the sequence similarity between different species, the hydrophilic C-terminus of TMEM70 protein is expected to be directly involved in its function. Therefore we analysed in detail the topology of TMEM70 in the inner mitochondrial membrane. We used C-terminal tagged forms of TMEM70 protein (TMEM70-GFP, TMEM70-MYC-FLAG and TMEM70-FLAG) as well as endogenous TMEM70 and tested the accessibility of the C-terminus to external protease (trypsin) or to the dye (trypan blue) quenching of the GFP fluorescence, under conditions when the reagent can reach just the intermembrane space or also the mitochondrial matrix. Both approaches revealed that the C-terminus of TMEM70 protein is oriented into the matrix. The previous indication of the higher structures of TMEM70 protein, such as dimers and higher oligomers, was further confirmed by native electrophoresis of different forms of TMEM70 and mainly by coimmunoprecipitation of TMEM70-GFP and TMEM70-FLAG from double transfected cell lines. However, neither extensive immunoprecipitation experiments nor electron microscopy studies with immunogold labelling revealed a direct interaction of TMEM70 protein with ATP synthase.

Together with the prediction of two transmembrane domain and the fact, that N-terminal signal sequence is cleaved after import, we can conclude that TMEM70 protein has a hairpin structure with both N- and C-termini oriented towards the mitochondrial matrix. TMEM70 was detected in multiple forms, not directly interacting with ATP synthase and thus the function of TMEM70 protein in ATP synthase biogenesis may be mediated through interaction with some other protein(s).

5. **Nuclear genetic defects of mitochondrial ATP synthase.** Kateřina Hejzlarová, Tomáš Mráček, Marek Vrbacký, Vilma Kaplanová, Vendula Karbanová, Hana Nůsková, Petr Pecina and Josef Houštěk. *Physiological Research*, 2014, 63(Suppl. 1): S57–S71. **IF = 1.487 (2013)**

In this review article, genetic bases of isolated ATP synthase defects are discussed. Mitochondrial DNA mutations in *MT-ATP6* and *MT-ATP8* genes are briefly summarised, same as the structure and function of ATP synthase, but the main focus is on the nuclear DNA mutations. There are only three other nDNA encoded proteins besides TMEM70 that associate with ATO synthase deficiencies in humans. They include two genes coding for subunits of F_1 part, *ATP5A1* (α subunit) and *ATP5E* (ε subunit), and one gene for F_1 assembly factor *ATPAF2* (Atp12p), with one mutation in each protein described so far. In case of *ATP5A1* two patients were reported, and only one in case of *ATP5E* or *ATPAF2*. Contrary, in *TMEM70* gene 11 different mutations and at least 44 patients were described in the time this review was published. Apparently, *TMEM70* gene is highly prone to mutagenesis and this type of a rare mitochondrial disease has a rather frequent incidence. Although the clinical manifestations and the severity of the disease in the individual patients are different, some phenotypes are more frequent than others. Here belongs metabolic distress represented by hyperammonemia, lactic acidosis and 3-methylglutaconic aciduria, hypotonia, cardio-myopathy or dysmorphic features. Apart from clinical phenotype, the possible mechanism of TMEM70 protein function was discussed as well. In the study where the first patient was described, the impairment of early stages of ATP synthase assembly was suggested by Houštěk et al. Recently, Torraco et al. proposed the role of TMEM70 in stabilisation of F_1 thus assisting further steps of enzyme biogenesis including the ultimate incorporation of mtDNA encoded subunits *a* and A6L.

6. **Alteration of structure and function of ATP synthase and cytochrome *c* oxidase by lack of F_o-a and Cox3 subunits caused by mitochondrial DNA 9205delTA mutation.** Kateřina Hejzlarová, Vilma Kaplanová, Hana Nůsková, Nikola Kovářová, Pavel Ješina, Zdeněk Drahota, Tomáš Mráček, Sara Seneca and Josef Houštěk. *Biochemical Journal,* 2015, 466: 601–611. **IF = 4.779 (2013)**

In the last publication, the consequences of mtDNA *m.9205delTA* mutation in *MT-ATP8/MT-ATP6/MT-CO3* gene were studied. This very rare mutation was found in two unrelated patients with very distinct severity of the disease progression. Both cases were reported to be homoplasmic and therefore we speculated that an additional nuclear-encoded ancillary factor might be involved in processing of MT-ATP8/MT-ATP6/MT-CO3 transcript and modulating the deleterious effects of *m.9205delTA* mutation. It was of interest to compare the cells from both cases and we identified heteroplasmy of *m.9205delTA* mutation in the fibroblasts of high passage from the first patient with milder phenotype, indicating that negative segregation of the mutation occurred during the prolonged cultivation and unmasked the mutation heteroplasmy.

To elucidate, whether the phenotypic differences between the two patients may be caused by different load of mtDNA mutation, we prepared the series of transmitochondrial cybrid cells with varying heteroplasmy (52–93 %) and studied the functional parameters of these cell lines. Control cybrid cells homoplasmic to wild-type mtDNA and cybrid cells homoplasmic to *m.9205delTA* mutation (99 %) were used as well. The content of subunits *a* and Cox3 was determined, and ADP-stimulated respiration, ATP production and changes of mitochondrial membrane potential were measured. All the parameters were heteroplasmy-dependent and displayed pronounced threshold effect above ~90 % mutation heteroplasmy. By comparing the control and homoplasmic cell lines we found, that the mutation strongly reduces the levels of both *a* and Cox3 proteins. Lack of subunit *a* alters the structure but not the content of ATP synthase, which assembles into a labile, ~60 kDa smaller complex retaining ATP hydrolytic activity but unable to synthesize ATP. To the contrary, lack of Cox3 limits the biosynthesis of COX but does not alter the structure of the enzyme. Consequently, the diminished mitochondrial content of COX and nonfunctional ATP synthase prevent most of mitochondrial ATP production.

The distinct phenotypic presentation of the two cases thus apparently resulted from differences in the mutation load with a critical threshold for disease manifestation lying at a very high heteroplasmy level. We observed linear relationship between the decrease in subunit *a* or Cox3 content and functional presentation of the defect. Therefore we could conclude that the threshold effect originated from a gene–protein level.

Contribution of dissertant to these publications:

The results presented have been achieved by a team effort of all coauthors of the above publications. The contribution of dissertant to experiments described in the presented articles is as follows:

1. Evaluation of mitochondrial functions, including spectrophotometric measurements of ATP synthase and respiratory chain enzymes activities, respiration and ATP production measurements.

2. Structural analysis and quantification of respiratory chain complexes, ATP synthase and TMEM70 using native, SDS and 2D electrophoreses, and Western blot immunodetection.

3. TMEM70 import and orientation studies using tagged analogues of the TMEM70 protein.

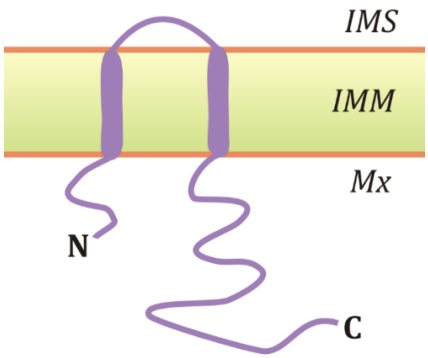
4. Cell cultures of fibroblasts and transmitochondrial cybrids, isolation of mitochondria and determination of *m.9205delTA* mutation load.

4. Conclusions

From our studies on molecular pathogenesis of severe mitochondrial diseases that were focused on the mechanisms underlying the isolated deficiency of ATP synthase due to nuclear or mitochondrial DNA mutations, we conclude, that:

1. Mutations in *TMEM70* gene are the most frequent cause of isolated ATP synthase deficiency manifesting as severe, mostly fatal neonatal encephalo-cardio-myopathy (OMIM 614052). Most common is homozygous *c.317–2A>G* mutation, preventing synthesis of TMEM70 protein, heterozygous mutations usually present with milder phenotypes. TMEM70 mutations downregulate biogenesis of ATP synthase complex, increase mitochondrial membrane potential and diminish mitochondrial ATP synthesis. Complementation of mutated TMEM70 with the wild type TMEM70 protein fully rescues the pathogenic phenotype *in vitro*. In the patients' fibroblasts ATP synthase deficiency leads to compensatory-adaptive upregulation of respiratory chain complexes III and IV which is enabled by posttranscriptional events in mitochondrial biogenesis.

2. TMEM70 protein is a novel ancillary factor involved in the biogenesis of mitochondrial ATP synthase, and the first one uniquely specific for higher eukaryotes. TMEM70 is localised in the inner mitochondrial membrane and forms higher oligomers. Upon import to mitochondria the mature, membrane bound TMEM70 of a 21 kDa has a hairpin structure with the N- and C-termini oriented into the mitochondrial matrix. As C-terminus of the protein appears to be crucial for TMEM70 functioning, the protein may be involved in ATP synthase assembly events associated with the matrix side of the membrane. However, the proper mechanism of TMEM70 protein function in the biogenesis of mitochondrial ATP synthase remains to be elucidated as this factor does not interact directly with ATP synthase.



3. Distinct phenotypes of two patients with mtDNA *m.9205delTA* mutation affecting synthesis of subunits *a* and Cox3 and thus the function of ATP synthase and COX are explained by different mutation load, with steep decline of structural and functional changes close to mutation homoplasmy. Threshold about 90 % of heteroplasmy, originating from the gene–protein level, has to be exceeded for the disease manifestation. In cells lacking subunit *a* the amount of ATP synthase is unchanged but the incomplete enzyme is unstable and unable to produce ATP, while the lack of subunit Cox3 strongly reduces the synthesis of COX complex. The pathogenic mechanism of *m.9205delTA* mutations is thus caused by a combined defect of both COX and ATP synthase enzyme, perhaps the COX deficiency being primary and more critical for the overall decline in energy provision.

5. Summary

Discovery of *TMEM70* mutations in the group of patients with isolated ATP synthase deficiency of nuclear origin appeared to be very important step in unravelling the genetic determinants of severe mitochondrial diseases of paediatric patients. TMEM70 turned to be the third ancillary factor of mammalian ATP synthase and the first one specific for higher eukaryotes. TMEM70 is a 21 kDa protein assembled as a hairpin in the inner mitochondrial membrane and it is usually absent in the patients. The mechanism of action of TMEM70 protein in the biogenesis of ATP synthase remains unclear, however it is highly probable that it depends on TMEM70 C-terminus exposed to the mitochondrial matrix.

The characterisation of unique *m.9205delTA* mtDNA mutation revealed a combined deficiency of ATP synthase and cytochrome *c* oxidase, both sharing high mutation threshold for the biochemical manifestation originating from the lack of subunits *a* and Cox3. While the biogenesis of cytochrome *c* oxidase becomes strongly diminished, the content of ATP synthase is enhanced, but the incomplete enzyme complex is unstable and loses the ATP synthetic activity.

Shrnutí

Odhalení mutací v genu *TMEM70* u skupiny pacientů s izolovaným defektem ATP syntázy jaderného původu se ukázalo jako velmi významný krok v objasnění genetických příčin závažných mitochondriálních onemocnění u pediatrických pacientů. TMEM70 představuje třetí pomocný faktor biogeneze savčí ATP syntázy, a vůbec první specifický pro vyšší eukaryota. TMEM70 o velikosti 21 kDa je proteinem vnitřní mitochondriální membrány, zabudovaným ve tvaru vlásenky. Protein u pacientů obvykle chybí. Mechanizmus působení proteinu TMEM70 v biogenezi ATP syntázy je stále nejasný, ale zřejmě závisí na C-koncové sekvenci lokalizované v mitochondriální matrix.

Charakterizace unikátní mtDNA mutace *m.9205delTA* ukázala, že výsledná deficience ATP syntázy i cytochrom *c* oxidázy sdílí vysokou prahovou hodnotu mutace mtDNA pro biochemickou manifestaci vyvolanou nedostatečnou tvorbou podjednotek *a* a Cox3. To má za následek inhibici biogeneze cytochrom *c* oxidázy, zatímco množství ATP syntázy se zvyšuje, ale nekompletní enzymový komplex je labilní a není schopen syntetizovat ATP.

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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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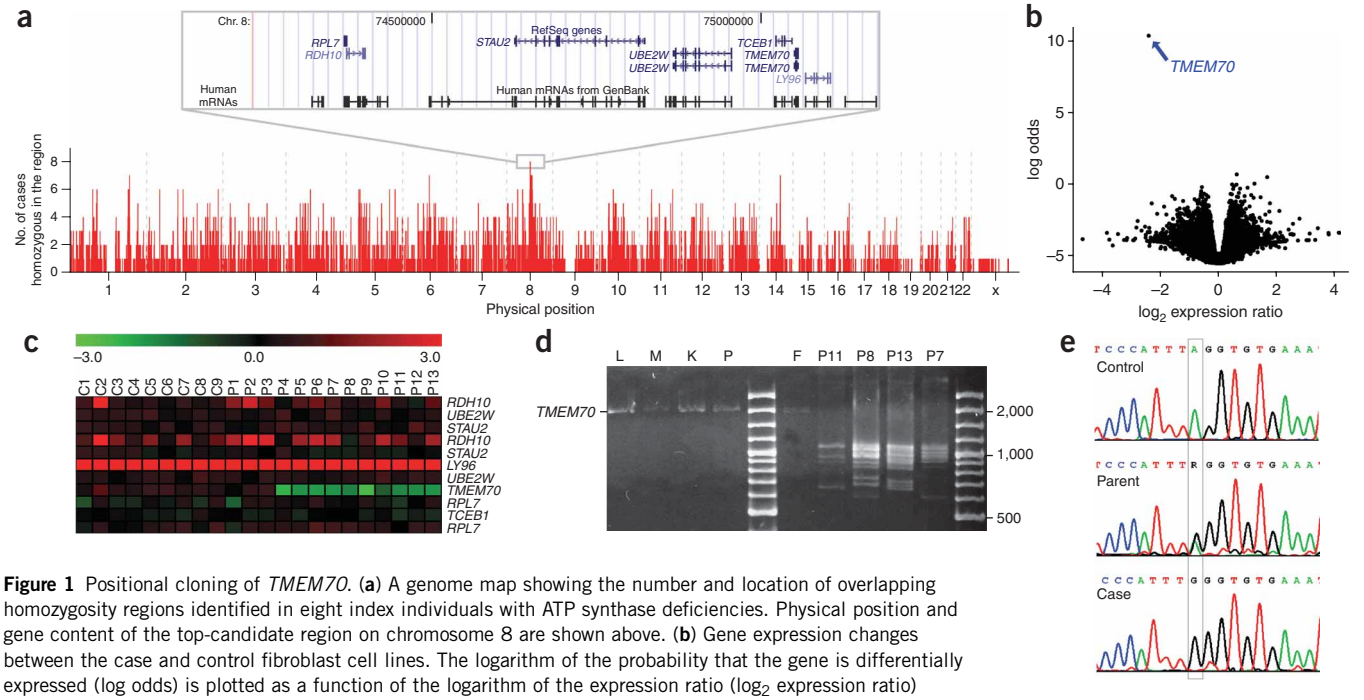

Figure 1 Positional cloning of *TMEM70*. (**a**) A genome map showing the number and location of overlapping homozygosity regions identified in eight index individuals with ATP synthase deficiencies. Physical position and gene content of the top-candidate region on chromosome 8 are shown above. (**b**) 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,

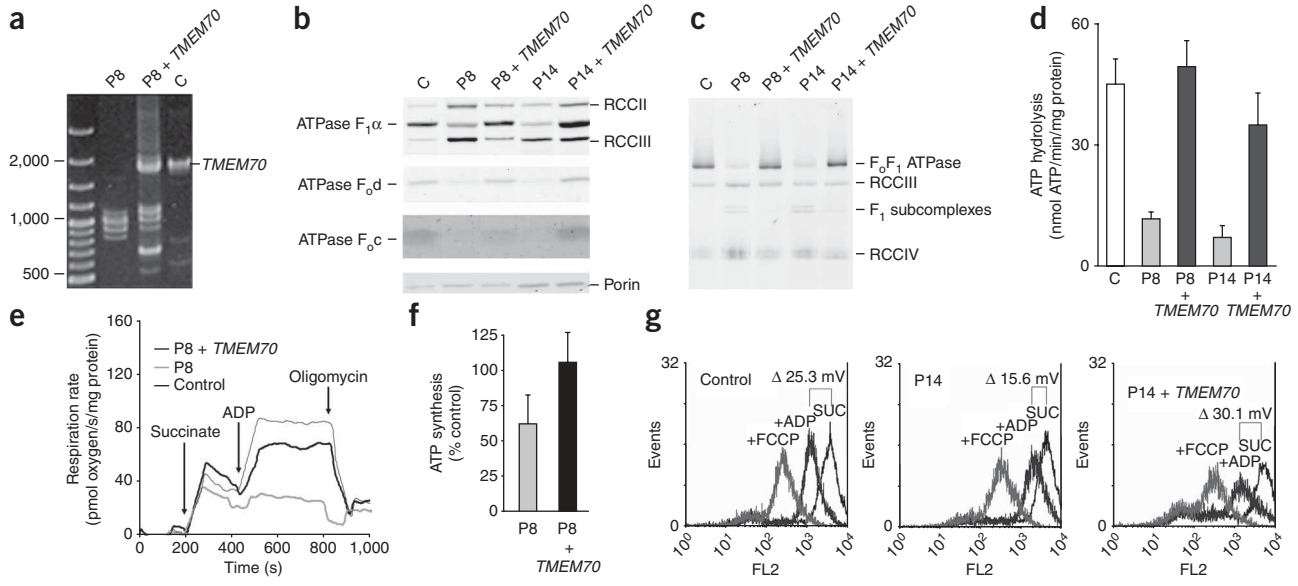

Figure 2 *TMEM70* complementation of ATP synthase deficiency. (**a**) *TMEM70* cDNA is present after transfection. (**b**) SDS-PAGE protein blot of fibroblasts shows a specific increase of the content of ATP synthase subunits relative to the respiratory chain complexes and porin. (**c**) BN-PAGE protein 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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SUPPLEMENTARY INFORMATION

(Supplementary Figures 1-5; Supplementary Tables 1 and 2; Supplementary materials and methods; Supplementary references)

TMEM70 mutations cause isolated ATP synthase deficiency and neonatal mitochondrial encephalo-cardiomyopathy.

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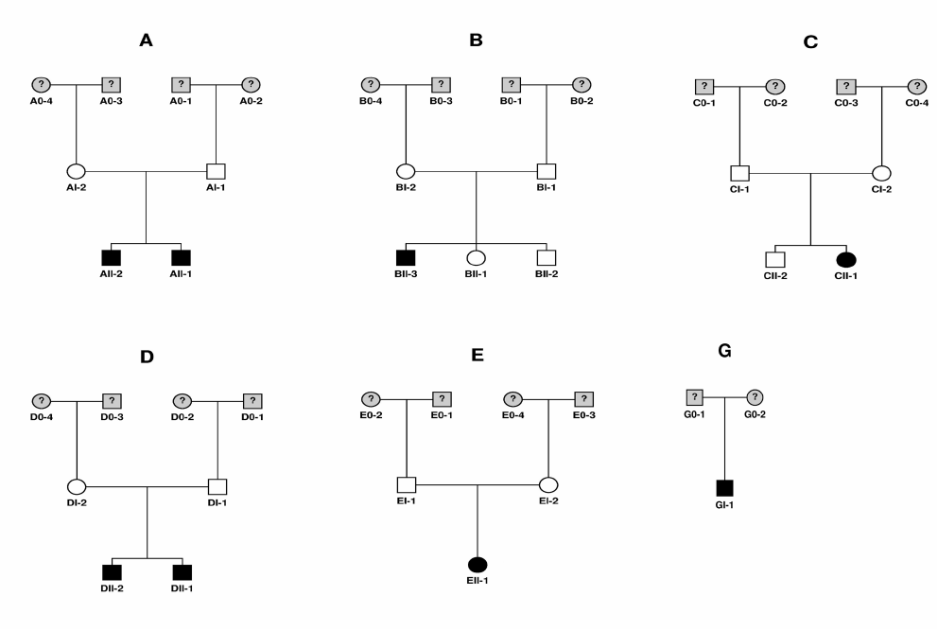
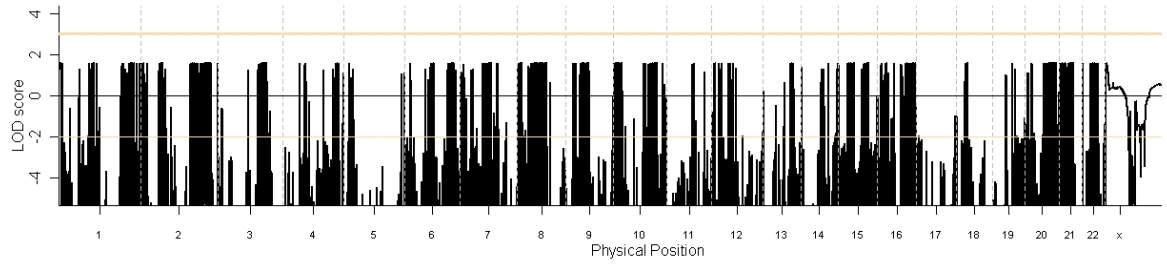
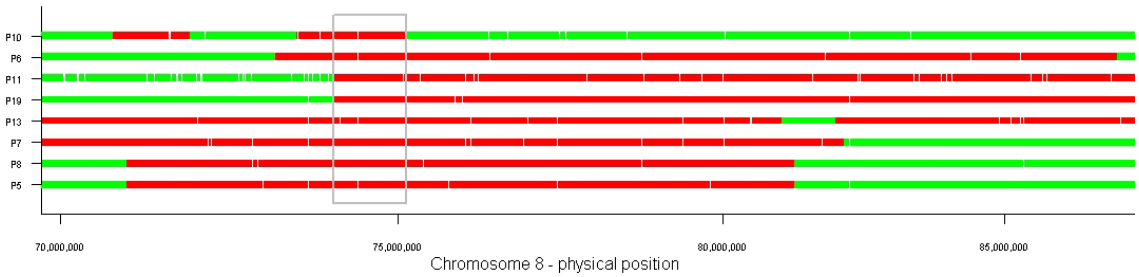
Supplementary Figure 1 Pedigree diagrams of the genotyped families and individuals. Black symbols denote affected individuals, open symbols denote unaffected individuals.



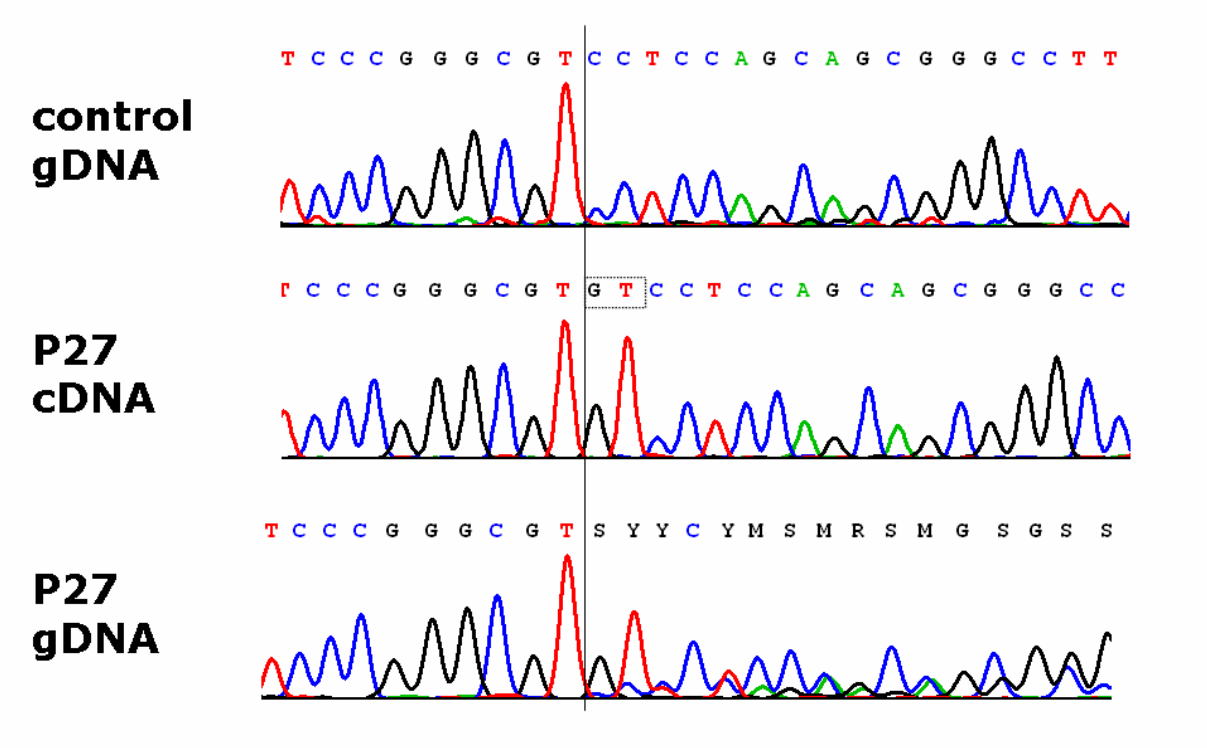
Supplementary Figure 2 Parametric linkage analysis



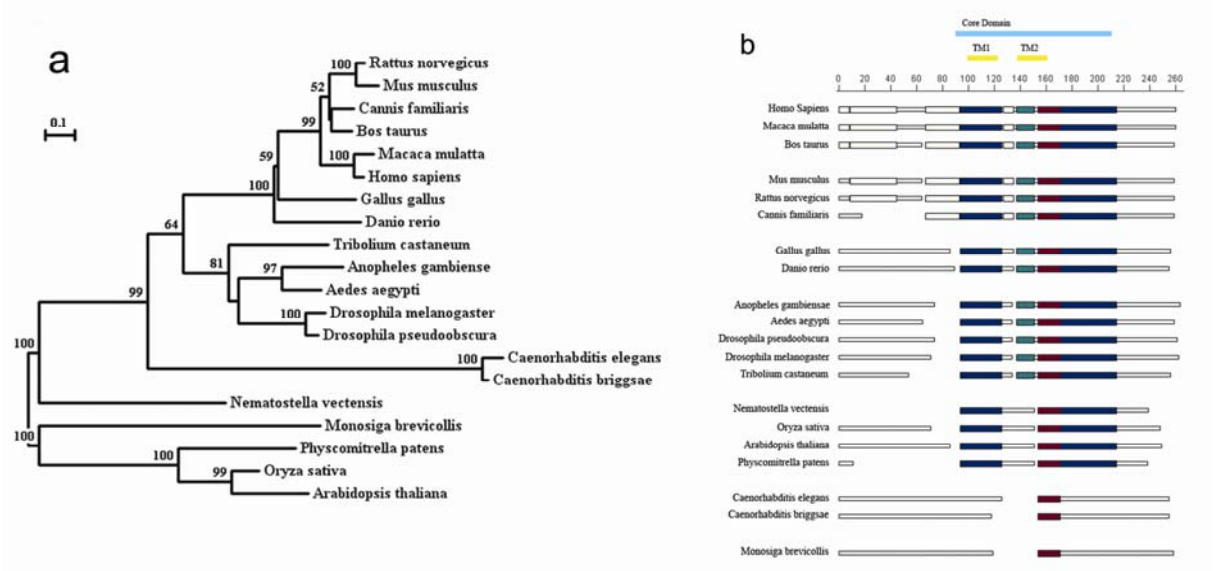
Supplementary Figure 3 Shared homozygosity region (red) on chromosome 8. The candidate region is delimited by SNP markers A-2085138 and A-2263684 (genomic positions chr8:74,229,948 and 75,177,995 bp, respectively).



Supplementary Figure 4 Chromatograms of *TMEM70* genomic and cDNA sequences showing insertion c.118_119insGT identified in compound heterozygous patient P27



Supplementary Figure 5 a) Phylogenetic analysis, and **b)** A scheme of multiple sequence alignment of TMEM70. Color of the boxes indicates the level of homology between different organisms (red, blue, green, and white; from most homologous to the least, respectively). TM1, TM2 denote putative transmembrane regions. Core domain represents central, the most homologous part of the protein.



Supplementary Table 1 Oligonucleotides used in this study

position	upper primer	lower primer
10K of promotor region		
target 1 : 514 to 1570	CAATTTTGTGCCTATGTGCAG	CCTCTGGTCTGGAGGACATC
target 2 : 965 to 2003	CTCCAAACTCCCTTGTTTCAG	TGGTGGGAGACAAGATCAGAG
target 3 : 1934 to 3126	CAAGCTACCATGTCTCAACTGG	TCTGGAGATACAATGGCAAGG
target 4 : 2948 to 4097	ACCATCTGGCAATGCATACAC	CGTCCAGTCTTGTCTGAAAGG
target 5 : 3982 to 5132	GCTAAATGGCACAAACTCCAC	GGCGTAACCCTAAATGAGAGG
target 6 : 5045 to 6180	GCATCCTGGGACTTGTAGTTTC	TGTGGTCAGGAGTTTGAGACC
target 7 : 6063 to 7172	CCCCTGAGTAGCTGGGATTAC	TTGGCAACTCCAAATACTGTC
target 8 : 6984 to 8157	CAGGAGTTTGAGACCAACCTG	TATTGCCAGGACTGAGCTAGG
target 9 : 8065 to 9249	TTTCAGGTATTTTCCTCCACTG	CCCTCTGGATTTGGAACTACC
target 10 : 9143 to 10256	GGGCTTGCAGAAGTAGCAG	CTTGAGCCCAGGAGTTTAAGG
target 11 : 10112 to 11152	AGTATTTGTAAAAGGCCTGGAC	ACCATTGCAACCTCTGCTTC
target 12 : 10497 to 11704	TAGAATTAAGGCAAGCCATTC	AGTGTGGTGGTGCATACCTGT
genomic coding regions		
exon 1 : 611U21- 1378L21	ATTCTCGTACAGCCCTCTGCC	GTAAGCACTTTCCAGCCGTTC
exon 2 : 574U20- 1209L20	GCAATGGTGAGCTGAGATCG	GCCGAGGTGGGTGGGTTACT
exon 3 : 2139U2- 4105L2	TGAGGTAGGGCCACTTAAACC	TTGGCAAGAGTCAATCTCCAC
RFLP_ex3_Alu restriction	GCAGGGACATTGAAGTCAAAA	CGTAGAATAAGAGAAACATTTCACAGC
cDNA		
c*TMEM70* : 8U21 to 1896L22	GAAGCCGTGTCTCGCAGTCGT	CACTACTTACCATTTCCCGTAT

Supplementary Table 2 Clinical, biochemical and molecular description of patients with isolated ATP synthase deficiency analyzed in this study

patient/ gender	pedigree ID	Survival *	Clinical and biochemical presentation **	SNP typing	GE ***	TMEM70 mutation c.317-2A>G	laboratory	reference
Patients with mitochondrial DNA mutation 9205ΔTA								
P1-m		9y	PMR, M, LA	n.d.	+	n.d.	CZ	3,19
P2-f			GR, LA	n.d.	+	n.d.	Be	3,20
Patients with nuclear defect								
P3-f		20y	PMR, Hy, PNP, HCMP, LA, ↓ATPase	n.d.	+	not found	A	3,21
P4-m		†2d	HCMP, Dy, Hps, LA, 3MGA, ↓ATPase	A.250k	+	+/+	CZ	3,11,21
P5-m	AII.1	†4y	PMR, M,Hy, HCMP, Dy, Hps, LA, 3MGA, ↓ATPase	A.250k	+	+/+	CZ	3,21
P6-m	EII.1	4y	PMR, M, Hy, HCMP, Dy, Hps, LA, 3MGA, ↓ATPase	A.250k	+	+/+	CZ	3
P7-m	BII.3	†18m	PMR, M, Hy, HCMP, Dy, Hps, LA, 3MGA, ↓ATPase	A.250k	+	+/+	CZ	3
P8-m	AII.2	2y	PMR, M, Hy, HCMP, Dy, Hps, LA, 3MGA, ↓ATPase	A.250k	+	+/+	CZ	3
P9-f		17y	PMR, M, Hy, HCMP, Dy, LA, 3MGA, ↓ATPase	A.250k	+	+/+	CZ	3,21
P10-m	GI.1	†10d	PMR, M, Hy, HCMP, Dy, Hps, LA, 3MGA, ↓ATPase	A.250k	+	+/+	CZ	3
P11-m	DII.2	7y	PMR, M, Hy, HCMP, GR, Dy, LA, 3MGA, ↓ATPase	A.250k	+	+/+	CZ	3,21
P12-f		8y	PMR, Hy, HCMP, LA, 3MGA, ↓ATPase	I.370k	+	+/+	A/D	3,21,22
P13-f	CII.1	5y	PMR, M, He, GR, LA, 3MGA, ↓ATPase	A.250k	+	+/+	CZ	3,21
P14-m		4y	PMR, Hy, HCMP, LA, 3MGA, ↓ATPase	I.370k		+/+	A/D	21
P15-f		†2y	PMR, HCMP, LA, 3MGA, ↓ATPase	I.370k		+/+	A/D	NR
P16-m	DII.1	†1m	HCMP, GR, LA, ↓ATPase	A.250k		+/+	CZ	NR
P17-m		3y	PMR, Hy, HCMP, Hps, LA, 3MGA, ↓ATPase	I.370k		+/+	A/D	NR
P18-f		5y	PMR, Hy, HCMP, GR, Dy, LA, ↓ATPase	I.370k		+/+	A/D	21
P19-f		†12d	PMR, Hy, HCMP, He, LA, 3MGA, ↓ATPase	n.d.		+/+	CZ	NR
P20-m		†3y	M, Hy, HCMP, GR, LA, ↓ATPase	n.d.		+/+	CZ	NR
P21-f		†2d	PMR, M, Hy, HCMP, He, GR, LA, 3MGA, ↓ATPase	n.d.		+/+	CZ	NR
P22-f		†4d	Hy, He, GR, LA, 3MGA, ↓ATPase	n.d.		+/+	CZ	NR
P23-f		†2d	HCMP, GR, Dy, LA, ↓ATPase	n.d.		+/+	CZ	NR
P24-m		†6w	PMR, M, Hy, HCMP, He, GR, LA, 3MGA, ↓ATPase	n.d.		+/+	CZ	NR
P25-f		17m	PMR, Hy, HCMP, He, GR, LA, 3MGA, ↓ATPase	n.d.		+/+	CZ	NR
P26-m		†1d	Hy, He,GR, LA, 3MGA, ↓ATPase	n.d.		+/+	CZ	NR
P27-f		9y	PMR, HCMP, LA, 3MGA, ↓ATPase	n.d.		+/c.118_119insGT	A	NR

* - present age/† age at the death; ** - clinical symptoms: PMR – psychomotor retardation, M – microcephaly, Hy – hypotonia, PNP – polyneuropathy, HCMP – hypertrophic cardiomyopathy, He – hepatopathy, GR – growth retardation, Dy – dysmorphy, Hps – hypospadia, LA – lactic acidosis, MGA-3-methylglutaconic aciduria, ↓ATPase - isolated deficiency of ATP synthase complex; SNP typing – A.250k and I.370 denotes analysis on Affymetrix and Illumina SNP Mapping arrays, respectively; *** GE – gene expression analysis; + denotes patients in whom gene expression analysis on Agilent 44k has been performed.

Materials and Methods

Patients and pedigrees

Clinical, biochemical and molecular description of patients analyzed in this study is provided in **Supplementary Table 2**. The cohort includes 23 cases with isolated ATP synthase deficiency due to low enzyme content, with no mtDNA mutations. For comparison, 2 cases with ATP synthase dysfunction due to mtDNA mutation 9205ΔTA in *ATP6* gene were included. Pedigree diagrams of the genotyped families are shown in **Supplementary Figure 1.** The project was approved by the Scientific Ethics Committee of the 1st Faculty of Medicine of Charles University of Prague. Patient participation in the project was made on a voluntary basis after oral and written information and consent according to the Helsinki V Declaration.

Genotyping, linkage analysis and homozygosity mapping

Genomic DNA was isolated by standard technology. DNA samples were genotyped using Affymetrix GeneChip Mapping 250K Nsp mapping arrays (Affymetrix, Santa Clara, CA) according to the manufacturer's protocol. Raw feature intensities were extracted from the Affymetrix GeneChip Scanner 3000 7G images using the GeneChip Operating Software. Individual SNP calls were generated using GeneChip Genotyping Analysis Software (GTYPE) 4.0.

Multipoint nonparametric and parametric linkage analysis along with determination of the most likely haplotypes was performed in a version 1.1.2 of Merlin software [1]. Parametric linkage was carried out under the assumption of an autosomal recessive mode of inheritance with a 1.00 constant, age independent penetrance, 0.00 phenocopy rate and 0.0001 frequency of disease allele. The results were visualized in a version 0.295 of the HaploPainter software [2] and in a version 2.6. of R-project, **Supplementary Figure 2**.

Extended homozygosity regions were identified in Affymetrix GeneChip Chromosome Copy Number Analysis Tool version 4.0 (CNAT) using the algorithm comparing values from the user's sample set and SNP-specific distributions derived from a reference set of two hundred ethnically diverse individuals. Distribution of extended homozygosity regions in affected and healthy individuals was analyzed and visualized using custom R-script. Detail of the shared homozygosity region on chromosome 8 is shown in **Supplementary Figure 3.**

Gene expression analysis

Sample and reference RNA isolations and RNA quality control were performed as previously described [3]. Total RNA (500 ng) was reverse transcribed, labeled and hybridized onto Agilent 44k human genome microarray using Two-color Microarray Based Gene Expression Analysis Kit (Agilent, Santa Clara, CA). All 13 patient samples and 9 controls (Cy5-labeled) were hybridized against to common Cy3-labeled reference RNA isolated from HeLa cell lines. The hybridized slides were scanned with GenePix 4200A scanner (Axon Instrument, Union City, CA) with PMT gains adjusted to obtain highest intensity unsaturated images. Gene PixPro software (Axon Instruments) was used for image analysis the of TIFF files, as generated by the scanner. Comparative microarray analysis was performed according to MIAME guidelines [4]. Normalization was performed in R statistic environment (http://www.r-project.org) using Limma package [5], which is part of Bioconductor project (http://www.bioconductor.org). Raw data from individual arrays were processed using loess normalization and normexp background correction. Gquantile 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 Benjamini & Hochberg method [6]. Gene expression data are deposited in the GEO repository under accession number GSE10956.

DNA analysis

Genomic fragments covering 10 kb of the promoter region and all of the exons with their corresponding exon-intron boundaries of the *TMEM70* were PCR amplified from genomic DNA and sequenced as previously described [7]. Presence of the c.317-2A>G mutation in probands and its segregation in the families was assessed in genomic DNA by PCR-RFLP analysis employing mutation induced loss of a primer mismatch created AluI restriction site. A 1910-bp fragment of the human *TMEM70* cDNA (nt 21 to 1931 of the GeneID: 54968 cDNA sequence) was PCR amplified from cDNA obtained either by reverse transcription of fibroblast RNA or from commercially available human cDNA panel (BD Biosciences, San Jose, CA). Primer sequences are provided in **Supplementary Table 1**.

TMEM70 expression vector

TMEM70 cDNA clone MHS1011-60493 was obtained from Open Biosystems, Huntsville, AL. Following the sequence verification, the insert was transferred into the mammalian expression vector pEF-DEST51 using the Gateway technology (Invitrogen, Paisley, UK). Resulting plasmids TMEM70-pEF-DEST51 were propagated in *E.coli*, isolated and fully sequenced before the transfection.

Cell cultures, transfection, isolation of mitochondria.

Fibroblast cultures were established from skin biopsies and cells were grown in DMEM medium (Sigma, Prague, Czech Republic) containing 10 % fetal calf serum (Sigma), penicillin (100 IE/ml) and streptomycin (100 μg/ml) at 37°C in 5 % CO_2 in air. Confluent cells were harvested by trypsinization and washed twice with PBS.

TMEM70 and empty DNA vectors have been transfected into cells (2 μg of DNA/ $5x10^5$ cells) using Nucleofector device and NHDF nucleofection kit (Amaxa, Cologne, Germany). Stably transfected cell lines were selected by blasticidin (10 μg/ml). Mitochondria were isolated from cultured cells by hypotonic shock method [8].

Polyacrylamide gel electrophoresis.

Sedimented mitochondria (15 000 g, 10 min) were solubilised on ice with dodecylmaltoside (4 mg/mg protein) in 1.75 M aminocaproic acid, 75 mM Bis-Tris, pH 7.0, 2 mM EDTA and centrifuged 20 min at 30 000g. Supernatants were supplemented with ServaBlue G (0.5 mg/mg dodecylmaltoside) and mitochondrial OXPHOS complexes were resolved by BN-PAGE [9] on a 6-15 % linear gradient of polyacrylamide slab minigels.

Tricine SDS-PAGE [10] on a 10 % polyacrylamide slab minigels was used for analysis of individual subunits of ATPase and respiratory chain complexes I-IV. The samples of mitochondria or fibroblasts were boiled for 3 min in 2 % mercaptoethanol, 4 % SDS, 10 mM Tris-HCl and 10 % glycerol before use.

Western blot analysis.

Proteins from the slab gels were blotted onto PVDF membrane by semidry electrotransfer (0.7 mA/cm^2, 1 h) and the membrane was blocked in PBS with 0.2 % Tween 20 (PBST). Membranes were incubated for 2.5 h with primary antibodies diluted in PBST containing 2 % bovine serum albumin (PBSTA), followed by incubation for 1.5 h with infrared IRDye®-labeled secondary antibodies (LiCor Biosciences, Lincoln, Ne) and detection on an Odyssey Infrared Imager (LiCor). The following subunit-specific mouse monoclonal antibodies from MitoSciences, Eugene, OR, were used: Anti-Core1 subunit of complex III (0.5 μg/ml,

MS303), anti-SDH70 subunit of complex II (1 μg/ml, MS 204), anti-F_1-α subunit of ATP synthase (1 μg/ml, MS502), anti F_o subunit d of ATP synthase (1.4 μg/ml, MS504) and anti Porin (5 μg/ml, MSA03). In addition, rabbit polyclonal antibody to F_o subunit c (1:1000) [11] of ATP synthase was used. For quantification of the signal we used Aida 2.11 Image Analyzer software (Raytest, Straubenhartd, Germany).

High resolution oxygraphy, ATP synthesis and ATPase activity

Oxygen consumption was determined at 30 °C using an Oxygraph-2k (Oroboros, Austria). 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) at 1 mg protein/ml and were permeabilised by 0.1 mg digitonin/mg protein. Oxygen consumption was measured in the presence of 10 mM succinate, 0.1 mM ADP and 1μM oligomycine as indicated and was expressed in pmol oxygen/s/mg protein. For determination of ATP synthesis, 10 μl samples were collected during respiratory measurements and quenched with 10 μl dimethylsufoxide. ATP content was determined by a luciferase assay [12]. Oligomycin-sensitive ATP synthase hydrolytic activity of cultured cells was measured in ATP-regenerating system [13]. The ATP production was expressed in nmoles ATP/min/mg protein.

Cytofluorometric analysis of mitochondrial membrane potential

Measurements of $\Delta\Psi_m$ according to [14] were performed on a PASIII cytofluorometer (Partec, Germany). Cells suspended (1 mg protein/ml) in a KCl medium with 10 mM succinate were permeabilised by 0.1 mg digitonin/mg protein, diluted 5-times and incubated with 20 nM tetramethylrhodamine methyl ester (TMRM, Molecular Probes, Invitrogen, Paisley, UK) for 15 min. ADP (0.01 mM or FCCP (1 μM) were added 1 min before cytofluorometric analysis. 10 000 cells were used for each measurement. Data were acquired on a log scale using CellQuest (FloMax, Partek) and analyzed with WinMDI 2.8 software (Trotter, J., TSRI, La Jolla, CA). Arithmetic mean values of fluorescence signal in arbitrary units were determined for each sample for subsequent graphic presentation. TMRM fluorescence changes were calculated in mV according to [15].

Phylogenetic analysis

Protein sequences were aligned using the T-COFFEE software with default parameters [16]. The resulting alignments were modified manually using GeneDoc (http://www.psc.edu/biomed/genedoc), taking into account secondary structure predictions (putative transmembrane domains and reported PFAM domain). Conserved blocks of sequences were concatenated before the construction of the phylogenetic trees. Phylogenetic trees based on the resulting alignments were produced with the aid of the Treecon package [17] using the neighbor-joining (NJ) algorithm [18] with Poisson correction for distance estimation. Datasets were resampled using 500 bootstrap replications and bootstrap values were used in support of the inferred trees, as indicated in the figures.

Accession numbers

Information on TMEM70; transmembrane protein 70 is accessible in NCBI Gene Entrez database under GeneID: 54968.

Whole genome gene expression data are deposited in NCBI Gene Expression Omnibus database (GEO) under accession number GSE10956.

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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,

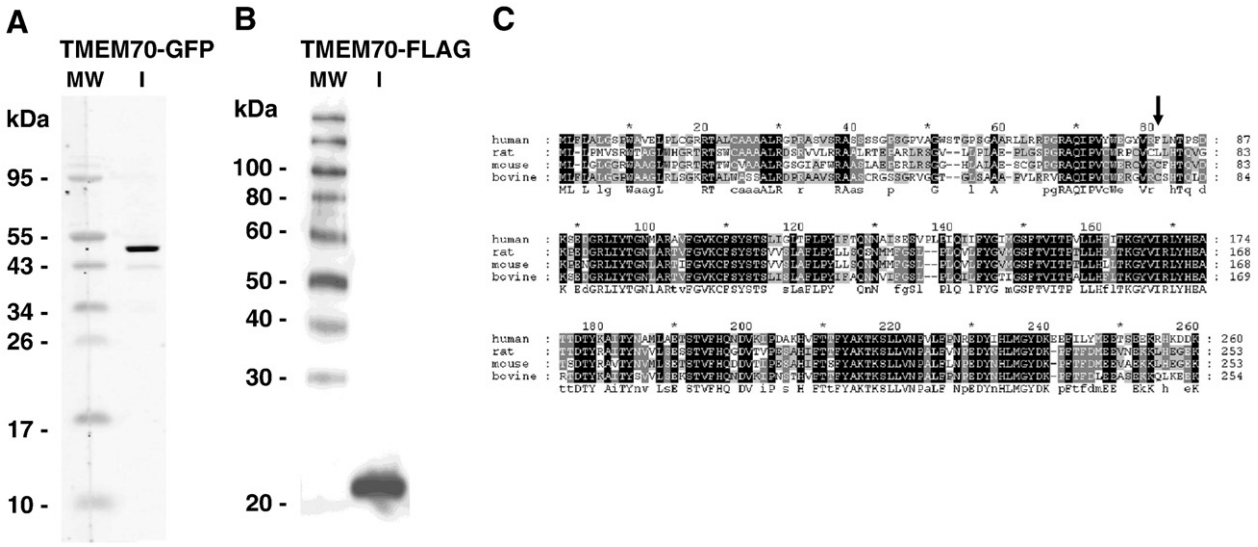

Fig. 1. The size of mature TMEM70. Western blot analysis of TMEM70-GFP (A) and TMEM70-FLAG (B) expressed in fibroblast and HEK293 cells. (C) Alignment of human (NCBI GI: 34147498), rat (157823940), mouse (15030135) and bovine (148878159) TMEM70 protein sequence; arrow indicates predicted cleavage site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

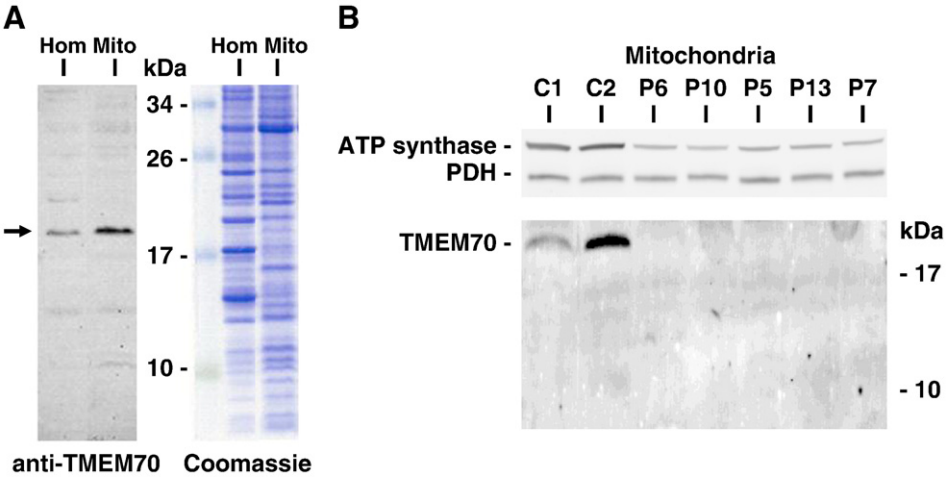

Fig. 2. Mature TMEM70 of ~21 kDa is absent in patients with ATP synthase deficiency. (A) Western blot detection of TMEM70 by polyclonal antibody in human heart homogenate and mitochondria. (B) Western blot detection of ATP synthase (β subunit), PDH (E1 α subunit) and TMEM70 in fibroblast mitochondria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

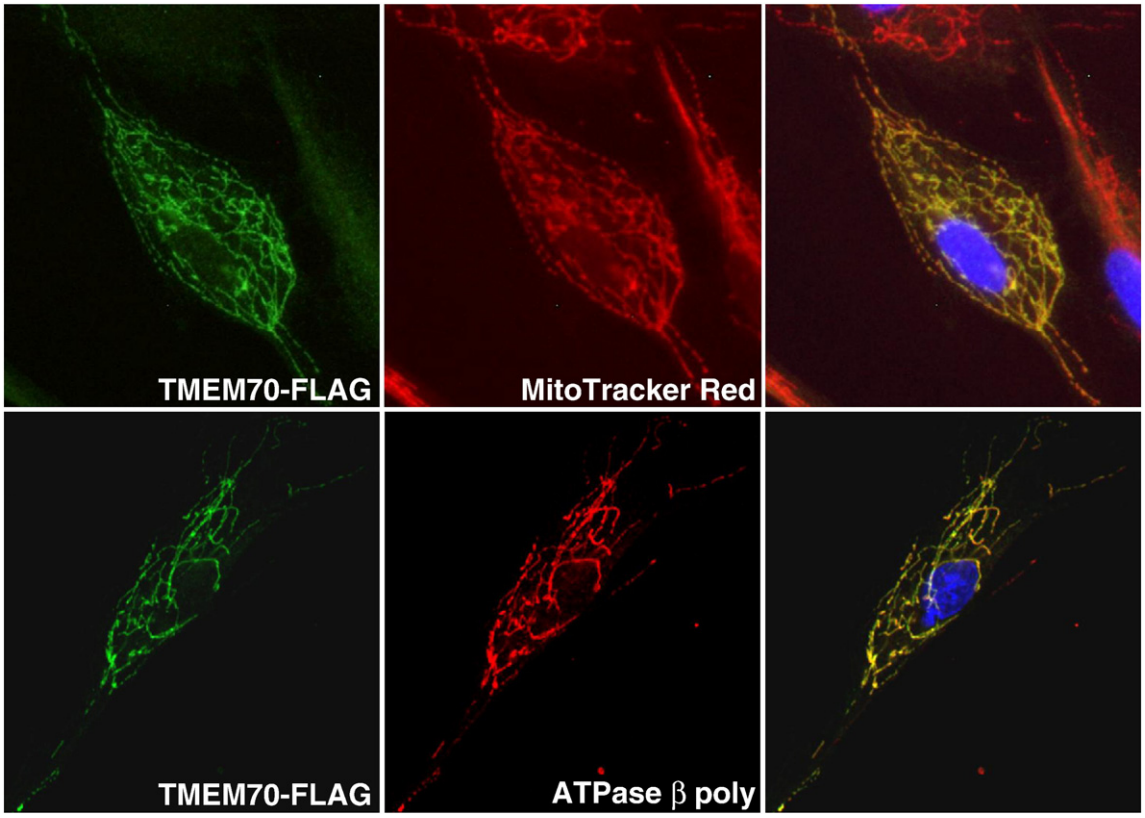

Fig. 3. Subcellular localization of TMEM70-FLAG with respect to MitoTracker Red or ATP synthase in human fibroblasts. Last column represents an overlay of the first two columns and shows cell nuclei stained with DAPI (blue).

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

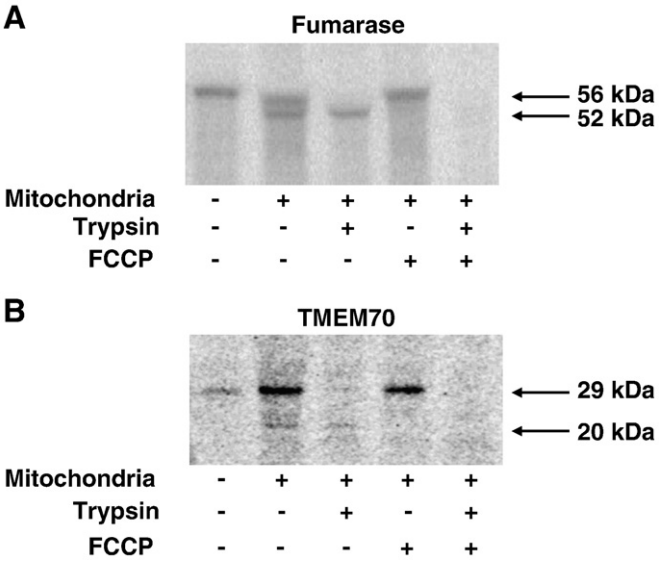

Fig. 4. Import of TMEM70 to mitochondria. *In vitro* translated human fumarase (A) and TMEM70 (B) were processed and imported to isolated rat liver mitochondria. Upon the import, the mature forms of both proteins resisted to trypsin and their import was prevented by uncoupler of oxidative phosphorylation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

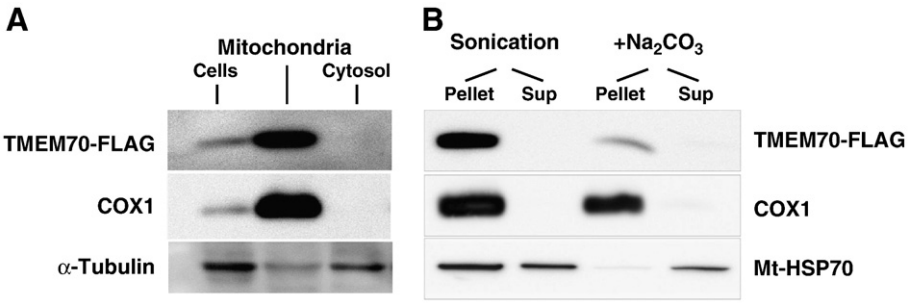

Fig. 5. TMEM70 is a membrane bound mitochondrial protein. HEK293 cells were transfected with *TMEM70-FLAG* and (A) cell homogenate, isolated mitochondria and cytosolic fractions were analyzed. (B) Isolated mitochondria were sonicated and extracted with Na_2CO_3 and 100,000*g* pellet and supernatant were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

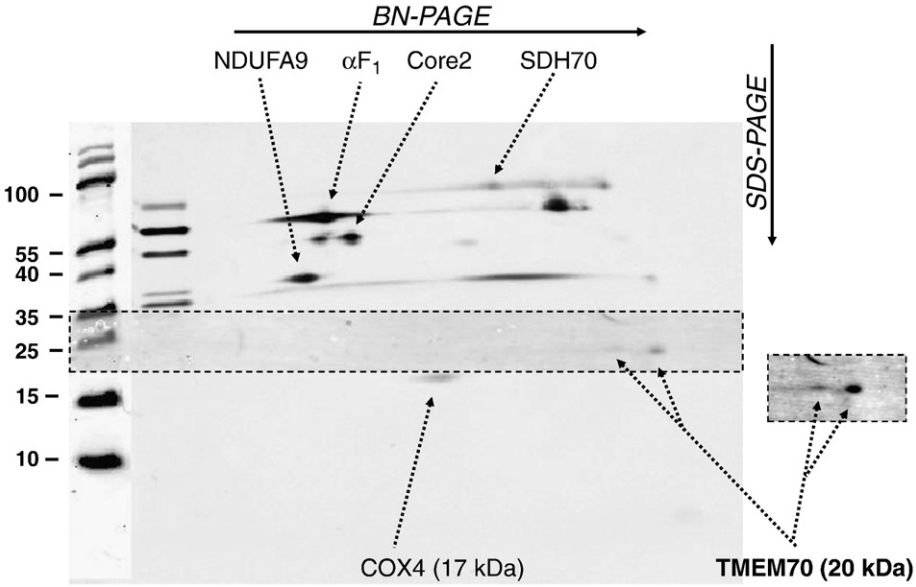

Fig. 6. DDM-solubilized mitochondrial proteins reveal a dimeric form of TMEM70. Fibroblast mitochondria were solubilized with DDM (1 g/g protein) and 30,000*g* supernatant was analyzed by two-dimensional electrophoresis (BN/SDS-PAGE) and WB using antibodies to indicated proteins. Dashed line frames the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

Acknowledgments

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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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Expression and processing of the TMEM70 protein

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Supplementary Data

Supplementary Figures

Figure S1. MS/MS identification of TMEM70 protein.

De-novo sequence of the unique HVFTTFYAK tryptic peptide corresponding to the 207-215 AA of human TMEM70 protein present in the gel slice of ca. 18-23 kDa of human heart mitochondria. MS/MS spectrum shows the fragmentation of the precursor ion 557.29, z = 2. Sequest scores were as follows: P(peptide) 3.22E-07, DeltaM (ppm) -0.06689, X-Corr 2.33, ions 12/16.

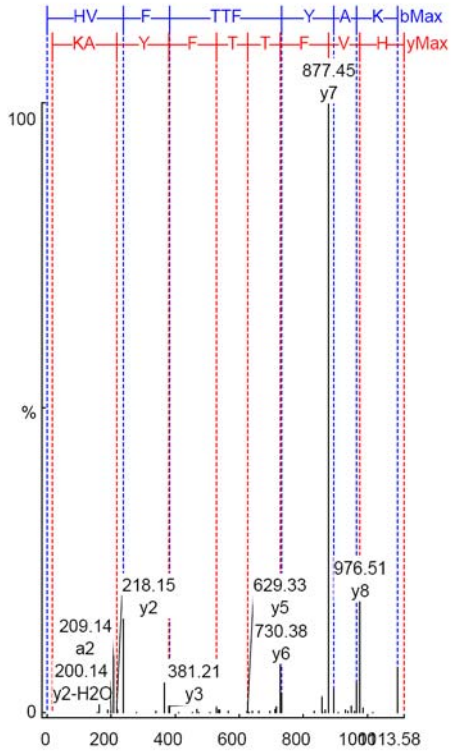
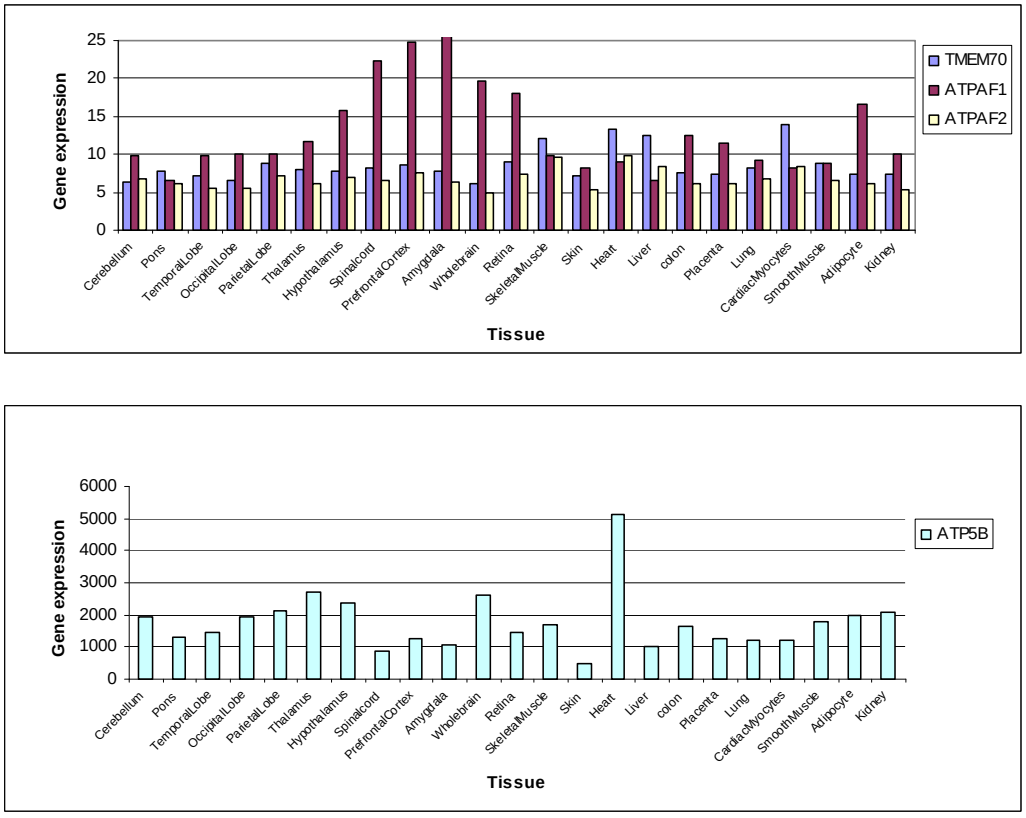


Figure S2. Gene expression profiles of *TMEM70*, *ATPAF1*, *ATPAF2* and *ATP5B* in human tissues. Gene expression pattern in selected human tissues is based on analysis by BioGPS using the U133A/GNF1H human GeneAtlas Data sets.



Supplementary Methods

Mass spectrometry analysis

Protein samples were resolved by SDS-PAGE, gel slices were excised (corresponding to approx.: 18-23, 23-28, 28-33 kDa regions) and in-gel trypsin digestion of DTT-reduced and iodoacetamide-modified proteins was performed as described [S1]. Extracted tryptic peptides were concentrated in a vacuum and dissolved in 0.1% (v/v) formic acid (FA). The resulting peptide mixture was analyzed by LC-MS/MS on an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Germany) coupled with a 2D capillary RP-HPLC system (Rheos 2000, Flux Instruments, Switzerland) with monolithic trap column (PS-DVB, Dionex, USA) and analytical capillary C18 column (PepMap C18, 75 μm × 150 mm × 3 μm, Dionex, USA). The column was equilibrated in 0.1% (v/v) FA and eluted with a gradient of a 98% (v/v) acetonitrile solution in 0.1% (v/v) FA. The precursor ions were generated from LC-MS/MS DDA proteomic analysis of tryptic digest of recombinant TMEM70. A data-dependent scan composed of one full MS scan (the resolution of 60,000) and five CID MS/MS scans (the resolution of 7,500) was used as the mass spectrometry method. The positive identification was realized via MS/MS analysis of selected peptide and comparing of retention time, exact mass of precursor ions and fragmentation spectra of peptides. The sequences were searched against the UniProt protein database (version accessed on June 2009) by the Bioworks Browser 3.3.1 SP1 and Sequest 2.0 software (Thermo Fisher Scientific, USA).

Gene expression profiles

Pattern of human gene expression across several tissues was analyzed by the BioGPS (http://biogps.gnf.org, [S2]) using the U133A/GNF1H human GeneAtlas Data

sets [S3]. Raw data for selected genes in the GeneAtlas U133A, gcrma were downloaded from BioGPS and visualized in Microsoft Excel.

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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

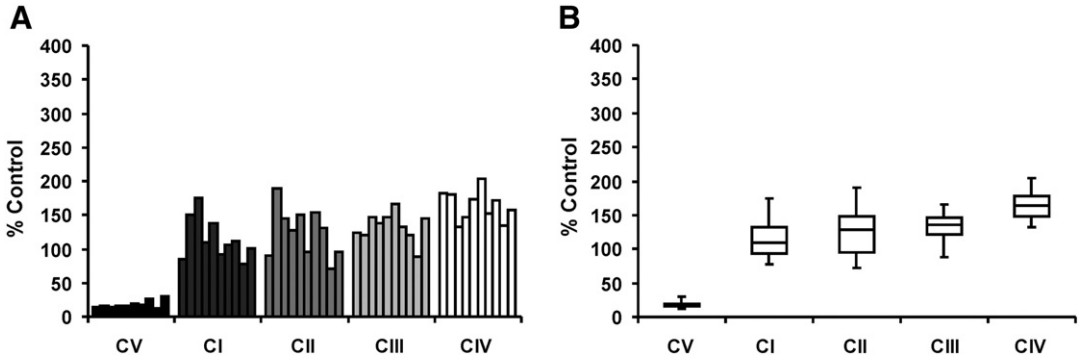

Fig. 1. The protein content of respiratory chain complexes in fibroblasts with *TMEM70* mutation. SDS-PAGE and Western blot analysis of fibroblasts were performed using subunit specific monoclonal antibodies. Detected signals were normalized to mitochondrial marker porin and expressed in % of control 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.

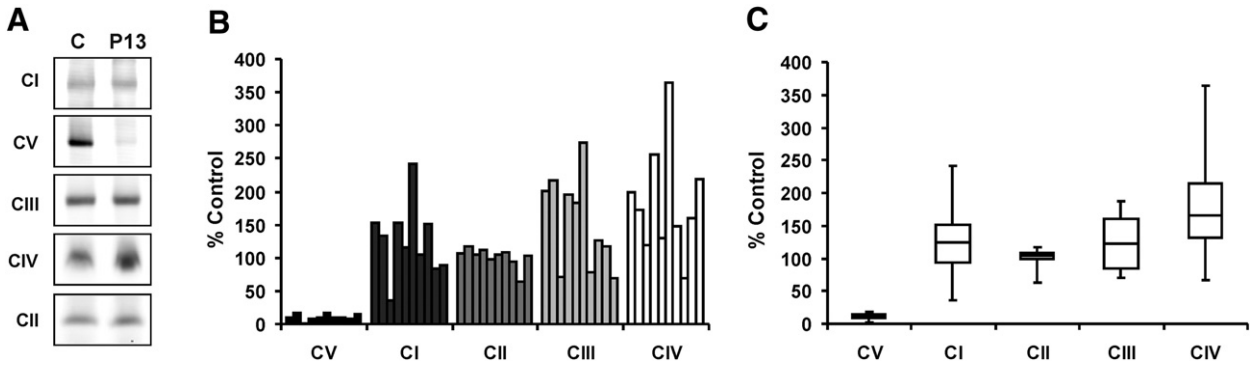

Fig. 2. The content of native respiratory chain complexes in fibroblasts with *TMEM70* mutation. BN-PAGE and Western blot analysis of DDM-solubilized fibroblasts were performed using subunit specific monoclonal antibodies. A—Analysis of control and P13 patient fibroblasts is shown. Detected signals were 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*,

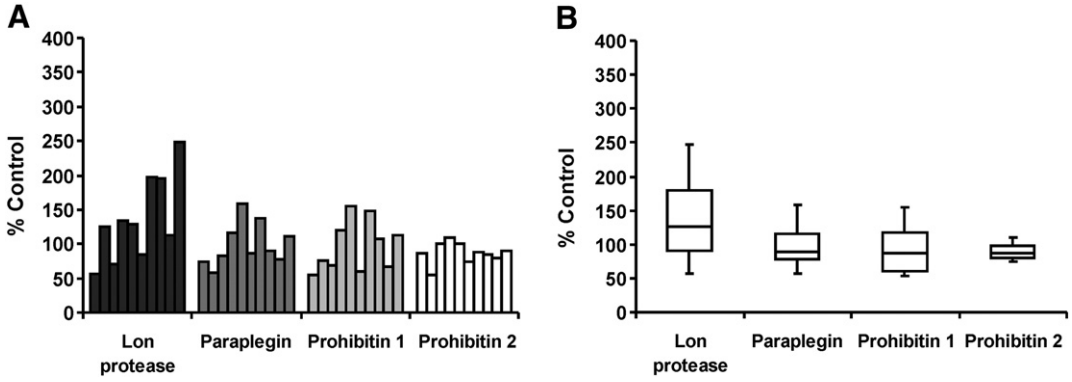

Fig. 3. The protein content of mitochondrial proteases in fibroblasts with *TMEM70* mutation. The content of Lon protease, paraplegin subunit of mAAA protease, prohibitin 1 and prohibitin 2 was analyzed in fibroblasts mitochondria by SDS-PAGE and WB using polyclonal antibodies. Detected signals in patient 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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Mitochondrial membrane assembly of TMEM70 protein

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ABSTRACT

Dysfunction of TMEM70 disrupts the biogenesis of ATP synthase and represents the frequent cause of autosomal recessive encephalocardiomyopathy. We used tagged forms of TMEM70 and demonstrated that it has a hairpin structure with the N- and C-termini oriented towards the mitochondrial matrix. On BN-PAGE TMEM70 was detected in multiple forms including dimers and displayed partial overlap with assembled ATP synthase. Immunoprecipitation studies confirmed mutual interactions between TMEM70 molecules but, together with immunogold electron microscopy, not direct interaction with ATP synthase subunits. This indicates that the biological function of TMEM70 in the ATP synthase biogenesis may be mediated through interaction with other protein(s).

1. Introduction

Mitochondrial ATP synthase is a multisubunit protein formed by F_1 catalytic part and membrane embedded F_o part, that are connected via two stalks. The biosynthesis and assembly of ATP synthase is a complex, stepwise process requiring several specific helper proteins (Ackerman and Tzagoloff, 2005). Although the structure of ATP synthase is highly similar between lower and higher eukaryotes, the biogenesis of the enzyme is rather different reflecting differences in number, expression and processing of mtDNA encoded subunits. Thus 12 specific factors involved in the biosynthesis and assembly of the mtDNA encoded subunits ATP6, ATP8 and ATP9 of the F_o part of the enzyme have been described in *Saccharomyces cerevisiae* (Ackerman and Tzagoloff, 2005; Osman et al., 2007; Zeng et al., 2007a, 2007b, 2008) and none of them has functional homologs in higher eukaryotes. In addition, 3 factors are involved in the assembly of yeast F_1 catalytic part of ATP synthase and two of them have their functional homologs in mammals — Atp11p and Atp12p chaperones for F_1-β and F_1-α subunits respectively (Wang et al., 2001). Recently TMEM70 protein has been described as another ancillary factor in the ATP synthase biogenesis (Cizkova et al., 2008). In contrast to the above-mentioned factors, TMEM70 is only present in higher eukaryotes and is lacking in *S. cerevisiae* and many other lower eukaryotes (Cizkova et al., 2008; Jonckheere et al., 2011).

The functional role of TMEM70 protein was discovered while searching for the disease causing gene responsible for the fatal mitochondrial disease caused by isolated deficiency of ATP synthase (Cizkova et al., 2008; Houstek et al., 1999). Mutation c.317-2A>G in *TMEM70* gene preventing the synthesis of the protein was found to be the cause of ATP synthase deficiency in the group of patients with severe neonatal encephalocardiomyopathy. Since then, several other *TMEM70* mutations have been described (c.118_119insGT, c.494G>A, c.336T>A, c.316+1G>T, c.238C>T, c.578_579delCA, c.211-450_317-568del, c.580G>A, g.2436–3789, c.628A>C and c.535C>T (Atay et al., 2013; Cameron et al., 2011; Cizkova et al., 2008; Jonckheere et al., 2011; Shchelochkov et al., 2010; Spiegel et al., 2011; Torraco et al., 2012; Tort et al., 2011)), demonstrating that *TMEM70* gene is rather prone to mutations and represents the most frequent cause of ATP synthase deficiency.

We have previously described that the 30 kDa precursor of TMEM70 is processed upon import to mitochondria to 21 kDa mature protein with two putative transmembrane domains, and it behaves as an inner mitochondrial membrane protein capable to form dimers or aggregates with some other protein(s) (Hejzlarova et al., 2011). While our previous studies pointed to the involvement of TMEM70 in the early stages of ATP synthase biogenesis, possibly the F_1 formation (Houstek et al., 1999, 2009), Torraco et al. (2012) recently suggested

Abbreviations: AA, amino acids; DDM, n-dodecyl-β-D-maltoside; F_1, catalytic part of ATP synthase; F_o, membrane embedded part of ATP synthase; TX-100, Triton X-100; PIC, protease inhibitor cocktail.

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the association of TMEM70 protein with ATP synthase subcomplex and the role of TMEM70 in the last steps of ATP synthase assembly — incorporation of the mtDNA encoded subunits F_o-a and A6L. However, the proper conformation of TMEM70 in the membrane as well as its function remain unknown.

In the present study we aimed to characterize structural properties of TMEM70 protein, namely its orientation in the inner mitochondrial membrane, presence in protein complexes under native conditions and its possible direct interactions with ATP synthase or other mitochondrial proteins.

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 bovine serum (Gold, PAA) at 37 °C in 5% CO_2 atmosphere.

2.2. Expression vectors and transfection

TMEM70-FLAG cDNA was inserted into vector pIRESpuro3 (Clontech) (Hejzlarova et al., 2011) and transfected into the HEK293 cell lines by nucleofection Kit V (Amaxa/Lonza). *TMEM70-GFP* cDNA expression vector (Calvo et al., 2006) was kindly provided by Prof. V.K. Mootha. To generate *TMEM70-MYC-FLAG*, *TMEM70* ORF was PCR amplified from *TMEM70-GFP* cDNA and cloned into the pCMV6-Entry plasmid (Origene). Plasmids were transfected into the HEK293 cell lines using METAFECTENE PRO (Biontex). *TMEM70-GFP* cDNA expression vector was transiently transfected into the HEK293 cells expressing TMEM70-FLAG by Express-In Transfection Reagent (Open Biosystems).

2.3. Isolation of mitochondria

Mitochondria were isolated from freshly harvested cells by hypo-osmotic shock method (Bentlage et al., 1996). Alternatively, for trypsin treatment experiment with TMEM70-FLAG, mitochondria isolated from cells homogenized by a Dounce homogenizer in isotonic medium (Stiburek et al., 2005) were used.

2.4. Trypsinization of the endogenous or C-terminal tagged TMEM70 protein in cells and mitochondria

Freshly harvested HEK293 cells constitutively expressing TMEM70-GFP, TMEM70-MYC-FLAG and control HEK293 cells were suspended in SEKT (0.25 M sucrose, 2 mM EGTA, 40 mM KCl and 20 mM Tris, pH 7.4) to a final protein concentration 5 mg/mL, mixed with the same volume of digitonin (1 mg/mL in SEKT) and incubated for 15 min on ice. The permeabilized cells were then incubated with trypsin (1 or 4 μg/100 μg of protein) in the presence or absence of 1% (v/v) Triton X-100 (TX-100) for 20 min at 37 °C. To stop digestion, SDS sample buffer (2% (v/v) 2-mercaptoethanol, 4% (w/v) SDS, 50 mM Tris (pH 7.0), 10% (v/v) glycerol) was added and the samples were incubated for 5 min at 90 °C. Samples were analyzed by 12% SDS-PAGE and WB using antibodies against TMEM70, FLAG, GFP, and compartment specific markers (intermembrane space — OPA1, matrix — PDH, loading control — porin). For details about antibodies used see Section 2.8.

To analyze the effect of trypsin on isolated organelle, mitochondria isolated from HEK293 cells constitutively expressing TMEM70-FLAG were exposed to hypotonic shock (Tang et al., 2009). Briefly, 150 μg of mitochondrial protein were resuspended in 100 μL of 5 mM MOPS-KOH, pH 7.2, and incubated for 20 min at 4 °C on a rotator. Swollen mitochondria with disrupted outer membrane were treated with trypsin (4 μg/100 μg of protein) for 20 min at 4 °C on a rotator in the presence or absence of 1% TX-100. Digestion was stopped with 100 μg/mL of soybean trypsin inhibitor and 1% (v/v) protease inhibitor cocktail (PIC, Sigma P8340). Samples were analyzed by 12% SDS-PAGE and WB using antibodies against FLAG and compartment specific markers (intermembrane space — OPA1, matrix — fumarate hydratase, inner membrane — Ndufb6).

2.5. Fluorescence microscopy analysis of TMEM70-GFP localization in intact cells and cells with permeabilized cytoplasmic and mitochondrial membrane

Selective permeabilization of the cytoplasmic and mitochondrial membranes by detergents as well as quenching of GFP fluorescence by Trypan blue was utilized as a tool to assess GFP tag orientation in mitochondria, essentially as in De Stefani et al. (2011). We used HEK293 cell lines transiently expressing TMEM70-GFP fusion protein, cells expressing GFP fusion protein localized in the cytosol (pMax-GFP, Amaxa/Lonza), or GFP fusion protein localized in the mitochondrial matrix (mt-GFP, Stratagene). Experiments were performed 48 h after transfection. The fluorescence signal was monitored on Leica DMI6000 wide field microscope with motorized z-stage equipped with Andor iXon897 photon counting camera. For each time point, image z-stack was collected (step optimized to 1 airy) and best focused plane was used for quantification. Cells were imaged in intracellular buffer containing 120 mM KCl, 3 mM HEPES, 5 mM KH_2PO_4, 3 mM $MgSO_4$ and 1 mM EGTA, pH 7.2. Each acquisition lasted for 10 min and subsequent additions were as follows: digitonin (0.2 g/g of protein, at 120 s), proteinase K (4 units, at 240 s), Trypan blue dye (0.05% (w/v), at 360 s) and TX-100 (1%, at 480 s). Total signal intensity from the whole cell at each timepoint was quantified in ImageJ software (FIJI package).

2.6. Immunoprecipitation

For TMEM70-FLAG immunoprecipitation, 1.3 mg protein of freshly isolated mitochondria from cultured cells were lysed with 0.35 mL buffer containing 0.5% TX-100, 150 mM NaCl and 50 mM Tris–HCl (pH 7.4) and 1% PIC for 30 min at 4 °C on a rotator and centrifuged at 12,000 *g* for 10 min. The lysate (0.32 mL) was incubated overnight at 4 °C with previously washed 40 μL of an EZ View ANTI-FLAG M2 affinity agarose resin (Sigma, F2426). Subsequently the resin was washed three times with buffer containing 150 mM NaCl and 50 mM Tris–HCl (pH 7.4). The bound protein was eluted by competition with 3× FLAG peptide (Sigma, F4799). The eluted immunoprecipitate was combined with SDS sample buffer and analyzed by 12% SDS-PAGE and Western blotting.

For ATP synthase immunoprecipitates, ATP Synthase Immunocapture Kit (Abcam, ab109715) was used. Freshly isolated mitochondria (0.45 mg protein) were solubilized with 2 g of n-dodecyl-β-D-maltoside (DDM)/g of protein in 90 μL PBS containing 0.2% PIC for 15 min on ice and centrifuged at 30,000 *g* for 20 min. Supernatant (80 μL) was mixed with ATP Synthase Immunocapture matrix. The mixture was incubated overnight at 4 °C on rotator and subsequently washed three times with PBS + PIC. Washed beads were mixed with SDS sample buffer and the sample subjected to 10% SDS-PAGE and Western blotting.

2.7. Polyacrylamide gel electrophoresis and Western blot immunodetection

SDS-PAGE (Schagger and von Jagow, 1987) was performed on 10% or 12% polyacrylamide minigels using Mini-Protean system (Bio-Rad).

BN-PAGE was performed on 8–15% polyacrylamide minigels (Schagger and von Jagow, 1991). Isolated mitochondria were solubilized with DDM (1 g/g of protein) or digitonin (2 g/g of protein) for 15 min on ice in 1.5 M aminohexanoic acid, 2 mM EDTA and 50 mM Bis-Tris, pH 7.0. The samples were centrifuged for 20 min at 4 °C and 30,000 *g* and Coomassie Brilliant Blue G-250 dye (Serva, 0.1 g/g of detergent) and 5% (v/v) glycerol were added to supernatants before electrophoresis.

For two dimensional BN/SDS-PAGE, strips of the first dimension gels were incubated in a 2D buffer containing 1% 2-mercaptoethanol and 1% SDS and then resolved in the second dimension on SDS-PAGE.

Western blot analysis using semidry electrotransfer of proteins was performed by standard protocols as previously described (Jesina et al., 2004; Stiburek et al., 2005). For immunodetection we used specific antibodies to FLAG (Sigma, F1804), OPA1 (BD Biosciences, 612606), fumarate hydratase (FH, Abnova, H00002271-M01), PDH E1-α subunit (Abcam, ab110334), GFP (Santa Cruz Biotechnology, sc-9996), TMEM70 (Proteintech Group, 20388-1-AP or 60195-1-Ig), Complex I subunit Ndufb6 (Abcam, ab110244), Complex II subunit Sdh70 (Abcam, ab14715), Complex III subunit Core2 (Abcam, ab14745), Complex IV subunit Cox1 (Abcam, ab14705), ATP synthase subunits F_1-α (Abcam, ab110273; or lot 20D6 (Moradi-Ameli and Godinot, 1983)), F_1-β (Abcam, ab14730), F_1-δ (GeneTex, GTX101503), F_o-c (Jesina et al., 2004), F_o-a (Jesina et al., 2004), OSCP (Abcam, ab110276), IF1 (Abcam, ab110277), F6 (Abcam, ab110279) and F_o-d (Abcam, ab110275). The immunoblots were detected with peroxidase conjugated secondary antibodies and SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific Pierce) using VersaDoc 4000 Imaging System (Bio-Rad), or with fluorescent secondary antibodies (Alexa Fluor 680, Life Technologies; IRDye800, Rockland Immunochemicals) on an Odyssey infrared imaging system (LI-COR).

2.8. Immunogold electron microscopy

Immunolabeling of TMEM70 protein and ATP synthase F_1-α at the ultrastructural level was performed directly on grids with the cell sections. Briefly, HEK293 cells grown on glass coverslips were fixed with freshly prepared formaldehyde, dehydrated and embedded in LR White resin. Ultrathin sections mounted on Formvar/carbon-coated nickel grids were incubated overnight with a mixture of primary antibodies: the rabbit polyclonal antibody to TMEM70 (Proteintech Group, 20388-1-AP) and the monoclonal antibody to ATP synthase F_1-α (Abcam, ab110273). After rinsing, the sections were incubated in secondary antibody for 30 min. Colloidal gold coupled secondary antibodies (anti-rabbit IgG — 6 nm gold particles, anti-mouse IgG — 12 nm gold particles, Jackson Immuno Research) were used. In the control specimens, sections were treated with the reaction mixture where the primary antibodies were omitted. Finally, the grids were contrasted with uranyl acetate followed by lead citrate. The labeled preparations were observed with a Zeiss 900 or Tecnai G^2 Sphera 20 electron microscopes at 80 and 100 kV, using a 30–40 μm objective aperture.

3. Results

3.1. Orientation of TMEM70 protein in the inner mitochondrial membrane

Previous studies demonstrated that TMEM70 is a mitochondrial protein localized in the inner mitochondrial membrane. Based on the two short and conserved transmembrane regions the protein has putative hairpin like fold with the short N-terminal and long C-terminal sequences oriented towards the same hydrophilic mitochondrial compartment (Jonckheere et al., 2011). To establish native orientation of TMEM70 protein in the inner membrane we tested accessibility of the C-terminal sequence to external protease- trypsin, at conditions when the protease can reach the intermembrane space or mitochondrial matrix. While the C-terminal sequence contains numerous lysines and arginines, no trypsin-cleavable sites are present in the predicted loop between the two transmembrane domains. For experiments we utilized HEK293 cells expressing the tagged forms of TMEM70 protein. Both tags used, i.e. MYC-FLAG and GFP, were attached to the noncleaved C-terminus of the sequence.

As summarized in Fig. 1, we tested the accessibility of TMEM70 in intact cells, after disruption of the plasma and outer mitochondrial membranes with digitonin and after permeabilization of the inner mitochondrial membrane by 1% TX-100. Low digitonin concentration (0.2 g/g of protein) is sufficient for permeabilization of the plasma and outer mitochondrial membranes but not for solubilization of the inner membrane proteins. Fig. 1A shows the behavior of the endogenous TMEM70 protein detected with specific antibody recognizing an epitope at the C-terminus of the protein. In digitonin-permeabilized cells addition of 1 or 4 μg of trypsin/100 μg of protein digested OPA1 protein localized in the intermembrane space and was without effect on matrix PDH as well as on TMEM70. Both TMEM70 and PDH became digested when TX-100 was added demonstrating that the TMEM70 antigen recognized by the antibody has to be located (similarly as PDH) in mitochondrial matrix. The same experiment was then performed with TMEM70-MYC-FLAG (Fig. 1B) and TMEM70-GFP (Fig. 1C). The MYC-FLAG attached to the TMEM70 C-terminus displayed analogous accessibility to trypsin and associated loss of the FLAG antigen; again in line with the matrix localization of the C-terminal sequence. This was further confirmed using TMEM70-GFP (Fig. 1C). The GFP signal at approx. 46 kDa was again resistant to trypsin unless Triton X-100 was added. Interestingly, the signal then moved to approx. 27 kDa and remained present even at the higher trypsin concentration (T4), while the TMEM70 signal completely disappeared. As GFP is highly resistant to trypsin digestion (Chiang et al., 2001) the 27 kDa signal most likely represents the whole GFP protein, which suggests that the cleavage site has to be at the very end of the TMEM70 C-terminus, close to the attached GFP molecule.

In further experiments we used isolated mitochondria from cells expressing TMEM70-FLAG to assess membrane topology of TMEM70. As shown in Fig. 2, when isolated mitochondria were swollen in hypotonic medium to disrupt the outer membrane, OPA1 protein of the intermembrane space was fully accessible and digested by trypsin, while the matrix enzyme, fumarate hydratase was protected from trypsin cleavage. At these conditions the C-terminal FLAG tag of TMEM70 protein was also unaffected by the protease. However, when TX-100 was added prior to trypsin treatment, both the fumarate hydratase and FLAG tag signals completely disappeared, thus confirming the matrix orientation of the TMEM70 C-terminus, in agreement with the data from the permeabilized cells.

As an independent approach to characterize the membrane topology of TMEM70 protein we used fluorescence microscopy of GFP protein and quenching of the GFP fluorescence by membrane impermeable dye Trypan blue. This approach was recently used to study the orientation of MCU protein from the inner mitochondrial membrane (De Stefani et al., 2011). As summarized in Fig. 3, we used HEK293 cells expressing TMEM70-GFP and compared them with HEK293 cells expressing two different forms of GFP — mt-GFP, i.e. a soluble GFP protein localized in the mitochondrial matrix, and pMax-GFP, which is localized in the cytosol. Cells were permeabilized with low digitonin concentration affecting only the cytoplasmic membrane and subsequently treated with proteinase K, Trypan blue and TX-100. As shown in Fig. 3A and B, in digitonin-permeabilized cells the cytosolic pMax-GFP fluorescence was completely lost by proteinase K digestion, as expected. In contrast, matrix mt-GFP and TMEM70-GFP were not affected by the addition of neither the proteinase K, which would digest proteins on the outer mitochondrial membrane nor by the Trypan blue, which would quench fluorescence of the GFP localized in the intermembrane space. However, rapid quenching of both the mt-GFP and TMEM70-GFP signals was induced by subsequent addition of TX-100 demonstrating that detergent solubilization of the inner mitochondrial membrane was essential for membrane impermeable Trypan blue and proteinase K to reach the GFP tag on TMEM70 or mt-GFP. Thus the morphological approach fully confirmed biochemical studies pointing to the matrix localization of the C-terminal sequence of TMEM70 protein.

3.2. Native organization of TMEM70 protein

We have previously found that TMEM70 was present on BN-PAGE as two spots possibly a monomer and dimer (Hejzlarova et al.,

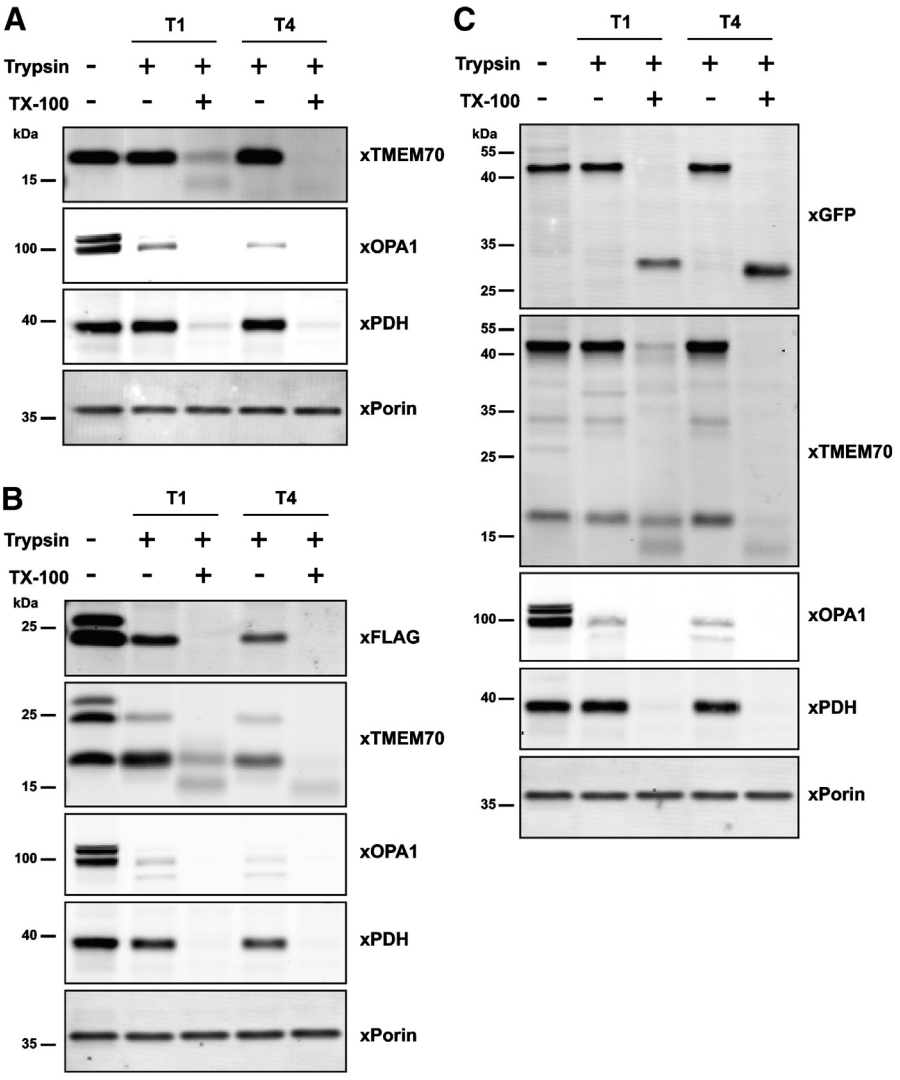

Fig. 1. Accessibility of TMEM70 to protease in digitonin-treated HEK293 cells expressing TMEM70, TMEM70-MYC-FLAG or TMEM70-GFP protein. After disruption of the cell and outer mitochondrial membranes by digitonin, HEK293 cells (A) with endogenous expression of TMEM70 protein, (B) constitutively expressing TMEM70-MYC-FLAG and (C) constitutively expressing TMEM70-GFP were treated with two different trypsin concentrations (T1, T4 — 1 and 4 μg of trypsin/100 μg of protein) in the presence or absence of 1% Triton X-100 (TX-100). Samples were separated by SDS-PAGE and probed with antibodies to TMEM70, FLAG, GFP, OPA1 (representative of intermembrane space proteins), PDH (representative of matrix proteins) and porin (loading control, as its beta barrel fold is well protected from protease digestion).

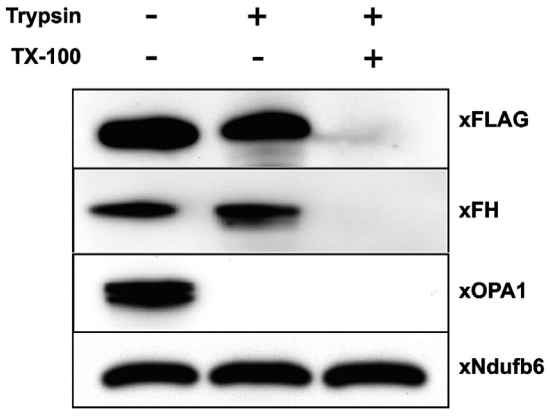

Fig. 2. Accessibility of the C-terminal FLAG to protease in mitochondria from HEK293 cells expressing TMEM70-FLAG. The outer membrane of isolated mitochondria was disrupted by hypotonic shock and mitochondria were treated with 4 μg of trypsin/100 μg of protein in the presence or absence of 1% Triton X-100 (TX-100) as indicated. Samples were separated by SDS-PAGE and probed with antibodies to FLAG, OPA1 (representative of intermembrane space proteins), fumarate hydratase (FH, representative of matrix proteins) and Ndufb6 (inner membrane protein protected from protease digestion).

2011). In further experiments we followed behavior of the detergent-solubilized tagged forms of TMEM70 by 2D analysis combining native and SDS electrophoreses and WB immunodetection with antibodies to the tags used (GFP, FLAG). As shown in Fig. 4A, the DDM-solubilized mitochondria from HEK293 cells expressing TMEM70-GFP (mature protein 46 kDa) revealed two distinct forms of the protein. Based on the migration pattern of free subunit F_1-α of 55 kDa, they should represent monomer and dimer of TMEM70-GFP. Relatively high portion of TMEM70-GFP could be detected in the high molecular weight region as an unfocused streak. In the cells expressing TMEM70-FLAG (mature protein 22 kDa) two major forms of the tagged protein were present (both migrating below F_1-α), while the migration of the single spot of TMEM70-MYC-FLAG (mature protein 24 kDa) was smaller than F_1-α subunit. These spots could represent TMEM70-FLAG monomer and dimer and TMEM70-MYC-FLAG dimer, respectively. A strong signal of TMEM70-MYC-FLAG was found also in the high molecular weight region, approximately between the position of ATP synthase monomers and dimers, but again this signal was rather unfocused. Distribution of tagged forms was further verified by using anti-TMEM70 antibody, which gave analogous patterns. Only discrepancy was in case of TMEM70-GFP, which was detected as two parallel lines of identical

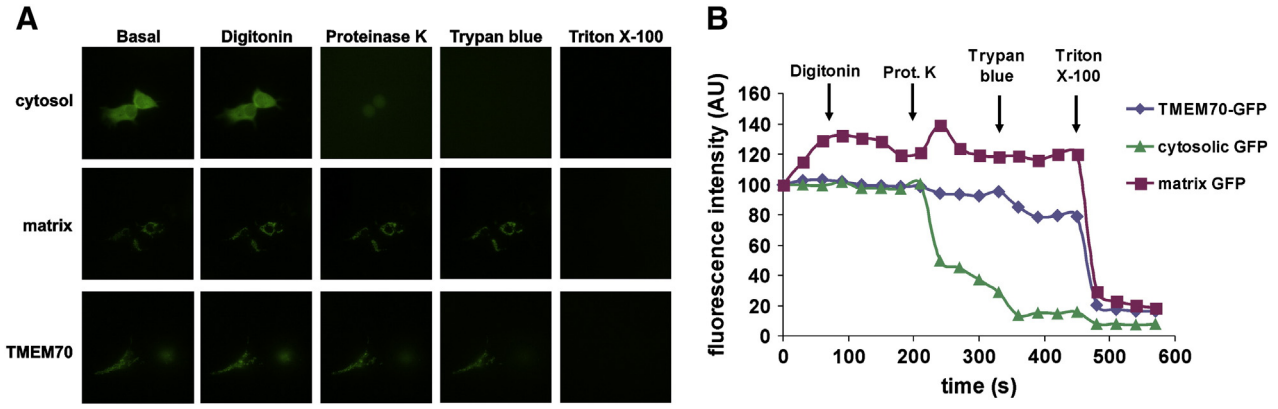

Fig. 3. Fluorescence detection of accessibility of C-terminal GFP to inner membrane impermeable Trypan blue dye in digitonin treated HEK293 cells expressing TMEM70-GFP protein. For experiment cell lines HEK293 pMax-GFP (GFP fusion protein localized in the cytosol), HEK293 mt-GFP (GFP fusion protein localized in the mitochondrial matrix) and HEK293 transiently expressing TMEM70-GFP were used. In the first step the cytoplasmic membranes of the cells were disrupted by low digitonin concentration treatment. Cells were subsequently incubated with proteinase K (digests cytosolic proteins) and Trypan blue dye. As a small molecule, Trypan blue gets into the intermembrane space inaccessible for proteinase K and quenches the GFP signal in this compartment. Afterwards Triton X-100 was added to disrupt all membranes. The fluorescence signal was monitored in HEK293 cells on Leica DMI6000 microscope. (A) GFP fluorescence images, (B) quantification of fluorescence data (n = 10 for each cell line).

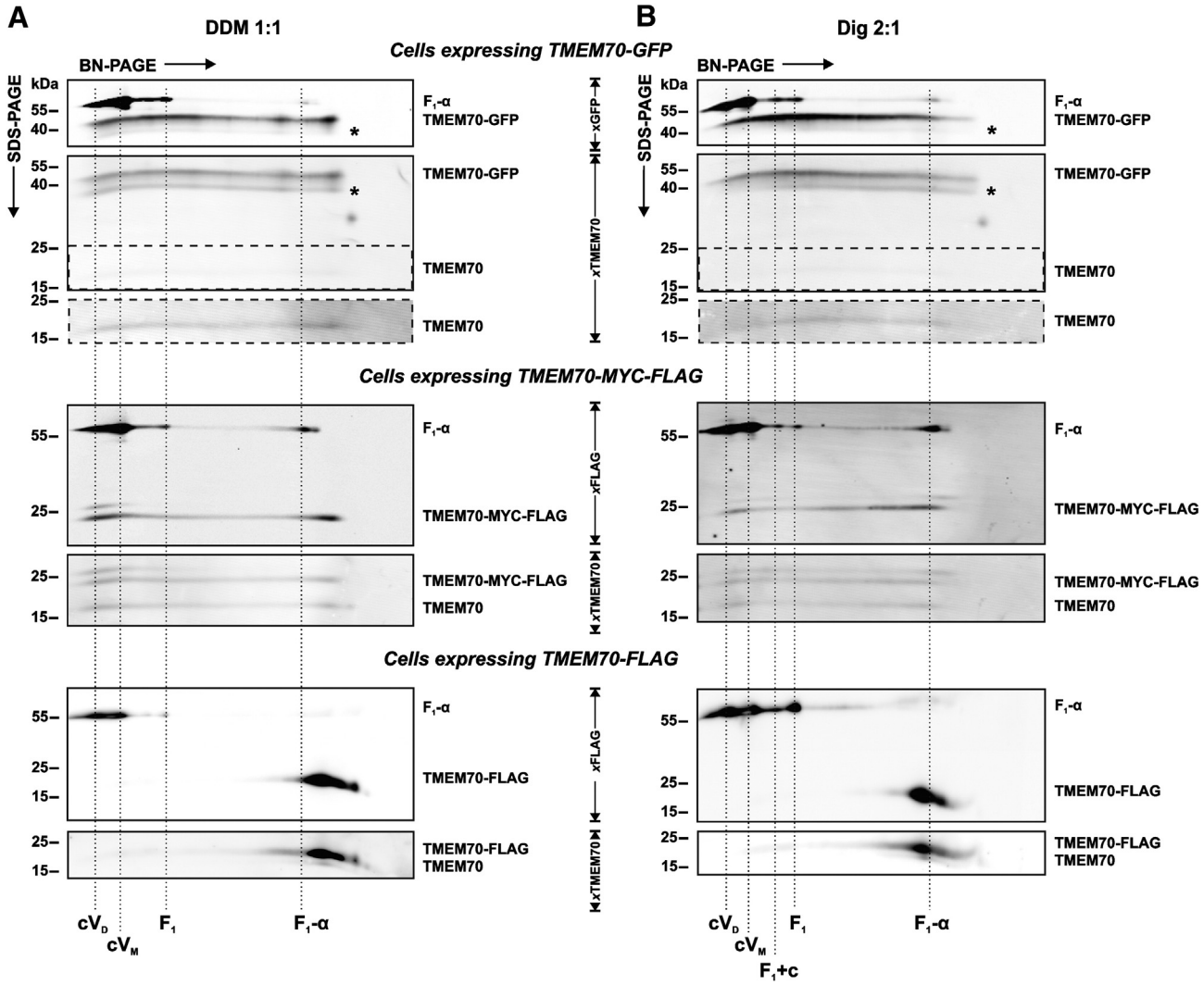

Fig. 4. Multiple forms of TMEM70 protein. Mitochondria isolated from HEK293 cells constitutively expressing TMEM70-GFP, TMEM70-MYC-FLAG or TMEM70-FLAG were solubilized with 1 g of DDM/g of protein (A) or with 2 g of digitonin/g of protein (B) and analyzed by two dimensional BN/SDS-PAGE and Western blot detection using antibodies to FLAG, GFP, TMEM70 and F_1-α subunit. Upper panels show detection of F_1-α subunit and GFP or FLAG, the lower panels show detection of TMEM70. cV_D, ATP synthase dimer; cV_M, ATP synthase monomer; F_1 + c, subcomplex of F_1 part and subunit c of ATP synthase; F_1, F_1 part alone; F_1-α, free F_1-α subunit. Dashed border line indicates higher intensity exposition of the relevant part of the membrane from the upper panel. Asterisks mark the proteolytic product of TMEM70-GFP.

profile. The lower (circa 40 kDa) line displayed only marginal reactivity with anti-GFP antibody and reflects some non-specific proteolysis of the GFP-tagged protein. Antibody to TMEM70 also clearly detected the endogenous TMEM70 protein. Its expression levels were considerably lower than those of the tagged forms, but showed similar distribution profile with low molecular weight forms as well as with the streaking signal up to the region of cV_D.

Since we detected all TMEM70-GFP, TMEM70-MYC-FLAG and native TMEM70 to partly overlap with ATP synthase (Fig. 4A), we used milder detergent digitonin instead of DDM for the solubilization of mitochondria, which may better preserve weak noncovalent interactions. In the region of putative monomers and dimers we observed a general shift to the higher molecular weights for TMEM70-GFP, TMEM70-MYC-FLAG as well as TMEM70, possibly due to the presence of the empty detergent micelles (Fig. 4B). More of the signal was found as unfocused streaks in the high molecular weight region, especially in the case of TMEM70-GFP. Nevertheless, no distinct spot pointing to specific association with the ATP synthase complex or the F_1 subcomplexes could be detected.

Expression of the tagged forms of TMEM70 was further used as an approach to find out whether TMEM70 can form oligomers. In Fig. 5 we have analyzed HEK293 cells expressing either TMEM70-GFP or TMEM70-FLAG alone or coexpressing both constructs simultaneously (TMEM70-FLAG + TMEM70-GFP). Mitochondria from these cells were solubilized with TX-100 and immunoprecipitation was performed with anti-FLAG antibody. As shown in Fig. 5, in the immunoprecipitate from cells coexpressing both tagged forms, the FLAG- and the GFP-tagged TMEM70 was detected. On the other hand, in cells expressing sole TMEM70-FLAG, only TMEM70-FLAG was detectable and no tagged TMEM70 was present in the immunoprecipitate from TMEM70-GFP expressing cells in accordance with the fact that anti-FLAG antibody was used for immunoprecipitation. Specific coimmunoprecipitation of TMEM70-GFP by anti-FLAG antibody from cells coexpressing both forms was further confirmed by the immunodetection with anti-GFP and anti-TMEM70 antibodies. In addition to the mature TMEM70-GFP (46 kDa) also a larger form, possibly yet unprocessed TMEM70-GFP was immunoprecipitated. These experiments clearly proved that TMEM70 protein can form oligomers, as previously suggested. On the other hand, they indicate that the interaction is weak and labile and/or may include only some copies of the protein.

3.3. No detectable interaction of TMEM70 with ATP synthase by immunoprecipitation and immunogold electron microscopy

Although the specific ancillary function of TMEM70 in the biosynthesis and assembly of functional ATP synthase is well established, its exact role in this process is still unclear (Cizkova et al., 2008; Honzik et al., 2010; Houstek et al., 1999; Torraco et al., 2012). An attractive hypothesis is that TMEM70 interacts directly with some of ATP synthase subunits, assembly intermediates or fully assembled enzyme. In Fig. 6 we addressed this question by further extension of immunoprecipitation experiments. We have expressed TMEM70-FLAG in HEK293 cells and used anti-FLAG antibody for immunoprecipitation of TX-100 solubilized mitochondria. The resulting immunoprecipitate was analyzed by SDS-PAGE and Western blot with a series of specific antibodies to subunits of ATP synthase. HEK293 cells expressing empty vector were used as a control. We have checked for the possible presence of F_1 subunits α, β, δ and IF1, as well as F_o subunits a, d, F6 and OSCP in the immunoprecipitate. However, none of them could be detected, although all antibodies were able to detect respective proteins in the whole mitochondria. In the case of subunit F_1-β, we found strongly reacting band that was somewhat higher than the mature F_1-β, but this reaction was unspecific as it was found in the immunoprecipitates from control cells not expressing TMEM70-FLAG as well (HEK293 cells with empty vector). In addition, we have also tested crossreactivity of the immunoprecipitates with antibodies to respiratory chain complexes II (Sdh70), III (Core2) and IV (Cox1), but with negative result. Efficiency of the immunoprecipitation was verified with antibodies to TMEM70 and FLAG.

In order to check whether the FLAG tag attached to TMEM70 did not affect its ability to interact with subunits of the ATP synthase in the FLAG-IP experiments, we took also the reverse approach. Here, we captured the ATP synthase by immunoprecipitation and looked for the presence of the endogenous TMEM70 protein. Fig. 7 illustrates, that

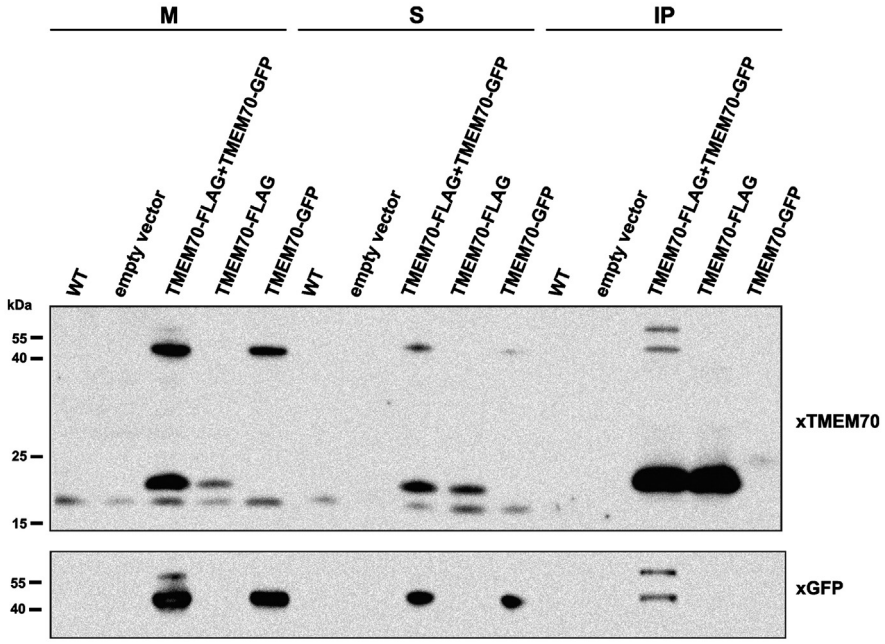

Fig. 5. Detection of TMEM70 oligomers by immunoprecipitation. Isolated mitochondria from parental HEK293 cells (WT), cells expressing TMEM70-FLAG, TMEM70-GFP, cells expressing both TMEM70-FLAG and TMEM70-GFP, as well as cells transfected with empty vector were solubilized with 0.5% Triton X-100 and for immunoprecipitation antibody to FLAG was used. Original mitochondria (M, 10 μg protein), solubilized proteins (S) and the immunoprecipitates (IP, obtained from ~290 μg protein of original mitochondria) were analyzed by SDS-PAGE and Western blot detection using antibodies to TMEM70 or GFP.

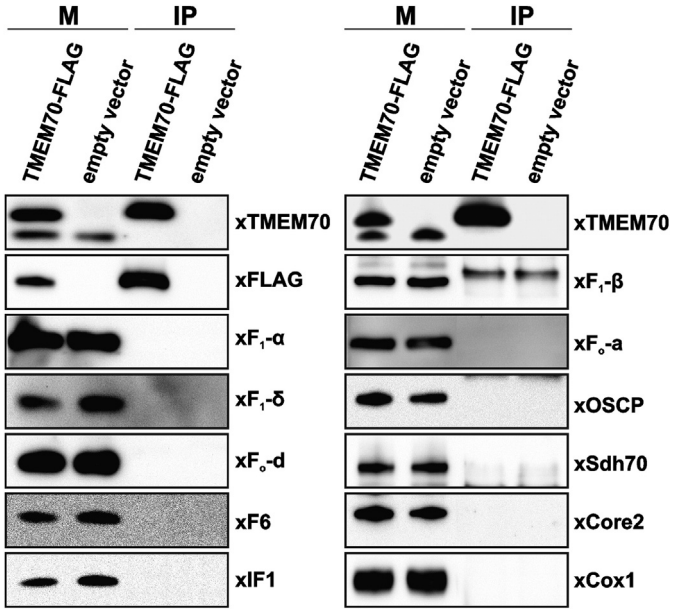

Fig. 6. Search for ATP synthase subunits in the TMEM70 immunoprecipitates. Mitochondria from HEK293 cells constitutively expressing TMEM70-FLAG and from HEK293 cells with empty vector were solubilized with 0.5% Triton X-100 and lysate proteins were immunoprecipitated using FLAG antibody. Original mitochondria (M, 10 μg protein) and the immunoprecipitate (IP, obtained from ~580 μg protein of original mitochondria) were analyzed by SDS-PAGE and Western blotting using indicated antibodies to TMEM70, FLAG, ATP synthase subunits (F_1-α, F_1-β, F_1-δ, IF1, F_o-a, F_o-d, OSCP, F6) and respiratory chain complexes II (Sdh70), III (Core2) and IV (Cox1).

using ATP Synthase Immunocapture antibody, ATP synthase was efficiently immunoprecipitated but no signal of TMEM70 protein could be observed, even when mild conditions of solubilization (2 g of DDM/g of protein) were used. The same results were obtained with other F_1 antibodies previously used for the immunoprecipitation (not shown) (Houstek et al., 1999).

Finally, to check for the possible interactions in the intact mitochondrial membrane, we have also performed electron microscopy studies by means of post embedding immunogold detection of TMEM70 protein and ATP synthase F_1-α. Immunolabeling of cellular constituents on ultrathin sections of intact cells is the method of choice for high resolution in situ localization of various proteins within the cell. Moreover,

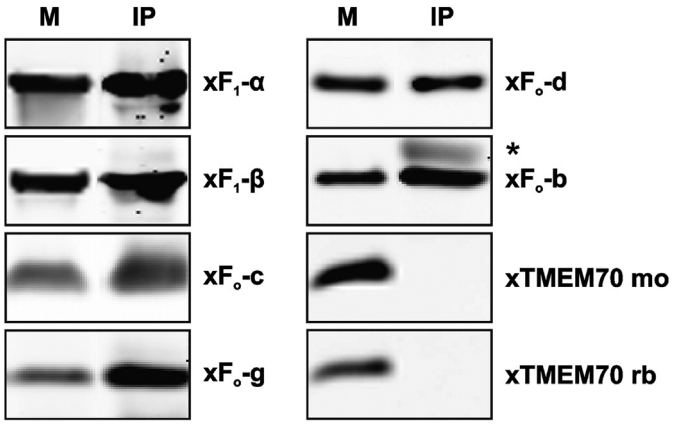

Fig. 7. Search for TMEM70 protein in the ATP synthase immunoprecipitates. Isolated mitochondria from wild type HEK293 cells were solubilized with 2 g of DDM/g of protein and immunoprecipitated with ATP Synthase Immunocapture Kit. Original mitochondria (M, 10 μg protein) and the immunoprecipitate (IP, obtained from ~40 μg protein of original mitochondria) were analyzed by SDS-PAGE and probed with mouse monoclonal (mo) or rabbit polyclonal (rb) antibody to TMEM70 and with antibodies to ATP synthase subunits F_1-α, F_1-β, F_o-c, F_o-g, F_o-d and F_o-b. Asterisk marks the light immunoglobulin chain.

such labeling circumvents the problem of the limited accessibility of antibodies into the cellular compartments, because the immunoreaction takes place on the surface of physically sectioned material. Fig. 8 shows the result of double immunogold analysis detecting ATP synthase with 12 nm colloidal gold particles and TMEM70 protein with 6 nm colloidal gold particles. Both signals were found inside of mitochondria, sometimes associated with the inner membrane. However, they were found in distinct regions and rather far from one another, thus arguing against close, intimate interaction of these two proteins.

4. Discussion

The ATP synthase biogenesis in mammalian mitochondria critically depends on TMEM70 protein, the lack of which diminishes the mitochondrial content of this enzyme far below the level needed for phosphorylation of ADP at physiological rates of substrate oxidation. While it is clear that TMEM70 protein is an important ancillary factor for de novo formation of ATP synthase complex, its exact role in this process remains unknown.

The aim of this study was to characterize the topology of TMEM70 protein within the inner mitochondrial membrane and to bring more light into the functional properties of TMEM70. We focused on its possible interaction with other protein(s), particularly ATP synthase subunits or assembly intermediates. The membrane topology of TMEM70 protein has previously been predicted by computational analysis (Jonckheere et al., 2011) of relatively high average accuracy (Klammer et al., 2009) but it was not experimentally verified. Therefore, in the first part of our study we have characterized the membrane topology of TMEM70 protein by several independent approaches using GFP and FLAG as the C-terminal tags of TMEM70 protein. Based on the accessibility to membrane impermeable protease or Trypan blue dye at differently permeabilized mitochondria we demonstrated that orientation of the C-terminus is towards the mitochondrial matrix.

TMEM70 protein sequence contains two highly conserved transmembrane domains spanning 21 amino acids (AA) each, separated by a short sequence of 18 mostly hydrophilic AA. Such structure should generate a hairpin like membrane assembly of the protein. Based on our results thus both the N- and C-termini of TMEM70 protein have to be located in the mitochondrial matrix. Such conformation is also in accordance with the previous computational prediction (Jonckheere et al., 2011). Additional evidence is also provided by the observation that the N-terminal presequence was cleaved upon import of nascent protein to the mitochondrial matrix (Hejzlarova et al., 2011), requiring the presence of the N-terminal part in the matrix during protein import. The matrix facing N-terminal is only 21 AA long and may not be critical for the protein function. On the other hand the matrix exposed C-terminal sequence is about 98 AA long with the first 40 AA highly conserved between species, indicating that this part of the sequence might be functionally important. TMEM70 could thus be involved in the matrix localized events in the ATP synthase biogenesis. Of interest is the phenotype of the recently reported c.578-579delCA mutation, predicted to yield truncated TMEM70 lacking the last 60 AA, but still containing the highly conserved region in the proximal part of the C-terminus. It was associated with rather long survival (24 years) of one of the siblings, which is unusual with other *TMEM70* mutations, although even here the phenotype was relatively severe (Spiegel et al., 2011).

Solubilization of membrane proteins in combination with analysis by native electrophoresis represents well established approach to characterize native forms of these proteins as well as their interactions with other components. Present analysis of different tagged forms of TMEM70 as well as previous studies of the wild type TMEM70 show that TMEM70 is presumably capable to form oligomers of different sizes, possibly dimers and larger forms; however, their pattern depends on solubilization conditions, attached tag, as well as electrophoretic system used for analysis. In contrast with our previous study (Hejzlarova

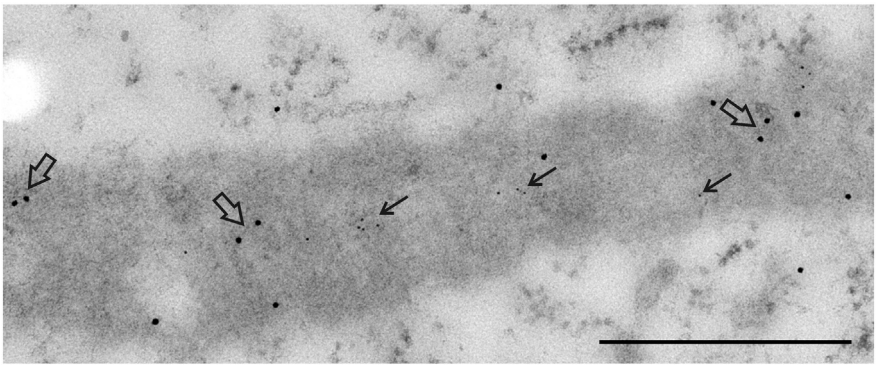

Fig. 8. Immunogold detection of TMEM70 protein and ATP synthase. Double immunolabeling for TMEM70 protein (small grains, arrows) and F_1-α subunit of ATP synthase (large grains, open arrows) on LR White resin sections showed that these factors do not colocalize inside of the mitochondrial space. Bar, 0.5 μm.

et al., 2011), which detected TMEM70 to form only monomers and dimers, here we used milder conditions for solubilization (half of the DDM concentrations or digitonin) and found also higher forms of TMEM70. Clearly a large portion of the solubilized TMEM70 that streaks up to the high molecular weight region (400–800 kDa) is mostly unfocused, especially in the digitonin solubilizates. However, such streaking is not uncommon for rather basic proteins and was observed for example also for AGP and MLQ subunits of ATP synthase (Meyer et al., 2007). We did not detect any specific overlap of TMEM70 with the signal of ATP synthase subunit F_1-α on 2D gels, where both proteins are very well detected. The direct interaction between TMEM70 molecules and formation of oligomeric forms was further confirmed by coimmunoprecipitation of TMEM70-FLAG and TMEM70-GFP from HEK293 cells coexpressing both tagged forms. The relative efficiencies of coimmunoprecipitation are similar to those observed by others in analogous experiments with other proteins of interest (Baughman et al., 2011) and demonstrate that oligomers of TMEM70 can be formed. On the other hand they do not provide hint about how quantitative this interaction is. Quite likely, only a part of all copies of TMEM70 protein is present as oligomers.

The key question to understand the biological role of TMEM70 is to uncover the physiological interaction with other components of the ATP synthase biogenesis, be it individual subunits, assembly intermediates or some other factors. Similar biochemical phenotype of ATP synthase deficiency, i.e. low content of otherwise functional enzyme, is observed not only in cells with *TMEM70* mutations (Cizkova et al., 2008), but also in the case of *ATP5A1*, *ATP5E* and *ATPAF2* mutations (De Meirleir et al., 2004; Jonckheere et al., 2013; Mayr et al., 2010). It suggests that the defect occurs at the early stage of the enzyme biogenesis, likely the formation and stabilization of F_1 part of the enzyme. Interestingly, very fast synthesis and degradation of the F_1-β subunit were found in the patient with *TMEM70* mutation (Houstek et al., 1999), suggesting the disturbance in the assembly of this subunit and thus of F_1 part of ATP synthase. The reason could be the problem at the level of the central stalk F_1 subunits γ, δ or ε, as their knockdown in HEK293 cells downregulates the ATP synthase biogenesis (Havlickova et al., 2010; Pecina et al., 2012). On the other hand the lack of F_1-γ, δ or ε subunits is associated with the accumulation of subunit F_o-c, a phenomenon absent in cells harboring *TMEM70* mutation (Havlickova et al., 2010).

The last stage in stepwise process of the ATP synthase assembly appears to be the addition of mtDNA encoded subunits. When the synthesis of subunits F_o-a and A6L (ATP6 and ATP8) is abolished due to the absence or depletion of mtDNA (Carrozzo et al., 2006), inhibition of mitochondrial protein synthesis (Nijtmans et al., 1995) or when *MT-ATP6* gene is mutated (Houstek et al., 1995; Jesina et al., 2004), a near complete but labile ATP synthase forms. However, it disintegrates upon solubilization and separation by BN-PAGE, which results in apparent accumulation of F_1 + c and F_1 subcomplexes. Recently the potential role for TMEM70 protein in the incorporation of mtDNA encoded subunits was suggested by Torraco et al. (2012). In TMEM70 patient cells they did not find full size ATP synthase, but by activity staining on BN-PAGE they detected a 550 kDa subcomplex, which should represent ATP synthase lacking mtDNA encoded subunits. However, the true nature of this complex is not obvious, since the authors detected it also in control cells, where it has not been shown in any of the previous studies. Also rather surprisingly, they did not observe OSCP signal in these subcomplexes, although OSCP should be present at least in the 550 kDa subcomplex. Nevertheless, they proposed that TMEM70 may bind to F_1, stabilize it to form F_1 + IF1 + c subassembly of 470 kDa and assist the insertion of subunits F_o-a and A6L at the ultimate stage of biogenesis. While this would support the above view of TMEM70 interacting with the whole F_1 or with F_1 subunits, neither they were successful in an attempt to identify any ATP synthase subunit directly interacting with TMEM70.

As revealed by our studies presented herein, some overlap between the endogenous or tagged TMEM70 protein and assembled ATP synthase or smaller F_1 containing assemblies can be found on native as well as 2D gels, which could be specific or just coincidental. Despite extensive attempts to find such specific interaction by means of several approaches, we were unable to detect a direct interaction with ATP synthase subunits. When we immunoprecipitated ATP synthase from the DDM solubilizates of mitochondria with different anti-F_1 antibodies there was no coimmunoprecipitation of TMEM70 protein. Vice versa, efficient immunoprecipitation of TMEM70 did not contain either F_1 or F_o subunits in the resulting immunoprecipitate. Finally, the third independent approach, morphological analysis by immunogold electron microscopy also indicated rather distant localizations of the ATP synthase subunit F_1-α antigen and the TMEM70 antigen in the mitochondrial membrane.

At the present stage the assembly of TMEM70 itself is well established while further studies are needed to find out TMEM70 interacting partners. These could be ATP synthase subunits, some chaperone, mitochondrial protease or some other protein through which TMEM70 participates in the biogenesis of ATP synthase.

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REVIEW

Nuclear Genetic Defects of Mitochondrial ATP Synthase

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Summary

Disorders of ATP synthase, the key enzyme of mitochondrial energy provision belong to the most severe metabolic diseases presenting as early-onset mitochondrial encephalo-cardiomyopathies. Up to now, mutations in four nuclear genes were associated with isolated deficiency of ATP synthase. Two of them, *ATP5A1* and *ATP5E* encode enzyme's structural subunits α and ε, respectively, while the other two *ATPAF2* and *TMEM70* encode specific ancillary factors that facilitate the biogenesis of ATP synthase. All these defects share a similar biochemical phenotype with pronounced decrease in the content of fully assembled and functional ATP synthase complex. However, substantial differences can be found in their frequency, molecular mechanism of pathogenesis, clinical manifestation as well as the course of the disease progression. While for *TMEM70* the number of reported patients as well as spectrum of the mutations is steadily increasing, mutations in *ATP5A1, ATP5E* and *ATPAF2* genes are very rare. Apparently, *TMEM70* gene is highly prone to mutagenesis and this type of a rare mitochondrial disease has a rather frequent incidence. Here we present overview of individual reported cases of nuclear mutations in ATP synthase and discuss, how their analysis can improve our understanding of the enzyme biogenesis.

Key words

Mitochondrial diseases • *TMEM70* • *ATPAF2* • *ATP5A1* • *ATP5E*

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Mitochondrial diseases

As the mitochondrial oxidative phosphorylation (OXPHOS) system is the main source of ATP in mammalian cells, it is not surprising that insufficient mitochondrial energy provision can lead to the deleterious dysfunction of organs and tissues with high energy demands such as heart, muscle and brain manifesting as mitochondrial diseases (Dimauro 2011). These OXPHOS disorders belong to the most severe inborn metabolic diseases primarily affecting newborns and small children, with no causal therapy yet available. Understanding of the molecular mechanisms leading to the pathologies and development of future therapeutic strategies require the identification of the disease-causing genes. OXPHOS system is genetically unique, because 13 of approximately 100 polypeptides constituting respiratory chain complexes and ATP synthase are encoded by maternally transmitted mitochondrial DNA (mtDNA) of 16.6 kb; the pathogenic mutations can therefore reside in both nuclear and mitochondrial genomes. The first described genetic defects of OXPHOS system were large deletions of mtDNA (Holt *et al.* 1988) and a point mutation in mtDNA *MT-ND4* gene for subunit 4 of complex I, NADH dehydrogenase (Wallace *et al.* 1988). Identification and characterization of mtDNA mutations dominated molecular genetic studies of mitochondrial diseases in successive two decades and at present more than 200 point mutations and hundreds of deletions are listed in MITOMAP inventory (www.mitomap.org, Ruiz-Pesini *et al.* 2007). Bourgeron *et al.* (1995) reported the first defect in a nuclear gene

resulting in a mitochondrial respiratory chain deficiency – a mutation in *SDHA* gene affecting complex II, succinate dehydrogenase. As most of OXPHOS disorders are transmitted as autosomal recessive traits, the number of recognized nuclear disease-causing genes was growing rapidly and by 2001, nuclear genetic defects had been reported in all electron transporting complexes of mitochondrial respiratory chain (Shoubridge 2001). Since then, increasing interest in nuclear genes constituting mitochondrial proteome further stimulated progress in identification of affected nuclear genes encoding either structural proteins or biogenetic and regulatory factors of OXPHOS machinery – representing so called "direct and indirect hits" (Dimauro 2011). Today more than 150 nuclear genetic defects have already been associated with disorders of mitochondrial energetic machinery (Vafai and Mootha 2012). They include also four genes responsible for the deficiency of mitochondrial ATP synthase, the key enzyme of mammalian ATP production.

ATP synthase structure and function

Proton-translocating mitochondrial ATP synthase (F_1F_o-ATPase, complex V, EC 3.6.3.14) is located in the inner mitochondrial membrane. It operates as a molecular motor utilizing proton motive force Δp of proton gradient generated by respiratory chain for phosphorylation of ADP. ATP synthase complex of ~600 kDa is composed of 17 different subunits organized into membrane-extrinsic F_1 catalytic part and membrane-embedded F_o part that are connected by two stalks (Walker 2013) (Fig. 1). Matrix oriented F_1 is formed by $\alpha_3\beta_3$ hexamer and by central stalk subunits γ, δ and ε. The stalk subunits attach the F_1 to subunit c-oligomer (c-ring), occupying about half of the F_o moiety. The F_o sector is further composed of subunits a, e, f, g, A6L and b subunit that extends to the matrix and together with the subunits d, F_6 and OSCP form the peripheral stalk connecting the F_o part to F_1. As the protons pass at the interface of the subunit a and c-oligomer, the proton gradient powers rotation of the c-ring and the central stalk thus induces cyclic conformational changes in $\alpha_3\beta_3$ hexamer. Consequently, ADP and P_i are bound and ATP synthesized and released through a cyclic binding-change mechanism (Boyer 1975). The efficacy of the process stems from the number of protons translocated per one rotation of the c-oligomer as for each copy of the c subunit one proton is utilized. The most efficient of all is mammalian enzyme containing 8 subunits, while 10-15 subunits are found in yeast and bacterial enzymes (Walker 2013). Small regulatory subunit IF_1 binds to F_1 at low pH and prevents the enzyme from a switch to hydrolytic mode and ATP hydrolysis. Other proteins described to be associated with ATP synthase are membrane proteins DAPIT (Diabetes-Associated Protein in Insulin-sensitive Tissue) and 6.8 kDa proteolipid (MLQ) (Chen *et al.* 2007, Meyer *et al.* 2007, Ohsakaya *et al.* 2011).

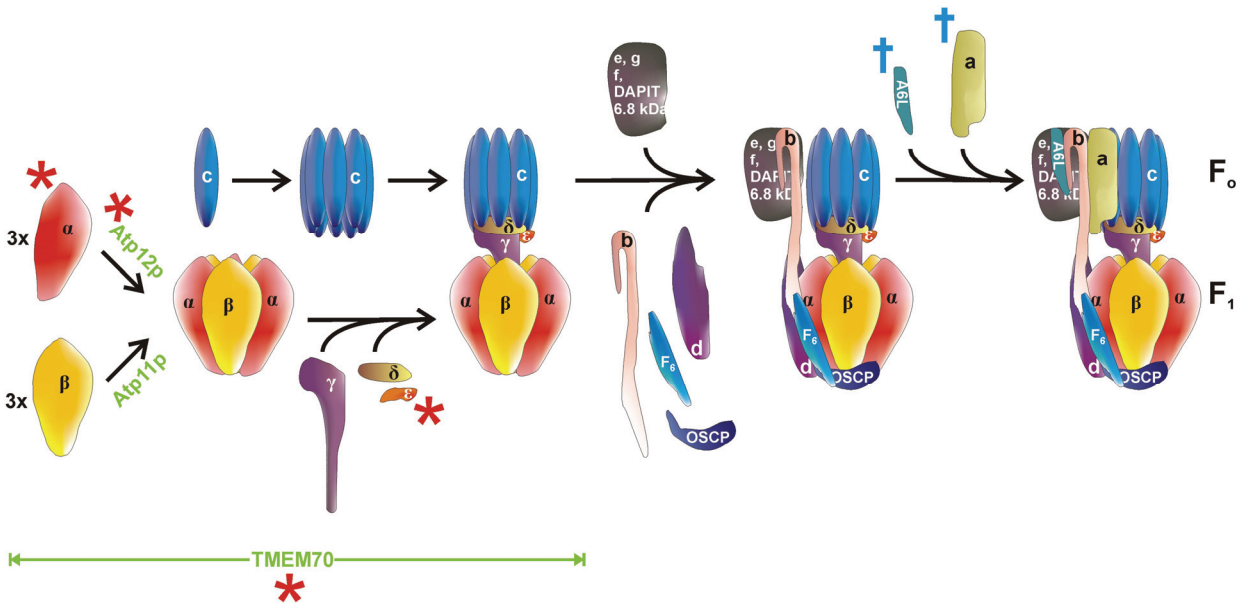

Fig. 1. Assembly scheme of mammalian ATP synthase. ATP synthase assembly starts with the $\alpha_3\beta_3$ hexamer formation, then central stalk subunits γ, δ and ε are added. The newly formed F_1 part is connected with the c-ring (F_1+c intermediate) and the assembly follows with subsequent addition of the F_o part subunits and peripheral stalk subunits. In the last steps mtDNA-encoded subunits a and A6L are incorporated. Red asterisks mark nuclear-encoded structural subunits and enzyme-specific biogenetic factors, the mutations of which are responsible for isolated deficiency of ATP synthase; blue crosses mark structural subunits with mtDNA mutations.

Biogenesis of ATP synthase

The biogenesis of ATP synthase is a very complex process that is still not fully understood. Current concept of the ATP synthase assembly is largely based on yeast model, *S. cerevisiae*, while the studies in mammalian cells are much less complex (see Ackerman and Tzagoloff 2005, Kucharczyk *et al.* 2009, Rak *et al.* 2009, 2011). Importantly, only subunits a (Atp6p) and A6L (Atp8p) are mtDNA-encoded in mammals, while in *S. cerevisiae* subunit c (Atp9p) is also mtDNA-encoded. The formation of ATP synthase from individual subunits is a stepwise procedure, proposed to proceed *via* assembly of several modules (Fig. 1), starting with an independent formation of F_1 and oligomer of c-subunits. After the F_1 attaches to the membrane-embedded c-ring, the subunits of peripheral arm (consisting of subunits b, d, F_6 and OSCP) and of membranous subcomplex (consisting of subunits e, f, g, DAPIT and 6.8 kDa protein) are added. In the last stage the enzyme structure is completed by incorporation of the two mtDNA-encoded subunits, a and A6L (Wittig *et al.* 2010). Both initial and end-stage of enzyme assembly appear to be identical in yeast and mammals, while the intermediate steps in mammalian enzyme biogenesis are partly hypothetical.

The ATP synthase biogenesis is assisted by numerous, enzyme-specific factors that partly differ between lower and higher eukaryotes. In *S. cerevisiae*, where the c subunit is encoded by mtDNA, several yeast-specific factors (Nca1–3p, Nam1p, Aep1–3p, Atp22p and Atp25p) are involved in the F_o biogenesis, namely in mRNA stability, translation and processing of mtDNA encoded subunits Atp6p and Atp9p (Tzagoloff *et al.* 2004, Ackerman and Tzagoloff 2005, Zeng *et al.* 2007a,b, 2008) or their assembly (Atp10p, Atp22p). However, none of them exists in mammals reflecting differences in structure of mitochondrial genes and expression of mtDNA-encoded subunits. Additional factor Atp23p (Osman *et al.* 2007, Zeng *et al.* 2007b) with metalloprotease/chaperone activity participates in processing of Atp6p and its association with c-oligomer, but there is only a partial homolog of Atp23p in mammals and its function remains unknown.

Assembly of the yeast F_1 part depends on three additional factors, Atp11p, Atp12p and Fmc1p (Ackerman and Tzagoloff 1990, 2005, Lefebvre-Legendre *et al.* 2001). Only the first two have their homologues in humans (Wang *et al.* 2001) interacting with the subunits β and α, respectively, during the $\alpha_3\beta_3$ hexamer formation (Fig. 1). Recently a 21 kDa protein called TMEM70 was described as a new and specific ancillary factor of mammalian ATP synthase and its absence strongly inhibits the enzyme biosynthesis (Cizkova *et al.* 2008, Houstek *et al.* 2009).

The functional monomers of ATP synthase can further interact together and it has been shown that ATP synthase is organized as dimers and higher oligomers in different types of energy-transducing membranes, including yeast and mammalian mitochondria (see Wittig and Schagger 2008, 2009, Wittig *et al.* 2008, 2010). Interaction between two monomers occurs *via* F_o and appears to involve subunits a, e, g, b and A6L. As the monomers interact in the dimers at 70-90° angle and the neighboring dimers at 20° angles, the supramolecular ATP synthase ribbons can shape the inner membrane at the apical regions and thus support cristae formation. Furthermore, this supramolecular organization could potentiate ATP synthesis and be functionally advantageous (Strauss *et al.* 2008).

ATP synthase disorders due to mtDNA mutations

Isolated disorders of ATP synthase manifest mostly as mitochondrial encephalo-cardiomyopathies, often severe with neonatal onset. However, as with other mitochondrial diseases, the spectrum of clinical phenotypes can be rather broad including mild and late manifestations (Schon *et al.* 2001, DiMauro 2004, Houstek *et al.* 2004, Jonckheere *et al.* 2012). Primary defects in the enzyme structure and function belong to two distinct groups depending on the affected genes. Maternally transmitted mtDNA mutations affect both mitochondrial-encoded subunits a and A6L. Here the function of F_o proton channel, stability of ATP synthase complex and possibly enzyme-enzyme interactions can be altered. The structural impairment due to missense mutations or lack of the subunit is rarely accompanied by major changes in ATP synthase content. Biochemical and clinical phenotypes depend on mtDNA mutation load and majority of cases manifest only when a genetic threshold of ~80-90 % of mutated mtDNA copies is reached. The second group of ATP synthase disorders are autosomal recessive nuclear genetic defects. Mutations are found in several genes encoding structural subunits of enzyme or biogenetic-assembly factors and common biochemical phenotype of altered enzyme biogenesis is a low content

of otherwise functional enzyme.

Nowadays, mtDNA mutations are routinely screened and relatively easily diagnosed. More than 30 different mutations in *MT-ATP6* and *MT-ATP8* genes have already been described (www.mitomap.org). The majority of them are *MT-ATP6* missense mutations; single mutations are associated with severe brain, heart and muscle disorders with early-onset, but also with deafness, multiple sclerosis, autism, optic neuropathy or diabetes. The most common are *m.8993T>G* (p.L156R) or *m.8993T>C* (p.L156P) transitions, manifesting usually as milder NARP (Neurogenic muscle weakness, ataxia, and retinitis pigmentosa) or more severe MILS (Maternally inherited Leigh syndrome). In general, the severity of *T>G* transition depends on the level of heteroplasmy, but other polymorphisms in the mtDNA as well as additional factors may also influence the disease pathology (Enns *et al.* 2006, D'Aurelio *et al.* 2010, Kara *et al.* 2012). *T>C* transition is less common, symptoms are milder and with later onset (Morava *et al.* 2006, Baracca *et al.* 2007, Craig *et al.* 2007). Similar features were described in the other relatively common transitions, *m.9176T>G* (p.L217R) and *m.9176T>C* (p.L217P) presenting as Leigh disease or Familial bilateral striatal necrosis (Carrozzo *et al.* 2001, Hung and Wang 2007, Vazquez-Memije *et al.* 2009). *MT-ATP6* mutations may also associate with some multifactorial polygenic diseases. For example, the *m.9176T>C* transition was found in late-onset hereditary spastic paraplegia (Verny *et al.* 2010) and *m.9176T>C* or *m.9185T>C* transitions were harbored in patients with mild Charcot-Marie-Tooth hereditary neuropathy (Pitceathly *et al.* 2012, Synofzik *et al.* 2012).

While the above discussed *MT-ATP6* transitions cause replacements of amino acids (AA) involved in the function of proton channel, a unique microdeletion of two base pairs at positions 9205/6 (*m.9205delTA*) at the interface of *MT-ATP6* and *MT-CO3* genes (Seneca *et al.* 1996) alters the splicing and maturation of their mRNAs and down-regulates the synthesis of subunit a (Jesina *et al.* 2004). Mitochondrial content of ATP synthase is unaffected but the enzyme is non-functional, because the proton channel in the F_o cannot operate in the absence of subunit a.

Mutations of *MT-ATP8* gene are a rarer cause of mitochondrial encephalo-cardiomyopathies. The *m.8529G>A* nonsense mutation was found in a patient with hypertrophic cardiomyopathy and neuropathy. ATP synthase complex was destabilized, which led to the strong reduction in holoenzyme content and accumulation of F_1 subcomplexes (Jonckheere *et al.* 2008). Later on, *m.8411A>G* missense mutation (p.M16V) in the conserved region of A6L protein in a patient with severe mitochondrial disorder (Mkaouar-Rebai *et al.* 2010) and *m.8528T>C* mutation changing conserved tryptophan 55 to arginine in A6L subunit as well as *MT-ATP6* initiation codon of subunit a in a patient with infantile hypertrophic cardiomyopathy (Ware *et al.* 2009) were reported.

Nuclear genetic defects

At present, inborn and isolated disorders of ATP synthase are associated with four nuclear genes, two of which code for the structural subunits α and ε while the other two encode biogenetic factors Atp12p and TMEM70 that are not part of the enzyme structure. All these mutations share a similar biochemical phenotype with pronounced decrease in the content of fully assembled and functional ATP synthase. However, their incidences, mechanism of molecular pathogenesis, clinical manifestation, as well as the course of the disease progression, differ substantially. Up to now, only two patients with mutations in *ATP5A1* and one patient each with mutations in *ATP5E* and *ATPAF2* genes were reported pointing to the level of rareness of these disorders. In contrast, nearly 50 cases of diagnosed patients with mutation in *TMEM70* gene have already been described and the number of reported patients and affected families as well as the spectrum of *TMEM70* mutations is steadily increasing. Apparently *TMEM70* gene is highly prone to mutagenesis and this type of rare mitochondrial disease has relatively frequent incidence.

Mutations in ATP synthase biogenetic factors

The first indication that ATP synthase dysfunction could be linked to a nuclear gene came from the study of Holme *et al.* (1992) who were unable to find any mtDNA mutation in a child with cardiomyopathy, lactic acidosis, persisting 3-methylglutaconic aciduria (3-MGA) and severely decreased activity of ATP synthase. Seven years later in another patient, we have demonstrated by the use of mitochondrial cybrids that mitochondrial disease presenting as early onset neonatal and fatal lactic acidosis, cardiomyopathy and hepatomegaly due to a 70 % isolated decrease of ATP synthase complex was of nuclear origin (Houstek *et al.* 1999). A number of similar patients have been described (Sperl *et al.* 2006) due to a joint effort of several

European mitochondrial centers that focus specifically on putative disorders of ATP synthase. DNA sequencing of those patients excluded mutations in genes for structural subunits, but De Meirleir *et al.* (2004) described one patient with severe neonatal encephalopathy who harbored missense mutation in Atp12p protein, an assembly factor essential for incorporation of α subunit into F_1-ATPase structure. Four years later, we used the homozygosity mapping and sequencing of candidate genes in other known patients and identified *TMEM70* as another disease-causing gene. The splicing site mutation in the second intron preventing the synthesis of TMEM70 protein was found in 24 cases including the first patient reported in 1999 (Cizkova *et al.* 2008, Honzik *et al.* 2010).

ATPAF2 mutation disease

A homozygous *c.280T>A* mutation in *ATPAF2* gene coding for Atp12p was discovered in a girl with dysmorphic features, cortical-subcortical brain atrophy followed by basal ganglia atrophy and metabolic acidosis, who died at the age of 14 months (De Meirleir *et al.* 2004). Activity and specific content of ATP synthase was strongly decreased, liver tissue was more affected than skeletal muscle. In liver mitochondria, severe reduction in native complex V without accumulation of F_1 containing subcomplexes was discovered. Significantly reduced content of individual ATP synthase subunits suggested that F_1 assembly could be disturbed at the early stage and the unassembled subunits were rapidly degraded. The *TGG>AGG* transition caused replacement of evolutionary conserved neutral tryptophan in position 94 to a basic arginine (p.W94R), probably affecting the Atp12p interaction with α subunit. Both parents (consanguineous, Moroccan origin) and unaffected sibling were heterozygous carriers of the mutation. With the help of a yeast model, it was later shown that this mutation affects the solubility of Atp12p protein, with the mutated form showing tendency to aggregate (Meulemans *et al.* 2010).

TMEM70 mutation disease

A search for affected nuclear gene in a large group of patients with ATP synthase deficiency, severe neonatal lactic acidosis and encephalo-cardiomyopathy led to the identification of *c.317-2A>G* mutation at the end of the second intron of *TMEM70* gene as a cause of the mitochondrial disease (Cizkova *et al.* 2008). Their fibroblasts showed decreased ADP-stimulated respiration, low ATP synthase activities and significantly reduced levels of fully assembled complex V with increased content of F_1 subcomplexes. All structural and functional changes in patient fibroblasts were complemented after transfection with wild type *TMEM70* (Fig. 2). As the defect affected assembly of the enzyme, TMEM70 was recognized as a new biogenetic factor of ATP synthase.

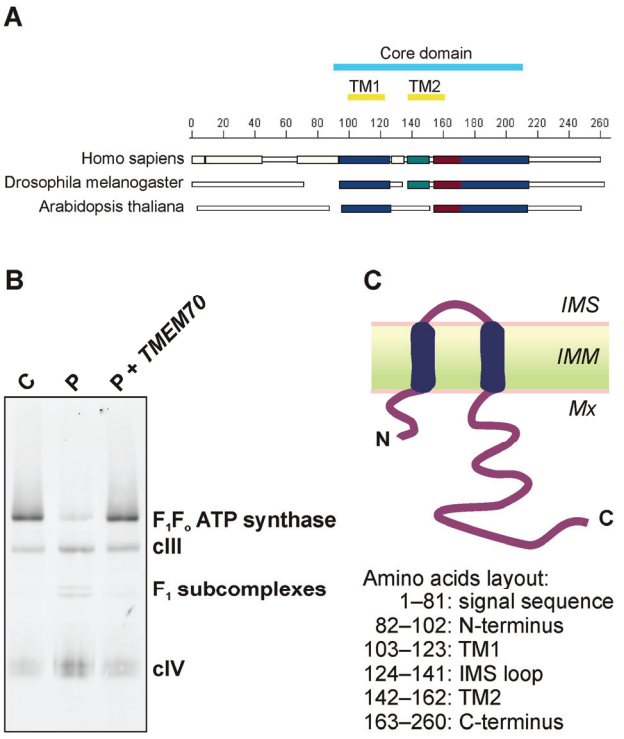

Fig. 2. Structure of TMEM70 protein and *TMEM70* complementation of ATP synthase deficiency. **A.** Sequence alignment of TMEM70 protein. Color of the boxes indicates the level of homology between different organisms (red, blue, green, and white; from the most homologous to the least, respectively). TM1, TM2 denote putative transmembrane regions. Core domain represents central, the most homologous part of the protein. **B.** Decrease of assembled ATP synthase (F_1F_o ATP synthase) and accumulation of F_1 subcomplexes in patient fibroblasts with *TMEM70* mutation (P) are reversed by complementation with wild type *TMEM70* (P+*TMEM70*); control cells (C); cIII and cIV are signals of respiratory chain complexes. **C.** Structure and orientation of mature TMEM70 protein in the inner mitochondrial membrane (IMM); the intermembrane space (IMS); the matrix (Mx).

TMEM70 – a novel ancillary factor of ATP synthase biogenesis

Mitochondrial localization of TMEM70 protein was first described by Calvo *et al.* (2006), later it was included in Mitocarta inventory (www.broadinstitute.org/pubs/MitoCarta/), the database of proteins most likely constituting human and mouse mitochondrial proteome (Pagliarini *et al.* 2008). *TMEM70* gene is located on chromosome 8, consists of

three exons and encodes a 260 amino acids protein that contains a conserved DUF1301 domain and two putative transmembrane regions indicating membrane-association of the protein (Fig. 2A). Analysis of human TMEM70 precursor protein of 30 kDa showed, that it is imported into mitochondria and upon removal of 9 kDa N-terminal signal sequence it is processed to 21 kDa mature TMEM70 protein, localized in the inner mitochondrial membrane (Hejzlarova *et al.* 2011). As predicted from the amino acid sequence, it contains two short transmembrane domains, 21 AA each, separated by even shorter 18 AA connecting sequence suggesting a hairpin like transmembrane fold of the protein (Fig. 2C). Short N-terminal (21 AA) and long C-terminal (98 AA) sequences of the mature protein should then be exposed to the same hydrophilic compartment. Their orientation was predicted to be towards the mitochondrial matrix (Jonckheere *et al.* 2011) and our recent studies with tagged forms of TMEM70 protein confirm this prediction (Kratochvilova *et al.*, in preparation).

The biological role of TMEM70 protein is directly linked to the biogenesis of ATP synthase but its exact function is not yet known. TMEM70 facilitates the formation of ATP synthase complex, but it may proceed, albeit at a low intensity, even in the absence of TMEM70 protein, as small, yet still significant, amounts of functional ATP synthase are found in all patients. The low content of ATP synthase complex in the TMEM70-deficient cells is accompanied by the severely reduced content of individual subunits including subunit c, suggesting fast degradation of unassembled subunits. Our previous studies indicated functional involvement of TMEM70 in the early stages of ATP synthase assembly (Houstek *et al.* 1999). Recently, Torraco *et al.* (2012) proposed the role of TMEM70 in stabilization of F_1 thus assisting further steps of enzyme biogenesis including the ultimate incorporation of the mtDNA encoded subunits a and A6L.

Transcript levels as well as protein detection by mass spectrometric analysis revealed very low abundance of TMEM70 protein, similar to the other ancillary factors (Hejzlarova *et al.* 2011). Detergent-solubilized protein resolved by Blue Native electrophoresis can be detected as a dimer or larger oligomers but its interacting partner(s) remain unknown. Genomic analysis found *TMEM70* homologues in genomes of all multicellular eukaryotes and plants, but only in some yeast or fungi a considerable homology was found in the transmembrane regions but not in N- and C-terminal regions (Cizkova *et al.* 2008, Jonckheere *et al.* 2011). Importantly, *S. cerevisiae*, the main yeast model for studies of mitochondrial ATP synthase biogenesis lacks *TMEM70* gene. Therefore, while more than a dozen of ATP synthase-specific biogenetic factors exist in yeast and are absent in mammals, TMEM70 protein is the first ancillary factor of mammalian ATP synthase that is, in contrary, absent in *S. cerevisiae*.

Pathogenic mutations of TMEM70 gene

Analysis of mutations in affected patients represents an important step towards better understanding of TMEM70 involvement in the biogenetic mechanism. Within the last couple of years, numerous other *TMEM70* mutations have been described with a broad spectrum of phenotypes (Table 1). *TMEM70* mutations associated with isolated ATP synthase deficiency are either homozygous or compound heterozygous, parents and healthy siblings of the patients are usually heterozygous carriers of the respective mutation(s).

The most common is the originally described homozygous *c.317-2A>G* mutation that removes the splicing site prior to the third exon and results in the generation of multiple incomplete and labile transcripts preventing synthesis of the protein. It has already been found in 30 patients (Cizkova *et al.* 2008, Wortmann *et al.* 2009, Honzik *et al.* 2010, Tort *et al.* 2011, Torraco *et al.* 2012, Stojanovic and Doronjski 2013) and other cases are known but have not been formally reported. Absence of TMEM70 protein in homozygous patients typically presents as early-onset and severe lactic acidosis, 3-MGA, hypertrophic cardiomyopathy, dysmorphism, hypotonia, ataxia, failure to thrive and psychomotor retardation. Out of 23 cases reported in 2008, about half died within the first few years and mostly in the first months of life. On the other hand some patients can survive significantly longer, with two of them currently reaching 12 and 17 years. As pointed out by a detailed retrospective clinical study (Honzik *et al.* 2010), if the patient survives the critical postnatal period of the first weeks and months of life, the metabolic problems and cardiac disorders may improve. Till now, only one patient was reported with rather late disease manifestation at the age of 3 years as a mild form of 3-MGA without hyperammonemia during the metabolic crisis (Stojanovic and Doronjski 2013). With one exception (Tort *et al.* 2011) the patients with homozygous *c.317-2A>G* mutation were of Roma origin but from several unrelated families (Wortmann *et al.* 2009, Honzik *et al.* 2010, Torraco *et al.* 2012, Stojanovic and Doronjski 2013).

Table 1. Nuclear DNA mutations associated with isolated deficiency of ATP synthase.

Gene	Mutation	Clinical phenotype	References
ATP5A1	*c.985C>T* missense (p.R329C) (*c.–49+418C>T* substitution)	Severe neonatal encephalopathy	(Jonckheere *et al.* 2013)
ATP5E	*c.35A>G* missense (p.Y12C)	Neonatal respiratory distress, LA, 3-MGA, severe peripheral neuropathy, exercise intolerance	(Mayr *et al.* 2010)
ATPAF2 (ATP12)	*c.280T>A* missense (p.W94R)	3-MGA, LA, neonatal encephalopathy, dysmorphism	(De Meirleir *et al.* 2004)
TMEM70	*c.317-2A>G* splicing	IUGR, neonatal LA, EOH, FD, HCMP, 3-MGA, cataract, encephalopathy, FTT, PMR	(Cizkova *et al.* 2008, Wortmann *et al.* 2009, Tort *et al.* 2011, Torraco *et al.* 2012, Stojanovic and Doronjski 2013)
	c.317-2A>G/c.118_119insGT frameshift	LA, 3-MGA, HCMP, FD, PMR	(Cizkova *et al.* 2008, Cameron *et al.* 2011)
	c.317-2A>G/c.494G>A missense (p.G165D)	LA, 3-MGA, HCMP, Reye-like syndrome, exercise intolerance	(Shchelochkov *et al.* 2010)
	c.336T>A frameshift (p.Y112X)	IUGR, LA, HCMP, FD, PMR	(Spiegel *et al.* 2011)
	c.316+1G>T splicing	IUGR, encephalopathy, HCMP, EOH, LA, FD	(Spiegel *et al.* 2011)
	c.238C>T frameshift (p.R80X)	IUGR, EOH, LA, 3-MGA, multiorgan failure	(Spiegel *et al.* 2011)
	c.578_579delCA frameshift (p.N198X)	IUGR, EOH, LA, 3-MGA, FD, cataract, encephalopathy, HCMP, PMR	(Spiegel *et al.* 2011)
	c.211–450_317–568del (2290bp deletion) frameshift	IUGR, HCMP, LA, 3-MGA, PMR	(Tort *et al.* 2011)
	g.2436–3789 in-frame deletion (1353bp)	IUGR, LA, HCMP, PMR, ptosis	(Jonckheere *et al.* 2011)
	c.317-2A>G/c.628A>C missense (p.T210P)	HCMP, LA, 3-MGA, arterial pulmonary hypertension, WPW	(Torraco *et al.* 2012)
	c.535C>T missense (p.Y179H)	IUGR, LA, EOH, FD, HCMP, bilateral cataract, PMR	(Atay *et al.* 2013)

3-MGA, 3-methylglutaconic aciduria; EOH, Early-Onset Hypotonia; FD, facial dysmorphism; FTT, Failure To Thrive; HCMP, Hypertrophic Cardiomyopathy; IUGR, Intrauterine growth retardation; LA, Lactic Acidosis; PMR, Psychomotor Retardation; WPW, Wolf-Parkinson-White pre-excitation syndrome.

Another homozygous splicing site mutation *c.316+1G>T* was reported in two patients from Arab Muslim family (Spiegel *et al.* 2011). Would the resulting transcript devoid of exon 2 be translated, the aberrant TMEM70 protein lacked 34 AA in the region 71–105, including last 10 AA of the mitochondrial signal sequence and first 3 AA of the first transmembrane domain. The consequence of this mutation could be inefficient import of TMEM70 into mitochondria or defective membrane assembly. The patients, two siblings of consanguineous parents presented with typical features described above but without 3-MGA and they died after 10 days and 5 months, respectively.

Different type of *TMEM70* mutation, homozygous *g.2436–3789* in-frame deletion resulting also in *TMEM70* transcript lacking exon 2 was described in one child of Iraqi consanguineous parents (Jonckheere *et al.* 2011). Contrary to *c.316+1G>T* patients, this patient with psychomotor retardation survives much longer; he was reported at the age of 6 years.

Four additional homozygous frameshift mutations creating premature stop codon and leading to the synthesis of potentially truncated TMEM70 have been reported. Two Arab Muslim patients (siblings) with symptoms similar to other TMEM70 patients plus bilateral cataract harbored *c.578_579delCA* deletion resulting in a putative 197 AA long protein lacking almost two third of the C-terminus. Surprisingly, their survival is remarkably different; one is 24 years old while the second died at 3.5 years. *TMEM70* mutation *c.336T>A* was found in another Arab Muslim patient, and it predicts synthesis of a 112 AA long TMEM70 protein (Spiegel *et al.* 2011) while *c.211–450_317–568del* mutation deleting the whole exon 2 (Tort *et al.* 2011) would result in a protein only 71 AA long that may be unstable and mislocalized. Both patients presented with hypertrophic cardiomyopathy, metabolic acidosis, mental retardation and facial dysmorphism at the age of 1 and 7 years, respectively. The last frameshift mutation *c.238C>T* resulting in a putative 80 AA long TMEM70 protein had severe phenotype with encephalopathy, hypotonia, metabolic acidosis and this Arab Muslim patient died 7 days old (Spiegel *et al.* 2011). All the patients reported by Spiegel *et al.* (2011) were from consanguineous families. The malfunction of the three short variants of TMEM70 protein is not surprising as even the longest (112 AA) only reaches till the first half of the first transmembrane domain.

The common *c.317-2A>G* mutation can also be found as compound heterozygous in combination with other *TMEM70* mutations. Two patients were described carrying *c.317-2A>G* and *c.118_119insGT* frameshift mutation resulting in premature stop codon and putative TMEM70 protein of 40 AA (Cizkova *et al.* 2008, Cameron *et al.* 2011). In one case the mother is Italian carrying the common splicing mutation and the father is Croatian carrying the insertion (Cameron *et al.* 2011). Both patients show typical TMEM70 symptoms and they are alive. Another three patients have a combination of *c.317-2A>G* with a missense mutations, such as *c.494G>A* changing neutral glycine 165 to acidic aspartate at the C-terminus (Shchelochkov *et al.* 2010) or *c.628A>G* changing highly conserved threonin 210 to prolin (Torraco *et al.* 2012). The clinical outcome of North European origin patient reported by Shchelochkov *et al.* (2010) and presenting as Reye-like syndrome was mild, while both Italian patients reported by Torraco *et al.* (2012) are strongly affected; in addition to metabolic acidosis and cardiomyopathy Wolf-Parkinson-White pre-excitation syndrome was found.

The only homozygous missense mutation described so far is *c.535C>T* mutation, which changes the highly conserved tyrosine to a histidine at position 179 at the beginning of the C-terminus (Atay *et al.* 2013). The patient of Turkish origin presented with cardiomyopathy, hypotonia, mild mental retardation, dysmorphism and bilateral cataract.

Although the changes in the quantity and structure of TMEM70 protein were not specifically addressed in most of the case reports, from the described mutations it appears that most of the genetic defects lead to the absence of this factor or the synthesis of incomplete truncated forms, lacking either a part of import sequence or a major part of the second transmembrane domain and/or C-terminal sequence. The p.Y179H amino acid replacement would point to a functional importance of conserved tyrosine at the beginning of the C-terminal sequence. A combination of splicing site and missense mutation could lead to the synthesis of sole TMEM70 protein with amino acid replacement if the splice variant mRNA was unstable and degraded. Interestingly, both such missense mutations, p.G165D and p.T210P affect the C-terminal region of the protein.

Mutations in ATP synthase structural subunits

It was not unexpected that isolated ATP synthase

deficiency, similarly as isolated disorders of other OXPHOS complexes can also be caused by a "direct hit". In 2008 our search for mutated gene in 25 patients with ATP synthase deficiency revealed *TMEM70* mutation in all but one patient (Cizkova *et al.* 2008) who also differed by a rather mild and distinct phenotype (Sperl *et al.* 2006). Subsequent sequencing of ATP synthase genes detected a mutation in ε subunit of F_1 part as the first mutation in nuclear-encoded subunit of the enzyme (Mayr *et al.* 2010). Three years later mutation in another structural subunit of F_1 was discovered, in this case affecting *ATP5A1* gene, coding for the α subunit (Jonckheere *et al.* 2013).

ATP5E mutation disease

A nuclear mutation affecting ε subunit of ATP synthase was found in 22 years old patient. Clinical phenotype started with early-onset lactic acidosis, 3-MGA, but no cardiac involvement, followed by mild mental retardation, exercise intolerance and peripheral neuropathy. Sequencing of *ATP5E* gene coding for subunit ε (Mayr *et al.* 2010) uncovered homozygous missense mutation *c.35A>G* replacing highly conserved tyrosine 12 with cysteine. Parents were healthy heterozygous carriers. In the patient fibroblasts decreased activities of ATP synthase and ADP-stimulated respiration with increased mitochondrial membrane potential at state 3-ADP were found, indicating an insufficient capacity of ATP synthase. The contents of both ATP synthase subunits and of fully assembled complex V were severely reduced. Surprisingly, the mutated subunit ε depressed biosynthesis of ATP synthase but it was incorporated in the enzyme complex without altering its synthetic and hydrolytic functions. The reduction of assembled 600 kDa complex V was not accompanied by the presence of F_1 subcomplexes. In the patient fibroblasts all enzyme subunits were correspondingly reduced with the exception of subunit c, which was not degraded. When *ATP5E* was knocked-down in HEK293 cells (Havlickova *et al.* 2010) a similar phenotype was found – isolated decrease of fully functional ATP synthase complex corresponding to the low levels of subunit ε and accumulation of subunit c. Changes in ATP synthase structure and function due to the mutation and downregulation of ε subunit therefore indicated an essential role of ε subunit in the biosynthesis and assembly of the F_1 part of ATP synthase. Moreover, ε subunit seems to be involved in the incorporation of subunit c into the rotor structure of the mammalian enzyme.

ATP5A1 mutation disease

Most recently, the first mutation was found in *ATP5A1* structural gene for subunit α in two siblings of non-consanguineous parents presenting with severe neonatal encephalopathy (Jonckheere *et al.* 2013). Both patients died at the first week of life and extensive damage of brain structures was accompanied by multiorgan lesions indicative of mitochondrial disease. Fibroblasts of both patients showed decreased oxygen consumption with unusually high activation by an uncoupler and isolated defect in ATP synthase hydrolytic activity. Strong reduction in specific content of fully assembled complex V without F_1 subcomplexes accumulation was accompanied by the reduced levels of individual ATP synthase subunits. In both siblings exome sequencing detected heterozygous *c.985C>T* mutation in *ATP5A1* gene that changed conserved basic arginine to neutral cysteine at position 329. The arginine 329 is predicted to be involved in the interaction between α and β subunits, thus Arg>Cys replacement can destabilize the $\alpha_3\beta_3$ hexamer. Observed missense mutation in subunit α indicates defect in the early stage of F_1-ATPase assembly when $\alpha_3\beta_3$ hexamer is formed. *ATP5A1* mutation revealed remarkable genetics. Healthy father was heterozygous carrier for the mutation, while mother expressed only the wild type sequence. Extensive analyses of the sequences and mRNA expression of *ATP5A1* alleles of both parents suggested that the maternal allele was inherited by both affected children but its expression was inhibited. Therefore the pathogenic phenotype was dominated by the paternal mutated allele. The reason for altered expression of the maternal allele remains unknown. The only polymorphism found was *c.–49+418C>T* variant in the first intron, approximately 5.4 kb from the core promoter of *ATP5A1* gene but there is no indication that it could affect the gene regulation.

Pathogenic mechanism of ATP synthase deficiency

Isolated defects of ATP synthase due to mutations in nuclear genes reported so far indicate that primarily affected is the initial stage of enzyme biosynthesis, i.e. the formation of F_1 part (Fig. 1) and the tissue content of the whole ATP synthase complex becomes reduced as a consequence. Despite different molecular genetic defects, in all these cases the mitochondrial pathology stems from the low content of ATP synthase in comparison to respiratory chain

complexes. Such decrease in relative ATP synthetic capacity is expected to limit mitochondrial ATP production *in vivo* depending on the extent of enzyme defect. In most patients the deficiencies are rather pronounced, ranging from 60-70 % decrease to a practically nondetectable levels of ATP synthase complex. Although this may reflect some methodological problems and differences in enzyme detection, it is clear that even relatively extreme defects can be tolerated to some extent, at least in terms of organs pathology and/or patients survival. Phenotypic manifestation of the genetic defects of OXPHOS system occurs only when a threshold level for a given reaction is exceeded (Rossignol *et al.* 2003). Physiological spare capacity of ATP synthase relative to respiratory chain enzymes was studied in detail in mouse tissues and demonstrated that for example in muscle, 80 % inhibition of ATP synthase still allowed for almost normal state 3-ADP respiration (Rossignol *et al.* 1999). Analogous studies do not exist for human tissues and most of the functional data from patients are obtained in cultured cells. Nevertheless, this may be the key explanation why mitochondrial energy provision in nuclear genetic defects of ATP synthase is less affected than would be predicted from the decrease in enzyme content.

The low capacity of ATP synthase subsequently leads to elevated levels of mitochondrial membrane potential ($\Delta\Psi$) at conditions of intensive coupled respiration, as demonstrated in *TMEM70* and *ATP5E* mutated fibroblasts (Cizkova *et al.* 2008, Mayr *et al.* 2010). The high levels of $\Delta\Psi$ in respiring mitochondria stimulate the electron leak within the respiratory chain thus increasing the generation of reactive oxygen species (ROS). This has been demonstrated in TMEM70-lacking fibroblasts (Houstek *et al.* 2004, Mracek *et al.* 2006) and upregulation of ROS also altered fibroblast viability in glucose-free, pyruvate-containing medium. Thus ATP synthase deficiency is connected with both altered energy provision and enhanced oxidative stress, similarly as dysfunction of ATP synthase due to mtDNA *MT-ATP6* mutations (Mattiazzi *et al.* 2004).

Mitochondrial biogenesis is subject of complex transcriptional regulation *via* PGC1/NRF axis and it is expected that mitochondria-nucleus retrograde signaling could respond to a metabolic disbalance due to complex V deficiency. In fibroblast cell lines with identical *TMEM70* homozygous *c.317-2A>G* mutation the decrease in complex V was accompanied by upregulation of respiratory chain complexes III and IV (Havlickova Karbanova *et al.* 2012) and similar upregulation was also found in patient tissues (Mayr *et al.* 2004). These compensatory adaptive changes were not connected with upregulation of mRNAs for corresponding structural subunits or biogenetic factors pointing to posttranscriptional regulatory events. Interestingly, analogous increase in electron transport complexes was also present in fibroblasts of *ATP5E* patient (Mayr *et al.* 2010), although these changes apparently cannot improve the energetic dysfunction of complex V-lacking mitochondria.

Morphological changes in structure of mitochondria also represent an important aspect of ATP synthase deficiency. The role of ATP synthase dimers in mitochondria cristae formation was described in yeast models, where a downregulation of ATP synthase (Lefebvre-Legendre *et al.* 2005) or altered formation of ATP synthase dimers due to deficiency of the subunits e or g (Paumard *et al.* 2002) led to the absence of cristae and mitochondrial appearance as onion-like structures. Analogous morphological changes, concentric arrangement of mitochondrial cristae were observed in skeletal muscle mitochondria of a patient with *TMEM70 c.317-2A>G* and *c.118_119insGT* mutation (Cameron *et al.* 2011) while in fibroblasts of patient with *TMEM70 g.2436–3789* in-frame deletion the swollen and irregularly shaped mitochondria with partial to complete loss of the cristae and a fragmented mitochondrial network were found (Jonckheere *et al.* 2011). The lack of ATP synthase dimers preventing formation of cristae may further potentiate functional defect in the synthesis of ATP, because the apical cristae structures with concentrated ribbon arrays of complex V appear to be associated with an increase in charge density and thus in the local pH gradient by approximately 0.5 units, leading to improved efficiency of ATP synthesis (Strauss *et al.* 2008, Davies *et al.* 2011).

Conclusions

Within the last ten years a substantial progress has been made in the characterization of molecular-genetic basis of ATP synthase disorders caused by mutations in nuclear genes. Improved diagnostics and genetic counseling of affected families represent an immediate outcome of this effort. However, equally significant is the new knowledge on basic mechanisms and factors involved in biosynthesis of enzyme components, their processing and assembly into native

structure of ATP synthase complex. In contrast to energetic function of ATP synthase that is already well characterized at the molecular level, process of the mammalian enzyme biogenesis is still far from being completely understood. Analysis of inborn genetic disorders represents an invaluable tool for deciphering of the biogenetic mechanisms as well as for the development of future therapeutic strategies.

Conflict of Interest

There is no conflict of interest.

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Abbreviations

3-MGA, 3-methylglutaconic aciduria; AA, amino acid; MILS, Maternally inherited Leigh syndrome; mtDNA, mitochondrial DNA; NARP, Neurogenic muscle weakness, ataxia, and retinitis pigmentosa; OXPHOS, oxidative phosphorylation system; ROS, reactive oxygen species.

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Alteration of structure and function of ATP synthase and cytochrome *c* oxidase by lack of F_o-a and Cox3 subunits caused by mitochondrial DNA 9205delTA mutation

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Mutations in the *MT-ATP6* gene are frequent causes of severe mitochondrial disorders. Typically, these are missense mutations, but another type is represented by the 9205delTA microdeletion, which removes the stop codon of the *MT-ATP6* gene and affects the cleavage site in the *MT-ATP8/MT-ATP6/MT-CO3* polycistronic transcript. This interferes with the processing of mRNAs for the Atp6 (F_o-a) subunit of ATP synthase and the Cox3 subunit of cytochrome *c* oxidase (COX). Two cases described so far presented with strikingly different clinical phenotypes – mild transient lactic acidosis or fatal encephalopathy. To gain more insight into the pathogenic mechanism, we prepared 9205delTA cybrids with mutation load ranging between 52 and 99 % and investigated changes in the structure and function of ATP synthase and the COX. We found that 9205delTA mutation strongly reduces the levels of both F_o-a and Cox3 proteins. Lack of F_o-a alters the structure but not the content of ATP synthase, which assembles into a labile, ∼60 kDa smaller, complex retaining ATP hydrolytic activity but which is unable to synthesize ATP. In contrast, lack of Cox3 limits the biosynthesis of COX but does not alter the structure of the enzyme. Consequently, the diminished mitochondrial content of COX and non-functional ATP synthase prevent most mitochondrial ATP production. The biochemical effects caused by the 9205delTA microdeletion displayed a pronounced threshold effect above ∼90 % mutation heteroplasmy. We observed a linear relationship between the decrease in subunit F_o-a or Cox3 content and the functional presentation of the defect. Therefore we conclude that the threshold effect originated from a gene–protein level.

Key words: ATP synthase, cytochrome *c* oxidase, mitochondrial diseases, mtDNA *MT-ATP6* mutation, oxidative phosphorylation, threshold effect.

INTRODUCTION

Mitochondrial diseases due to disorders of the oxidative phosphorylation system (OXPHOS) are frequently caused by mitochondrial DNA (mtDNA) point mutations in protein-coding genes [1]. They alter the amino acid composition or (less frequently) lead to the formation of truncated protein if a premature stop codon has been formed. Up to now, over 200 point mutations of mtDNA have been reported. By their nature, they can be either homoplasmic and/or heteroplasmic and affect different mitochondrially synthesized subunits (www.mitomap.org [2]). In 1996, Seneca et al. [3] found a new type of mtDNA mutation that affects the *MT-ATP6* and *MT-CO3* genes by microdeletion of two bases, TA, in mtDNA at positions 9205–9206 (9205delTA). This mutation removes the stop codon of the *MT-ATP6* gene and alters the splicing site for processing of the polycistronic *MT-ATP8/MT-ATP6/MT-CO3* transcript. The 9205delTA mutation can be expected to alter the levels of *MT-ATP6* and *MT-CO3* transcripts and thus the synthesis of the F_o-a (Atp6) subunit of ATP synthase and the Cox3 subunit of cytochrome *c* oxidase (COX), which could limit the biogenesis of these two respiratory chain complexes.

The first case with the 9205delTA mutation presented with a relatively mild phenotype – seizures with several episodes of transient lactic acidosis [3]. Analysis of patient fibroblasts with the reported homoplasmic mutation revealed no changes in *MT-ATP6* and *MT-CO3* mRNA processing, a significant increase in deadenylation of *MT-ATP8/MT-ATP6* bicistron [4], and relatively insignificant biochemical changes [5]. The second case of the 9205delTA mutation was a child with severe encephalopathy and hyperlactacidaemia [6]. In correspondence with the fatal clinical course, the patient fibroblasts showed a pronounced alteration of ATP synthase structure and a low activity and protein content of COX resulting in a ∼70 % decrease in mitochondrial ATP synthesis [7]. There was a marked and specific decrease in *MT-ATP8/MT-ATP6/MT-CO3* primary transcript processing. F_o-a subunit content and its *de novo* synthesis were reduced 10-fold when compared with the other ATP synthase subunits. Both cases were reported to be homoplasmic and therefore we speculated that an additional nuclear-encoded mitochondrial factor might be involved in processing of the *MT-ATP8/MT-ATP6/MT-CO3* transcript and modulate the deleterious effects of the 9205delTA mutation [7]. It was of interest to compare the cells from both cases. While both cases were supposedly homoplasmic,

Abbreviations: BNE, blue native electrophoresis; COX, cytochrome *c* oxidase; DDM, *n*-dodecyl-β-D-maltoside; DMEM, Dulbecco's modified Eagle's medium; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; hrCNE1, high-resolution clear native electrophoresis; LLS, Leigh-like syndrome; LS, Leigh syndrome; MILS, maternally inherited LS; NARP, neurogenic muscle weakness, ataxia and retinitis pigmentosa; OSCP, oligomycin-sensitivity conferral protein; OXPHOS, oxidative phosphorylation system; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; TPP^+, tetraphenylphosphonium; WB, Western blot.

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methodological limitations mean that they can only be claimed to have a mutation load >98 %. Later we identified heteroplasmy of the 9205delTA mutation in the fibroblasts of high passage from the first patient, indicating that negative segregation of the mutation occurred during the prolonged cultivation and unmasked the mutation heteroplasmy. The phenotypic differences between the two patients may therefore be caused by a threshold effect with a very steep dependence close to homoplasmy [8] and tissue-specific differences in heteroplasmy.

To gain more insight into the pathogenic mechanism of the 9205delTA mutation, we prepared cybrid cell lines with a varying load of the mtDNA 9205delTA mutation and investigated changes in the structure and function of ATP synthase and COX. We found that the 9205delTA mutation strongly reduces the levels of both F_o-a and Cox3 proteins, alters the structure of ATP synthase, decreases the content of COX, and prevents most of the mitochondrial ATP synthesis. All of the biochemical effects exerted a pronounced threshold effect above 90 % heteroplasmy. In addition, we found that a slightly smaller ATP synthase complex devoid of the F_o-a subunit is formed but it is rather labile and unable to synthesize ATP.

MATERIALS AND METHODS

Chemicals

Unless otherwise indicated, chemicals of the highest purity were obtained from Sigma–Aldrich.

Preparation of cybrids and isolation of mitochondria

Transmitochondrial cybrids were prepared according to [9]. Fibroblasts from the two patients P1 [3] and P2 [7] harbouring the 9205delTA mutation and from controls were enucleated by centrifugation in Dulbecco's modified Eagle's medium (DMEM, BioTech) containing 10 μg/ml cytochalasin B and then fused with mtDNA-less (ρ^0) 143B TK$^-$ osteosarcoma cells by adding a 50 % (w/v) solution of PEG with 10 % (v/v) DMSO. Cells were selected for 3 weeks in DMEM containing 5 % (v/v) fetal bovine serum, 0.1 mg/ml 5-bromodeoxyuridine and lacking uridine. The ring-cloned and subcloned cybrid cells were grown to ~90 % confluence, harvested using 0.05 % (w/v) trypsin and 0.02 % (w/v) EDTA, and washed twice in PBS before use.

Mitochondria from cybrid or fibroblast cells were isolated at 4 °C by a hypo-osmotic shock method [10]. The freshly harvested cells were disrupted in 10 mM Tris/HCl, pH 7.4, homogenized in a Teflon/glass homogenizer (10 % homogenate, w/v) and then sucrose was added to a final concentration of 0.25 M. Mitochondria were sedimented from the 600 ***g*** postnuclear supernatant by 10 min centrifugation at 10000 ***g***, washed, and resuspended in 0.25 M sucrose, 2 mM EGTA, 40 mM KCl and 20 mM Tris/HCl, pH 7.4.

In some experiments we also used the membrane fraction obtained by 10 min centrifugation of cell homogenate (10 % (w/v) in 83 mM sucrose and 6.6 mM imidazole, pH 7.0) at 15000 ***g*** [11]. Samples were stored at − 80 °C.

PCR and restriction analysis

To determine the amount of 9205delTA mtDNA, the isolated DNA was amplified by PCR using mismatch primers (bold) 5′-CCT CTA CCT GCA CGA CAA T**GC** A-3′ (forward) and 5′-CGT TAT G**C**A **T**TG GAA GTG AAA TCA C-3′ (reverse), corresponding to nt 9183–9329 (147 bp) [5]. Mismatch primers generated two *Nsi*I restriction sites in the case of wild-type mtDNA (fragments 116 + 22 + 9 bp) and one *Nsi*I restriction site in the case of mutated mtDNA (138 + 9 bp). PCR products were digested with *Nsi*I (Roche) for 3 h at 37 °C, the enzyme was inactivated for 15 min at 65 °C, and DNA fragments were separated on 1.5 % (w/v) agarose in TBE buffer (0.09 M Tris/HCl, 0.09 M H_3BO_3 and 2 mM sodium EDTA, pH 8.0). Ethidium bromide-stained gels were visualized on the transiluminator BioDocAnalyze (Biometra) and the signal was quantified using Aida 3.21 Image Analyzer. Heteroplasmy was expressed as a percentage of mutated mtDNA relative to the total signal of amplified mtDNA.

Electrophoresis, Western blot analysis, in-gel ATPase activity

SDS-PAGE [12] was performed on 10 % (w/v) polyacrylamide slab minigels (MiniProtean System, Bio-Rad Laboratories) at room temperature. Samples of whole cells or isolated mitochondria were heated for 20 min at 40 °C in a sample lysis buffer (2 % (v/v) 2-mercaptoethanol (Fluka), 4 % (w/v) SDS (Serva), 50 mM Tris/HCl (pH 7.0) and 10 % (v/v) glycerol).

Separation of native OXPHOS complexes by blue native (BNE) [11,13] or high-resolution clear native electrophoresis (hrCNE1 system) [14] was performed on polyacrylamide gradient (6–15 % for COX analysis, 4–13 % for ATP synthase analysis) minigels at 7 °C. Mitochondrial or membrane fraction proteins were solubilized with *n*-dodecyl-β-D-maltoside (DDM) or digitonin at the indicated detergent/protein ratio for 15 min on ice. The samples were centrifuged for 20 min at 4 °C and 30000 ***g***, and either Coomassie Brilliant Blue G dye (Serva Blue G-250, 0.125 g/g detergent) or Ponceau Red dye (0.005 %) and 5 % glycerol were added to the supernatants before electrophoresis. For two-dimensional (2D) analysis, strips of the first dimension native gels were incubated for 1 h in 1 % (w/v) SDS and 1 % (v/v) 2-mercaptoethanol at room temperature, washed in water and subjected to SDS-PAGE for separation in the second dimension.

Gels were blotted onto PVDF membrane (Millipore) by semi-dry electrotransfer (1 h at 0.8 mA/cm^2) and the membrane was blocked in 5 % defatted milk (Promil) in TBS (150 mM NaCl and 10 mM Tris/HCl, pH 7.5). The membranes were washed twice in TBST (TBS with 0.1 % (v/v) Tween-20) and immunodecorated with the following primary antibodies diluted in TBST: rabbit polyclonal antibodies to subunits F_o-c (1:1000) and F_o-a (1:500) [7], mouse monoclonal antibodies from Abcam to subunits F_1-α (1:1000, ab110273), F_1-β (1:2000, ab14730), F_o-d (1:700, ab110275), OSCP (oligomycin-sensitivity conferral protein) (1:250, ab110276), Cox1 (1:1000, ab14705), Cox2 (1:1000, ab110258), Cox4 (1:1000, ab110261), Cox5a (1:500, ab110262), Cox6c (1:500, ab110267), Core2 subunit (1:1000, ab14745) and pyruvate dehydrogenase (PDH, 1:1000, ab110334). Goat polyclonal antibody to Cox3 (1:200 in TBST with 3 % (w/v) BSA) was from Santa Cruz Biotechnology (sc-23986), rabbit polyclonal antibody to porin (1:1000) was a gift from Professor Vito de Pinto (Dipartimento di Scienze Chimiche - Università di Catania, Catania, Italy). For a quantitative detection, the following infra-red fluorescent secondary antibodies (Alexa Fluor 680, Life Technologies; IRDye 800, Rockland Immunochemicals) diluted in TBST were used: goat anti-mouse IgG (1:3000, A21058), goat anti-rabbit IgG (1:3000, A21109), donkey anti-rabbit IgG (1:3000, 611-732-127), and donkey anti-goat IgG (1:3000, A21084). The fluorescence was detected using ODYSSEY infra-red imaging system (LI-COR Biosciences) and the signal was quantified using Aida 3.21 Image Analyzer software.

ATPase hydrolytic activity was detected on native gels immediately after electrophoresis according to [15]. Briefly, gels were incubated for 1 h in 35 mM Tris/HCl, 270 mM glycine, 14 mM $MgSO_4$, 0.2 % (w/v) $Pb(NO_3)_2$ and 8 mM ATP, pH 8.3,

and white lead phosphate precipitates were documented by scanning.

High-resolution oxygraphy

Oxygen consumption by cybrid cells (0.75 mg protein/ml) was determined at 30 °C in a KCl medium (80 mM KCl, 10 mM Tris/HCl, 3 mM $MgCl_2$, 1 mM EDTA and 5 mM potassium phosphate, pH 7.4) as described previously [16], using Oxygraph-2k (Oroboros). Cells were permeabilized by 0.05 g of digitonin/g of protein. Respiration was measured with 10 mM succinate in the presence of 2.5 μM rotenone and 25 μM Ap5A (P^1,P^5-di(adenosine-5′)pentaphosphate), then 1.25 mM ADP was added. ADP-stimulated respiration was inhibited after 6 min with 1 μM oligomycin and after 2 min, 0.1 μM FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) was added. Activity of COX was measured with 5 mM ascorbate and 1 mM TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine) in the presence of 1 mg/ml antimycin A and was corrected for substrate autoxidation insensitive to 0.33 mM KCN. Oxygen consumption was expressed in pmol of oxygen/s/mg of protein.

Mitochondrial membrane potential $\Delta\psi_m$ measurements

$\Delta\psi_m$ was measured with TPP^+ (tetraphenylphosphonium)-selective electrode in 1 ml of KCl medium as described in [16]. Cells (2 mg of protein/ml) were permeabilized with digitonin (0.04 g/g of 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. negative decimal logarithm of TPP^+ concentration.

ATP synthesis

During respiration measurements, 10 μl samples were collected from the oxygraphic chamber (before and 6 min after ADP addition) and immediately mixed with the same volume of 100 % DMSO. ATP content was then determined in DMSO-quenched samples by a luciferin–luciferase reaction [17]. Bioluminescence was measured in the medium containing 25 mM tricine, 5 mM $MgSO_4$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.6 mM luciferin (Promega) and 6×10^7 luciferase units/ml luciferase (Promega), pH 7.8, using 1250 Luminometer (BioOrbit). Calibration curve was measured in the range 0–10 pmol of ATP. ATP production was expressed in nmol of ATP/min/mg of protein.

Ethics

The present study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Committee of Medical Ethics of Institute of Physiology Academy of Sciences of the Czech Republic. Informed consent from the parents of the patients was obtained.

RESULTS

Cybrids with mtDNA 9205delTA mutation

The cybrid cell lines used in the present study were derived from the fibroblasts of two patients (P1 and P2) with the 9205delTA mutation (Figure 1A) and included cybrid clones of varying mutation heteroplasmy. To estimate the relationship between biochemical consequences and the 9205delTA mutation load we used wild-type mtDNA homoplasmic control cybrids, several clones of 9205delTA heteroplasmic cybrids with the content of

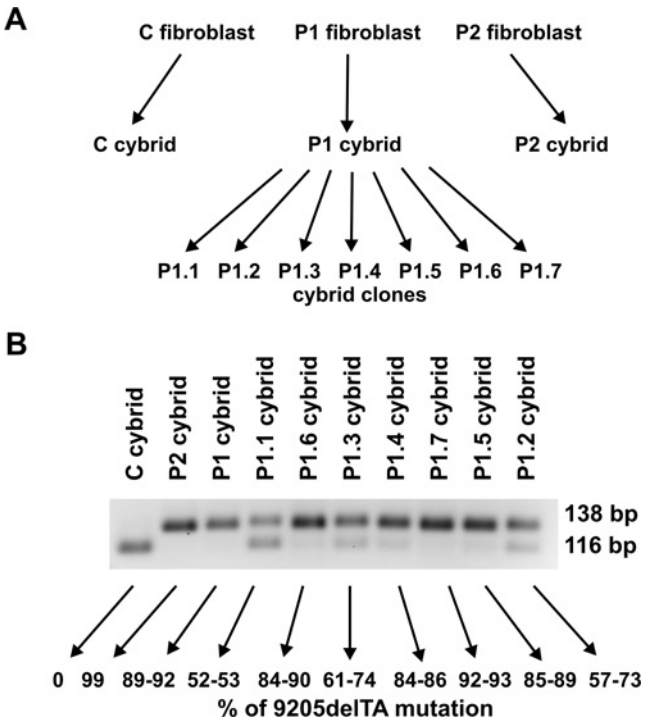

Figure 1 Cybrid cell lines used in the study

(**A**) Fibroblasts from two patients with the mtDNA 9205delTA microdeletion and from a control were enucleated and then fused with mtDNA-less (ρ^0) 143B TK^- osteosarcoma cells to produce transmitochondrial cybrid cell lines. (**B**) Mutation load in cybrid clones and subclones was analysed by restriction analysis with *Nsi*I of nt 9183–9329 mtDNA PCR products and was calculated from the amounts of 138 bp and 116 bp fragments corresponding to the mutated and wild-type mtDNA, respectively.

9205delTA mtDNA ranging between 52 and 92 % (derived from P1 fibroblasts) and 9205delTA cybrids with >99 % of mutated mtDNA (derived from P2 fibroblasts).

Throughout the course of the studies, individual cybrid cell lines maintained a stable heteroplasmy level, which was routinely checked by restriction analysis of PCR products and was expressed as a percentage of the mutated mtDNA relative to the total mtDNA (Figure 1B).

Changes in mitochondrial content and composition of ATP synthase and cytochrome *c* oxidase subunits in 9205delTA homoplasmic cells

Previous analysis of fibroblasts from the P2 patient demonstrated a very strong reduction in subunit F_o-a content [7]. The reduced content of COX subunits Cox1, Cox4 and Cox6c as well as altered maturation of Cox3 mRNA further indicated that the 9205delTA mutation may also disrupt the synthesis of subunit Cox3. To verify this assumption, we analysed cell homogenates and isolated mitochondria from control and 9205delTA homoplasmic cybrids by SDS-PAGE and Western blot (WB) (Figure 2A) using antibodies against several subunits of ATP synthase and COX. To quantify their specific content, the signals of individual subunits were normalized to those of porin and expressed as a percentage of control (Figure 2B).

The subunit F_o-a content was strongly reduced in 9205delTA homoplasmic cybrid cells; only a very low amount of F_o-a could be detected in isolated mitochondria. In contrast, F_1-α and F_1-β subunits of the catalytic part were present in near-normal levels

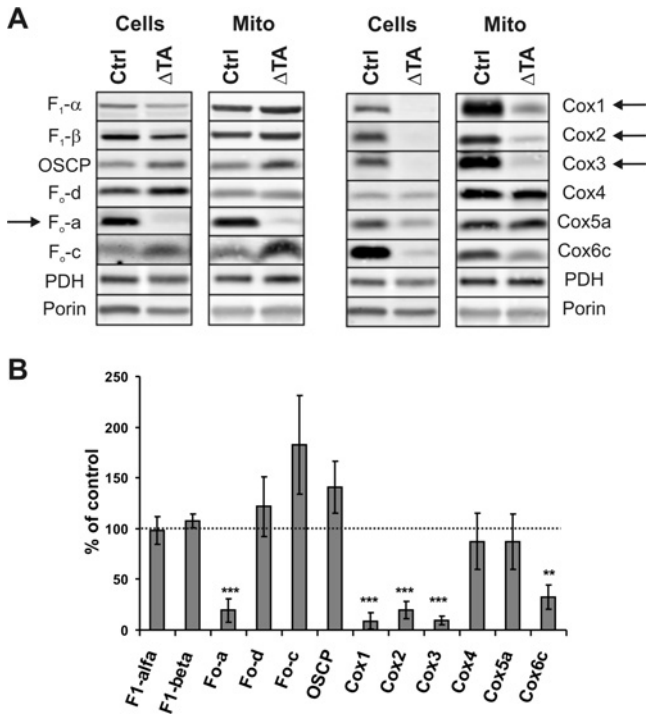

Figure 2 Specific content of ATP synthase and cytochrome *c* oxidase subunits in control and 9205delTA cybrid cells and isolated mitochondria

(**A**) Protein aliquots of cell homogenate (Cells, 15 μg) and isolated mitochondria (Mito, 10 μg) from control (Ctrl) and 9205delTA homoplasmic (ΔTA) cybrids were analysed by SDS-PAGE and WB with antibodies against indicated subunits. (**B**) Specific content of each subunit in 9205delTA samples was normalized for the signal of porin and expressed as a percentage of the content in the control. Data are the means $\pm$ S.E.M. for five experiments. $^{***}P < 0.001$, $^{**}P < 0.01$ (Student's *t* test).

in both cell homogenates and isolated mitochondria. Similarly, a normal or even increased content was found in the case of several subunits of F_o membrane part (F_o-d, OSCP and F_o-c; Figure 2B). Thus, with the exception of subunit F_o-a which was reduced to less than 20 %, all other ATP synthase subunits were present in normal or increased levels in homoplasmic 9205delTA cybrids when compared with the control cybrids.

The analysis of Cox3 clearly showed that the content of this subunit was strongly reduced due to the 9205delTA mutation. Interestingly, all mitochondrially encoded COX subunits (Cox1, Cox2 and Cox3) were similarly decreased in whole cells and isolated mitochondria (Figure 2A) and their respective content in 9205delTA homoplasmic cybrids was 8–20 % of the control (Figure 2B). Nuclear-encoded subunits were less affected and their content varied – Cox4 was almost normal in whole cells and isolated mitochondria, Cox6c was decreased to ~40 % of control in both samples, and Cox5a was decreased in the whole cells but not in isolated mitochondria (Figure 2A).

Changes in the assembled complexes of ATP synthase and cytochrome *c* oxidase in 9205delTA homoplasmic cells

Further, we were interested how the primary lack of F_o-a and Cox3 alters the properties of the assembled ATP synthase and COX. Mitochondria from the control and homoplasmic 9205delTA cybrids were solubilized by DDM or digitonin, resolved by BNE and visualized by WB using subunit-specific antibodies.

As shown in Figures 3A and 3B, in DDM-solubilized mitochondrial proteins of control cells, practically all F_1-β was recovered in ATP synthase monomer (complex V, cV) of approximately 600 kDa and a small amount, less than 10 %, was present in subcomplexes of 460 and 350 kDa. In 9205delTA cybrids, the pattern detected by anti-F_1-β antibody was completely different and revealed a strongly reduced amount of ATP synthase monomer (cV) but a high content of smaller sub-assemblies. The largest one, cV*, was approximately 60 kDa smaller than the cV monomer. Judging from the presence of subunits F_o-c and F_o-a, this could represent an almost complete cV without subunit F_o-a and possibly some other small subunit(s) (Figure 3A). This cV* was present in a similar amount as cV in 9205delTA cybrids but was completely absent from control cells. The majority of F_1-β was present in the three other, smaller, subcomplexes with the largest one being also the most abundant. None of those subcomplexes contained the F_o-a subunit. As similar subcomplexes were repeatedly described in *MT-ATP6* patients and ρ° cells [18–21], one may predict their composition. The 460 kDa subcomplex is thus expected to contain F_1 with the ring of F_o-c subunits (c-ring) and the inhibitory factor IF_1 (F_1IF_1c); the 380 kDa subcomplex corresponds to F_1IF_1, and the 350 kDa subcomplex represents F_1 alone. Judging from the F_1-β signal the relative content of these forms was 16:23:46:8:7 % for F_1:F_1IF_1:F_1IF_1c:cV*:cV, respectively. Importantly, the total amount of DDM-solubilized F_1-β signal, and thus of various cV assembly intermediates, in 9205delTA cybrids was the same or even higher than in control cells. The increase in DDM concentration from 2 g/g of protein to 4 g/g of protein did not affect the observed pattern of ATP synthase assembly forms (Figure 3A).

While it was previously proposed that ATP synthase subcomplexes observed in cells with *MT-ATP6* mutations do represent the breakdown products of assembled ATP synthase with mutated F_o-a [19], their formation may also be an artefact of the stringent conditions during BNE separation as was observed in ρ° cells [22]. Therefore, we used hrCNE1 to analyse ATP synthase assembly in 9205delTA cells. As shown in Figure 3C, when the DDM-solubilized proteins were resolved by hrCNE1, predominantly a single form of 9205delTA ATP synthase was present with a molecular mass of about 540 kDa that corresponded to the cV* detected on BNE. In-gel ATPase activity and WB analysis showed that this complex contains F_o subunits F_o-c and F_o-d but not F_o-a. A similar incomplete ATP synthase complex was described in ρ° cells, lacking both subunits F_o-a and A6L, with the mass around 550 kDa [22]. When the dye Coomassie Blue G was added to the 9205delTA sample before hrCNE1 (Figure 3C), the complex cV* broke down to the same 460 kDa and 350 kDa subcomplexes demonstrated in Figure 3A. Their composition detected by 2D analysis is shown in detail in Figure 3D. These experiments thus provide clear evidence supporting the view that mammalian ATP synthase can assemble even without the F_o-a subunit, but that the complex is unstable and dissociates easily.

When COX was analysed by BNE (Figures 4A and 4B) in control mitochondria solubilized by DDM (1 g/g protein), Cox1- and Cox4-specific antibodies detected most of the signal in the form of COX monomer (respiratory chain complex IV, cIV). A small amount was also present in higher structures – as COX dimer (cIV_2) and a supercomplex of two copies of complexes III and one copy of COX ($cIII_2cIV$), which was also detected by the antibody against cIII subunit Core2 (not shown). A small amount of both a 180 kDa subcomplex, which appears to represent the COX assembly intermediate S3, and free Cox1 subunit was also present. At a higher DDM concentration (4 g/g of protein), less supercomplex and cIV_2 but more S3 could be seen. In 9205delTA cybrid mitochondria (Figures 4A and 4B), we found no cIV_2 and strong reduction in other forms of COX compared with the control – $cIII_2cIV$, cIV and S3 were similarly decreased to 14 %,

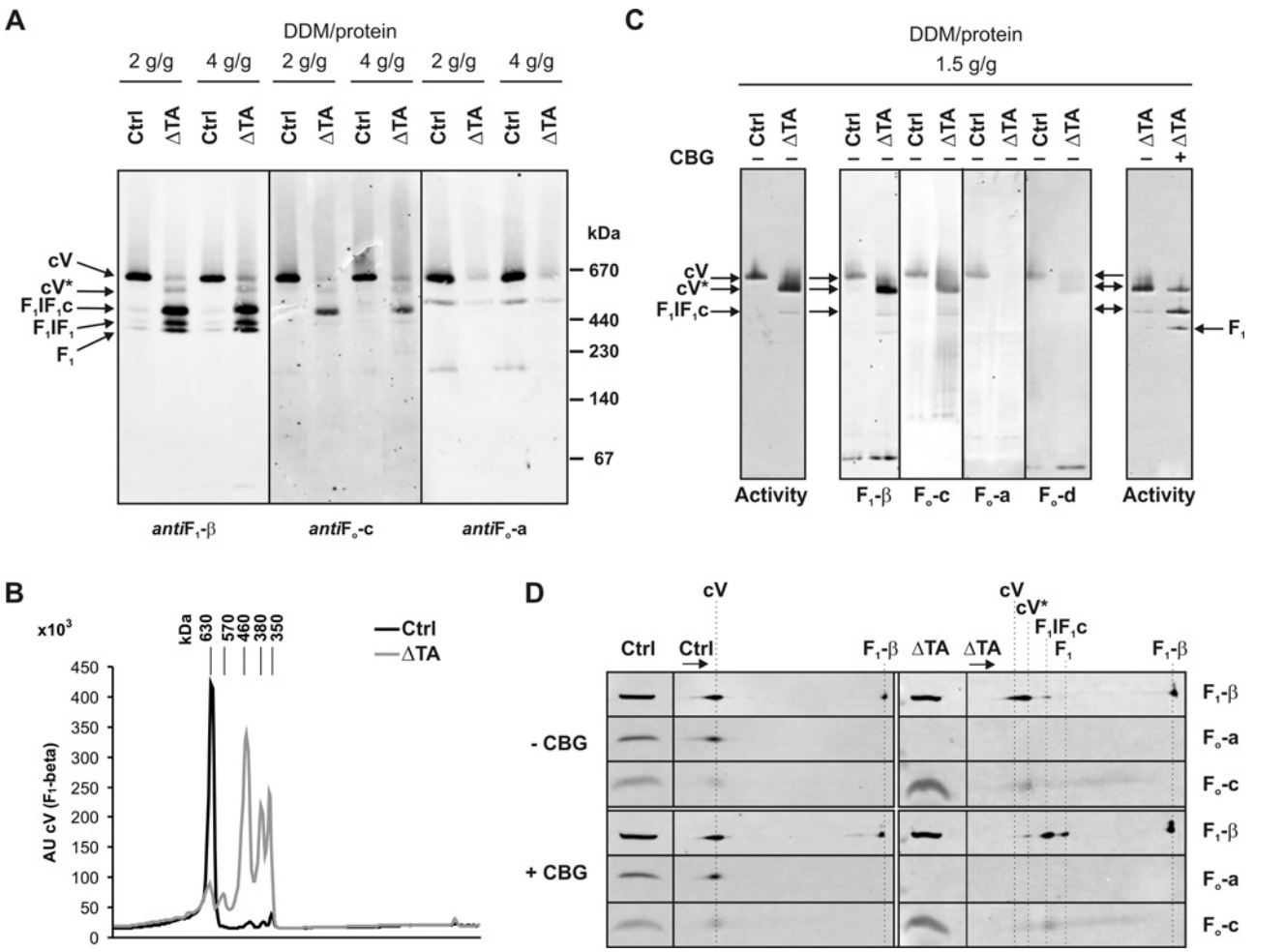

Figure 3 BNE analysis of ATP synthase complex in control and 9205delTA cybrid mitochondria

(**A**) Isolated mitochondria from control (Ctrl) and 9205delTA homoplasmic (ΔTA) cybrids were solubilized with indicated concentrations of *n*-dodecyl-β-D-maltoside (DDM) and analysed by BNE and WB using antibodies against indicated ATP synthase subunits. cV – ATP synthase complex; cV* – ATP synthase complex lacking subunit F_o-a; F_1IF_{1c} – subcomplex of F_1 with c-ring and IF_1 inhibitory factor; F_1IF_1 – subcomplex F_1 with IF_1; F_1 – F_1 alone. In (**B**) quantitative distribution of F_1-β subunit in samples solubilized at 4 g of DDM/g of protein is shown. (**C** and **D**) Mitochondrial membranes were solubilised with 1.5 g of DDM and samples with or without Coomassie Blue G dye (CBG) were analysed by hrCNE1 and 2D hrCNE1/SDS-PAGE. (**C**) ATPase activity staining and WB analysis of the hrCNE1 first dimension. (**D**) WB analysis of the hrCNE1/SDS PAGE second dimension. Aliquots of 15 μg of DDM-solubilized proteins were used.

20 % and 37 %, respectively. In contrast, the amount of free Cox1 subunit was comparable between 9205delTA and control cybrids suggesting that the early biogenesis of COX is not affected. Given the decrease in assembled enzyme, free Cox1 represented 55 % of the total Cox1 signal in 9205delTA cybrids and only 17 % in controls. Digitonin solubilization and subsequent BNE analysis achieves better resolution of supramolecular COX forms such as S2 intermediate of ∼100–140 kDa. While S2 is specifically increased in cells with COX deficiency due to *SURF1* mutations [23], Figure 4B clearly shows that this is not the case with 9205delTA cybrids.

9205delTA heteroplasmy-dependent variation in the subunit F_o-a and Cox3 content

It can be expected that the primary effect of the 9205delTA mutation is impaired synthesis of subunits F_o-a and Cox3, which leads to the formation of defective and unstable ATP synthase complex and decreased content of the fully assembled COX. To estimate how the subunit F_o-a content varies with the mutation load, we analysed several cybrid cell lines for the content of F_o-a (Figure 5A). The protein level of subunit F_o-a in 9205delTA cybrid cells did not change, until the heteroplasmy reached ∼90 %. When the mutation load exceeded this threshold, the F_o-a content progressively declined towards homoplasmy.

When we performed analogous analyses of the effect of 9205delTA mutation on the amount of Cox3 subunit (Figure 5B), again a pronounced threshold dependence could be observed. The normal amount of Cox3 subunit was present up to ∼90 % heteroplasmy, followed by a steep decrease in Cox3 content afterwards. Altogether, the contents of F_o-a and Cox3 were decreased 5 times and 10 times, respectively, in the homoplasmic cybrid cell line.

9205delTA heteroplasmy-dependent changes in the mitochondrial energetic function

9205delTA mutation affects both ATP synthase and COX, yet the functional outcome seems to be different. As shown in Figure 6, the mutation strongly affects both the generation of mitochondrial membrane potential by substrate oxidation and its utilization for ATP synthesis. In homoplasmic 9205delTA cybrids,

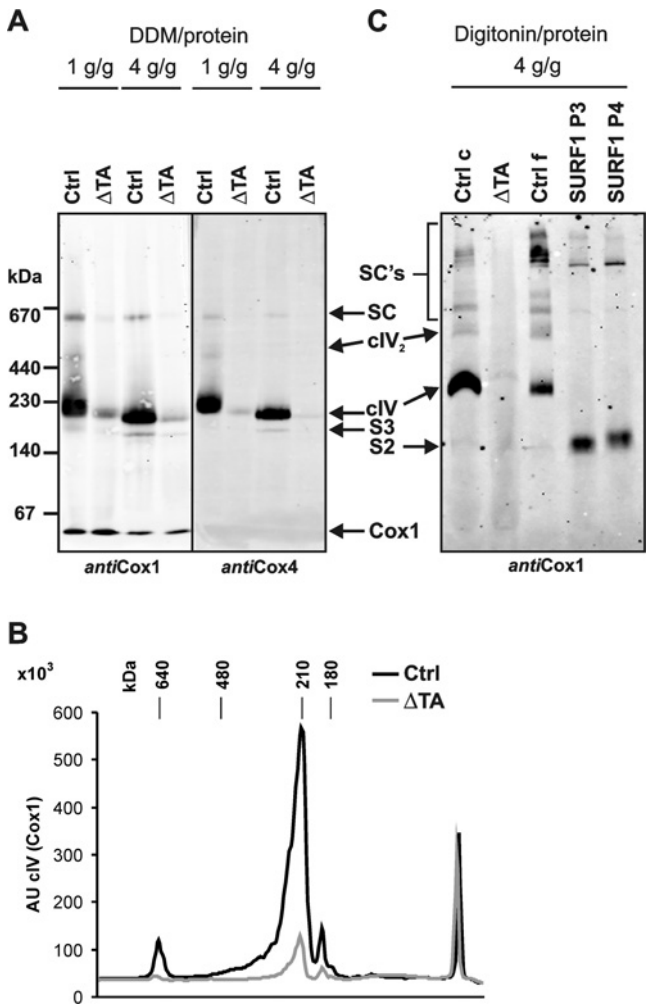

Figure 4 BNE analysis of cytochrome *c* oxidase complex in control and 9205delTA cybrid mitochondria

Isolated mitochondria of control (Ctrl) and 9205delTA homoplasmic (ΔTA) cybrids and of control (Ctrl f) and SURF1 patient (P3 and P4) fibroblasts were solubilized with given concentrations of (**A**) *n*-dodecyl-β-D-maltoside (DDM) or (**C**) digitonin, and analysed by BNE and WB using antibodies to indicated subunits of cytochrome *c* oxidase. SC's – COX supercomplexes, SC – $cIII_2cIV$ supercomplex of COX with two complexes III, cIV_2 – COX dimer, cIV – COX monomer, S3 and S2 – COX assembly intermediates. In (**B**) quantitative distribution of Cox1 subunit in samples solubilized at 4 g of DDM/g of protein is shown. Aliquots of 20 μg of DDM-solubilized proteins were used in (**A**). In (**C**) digitonin-solubilized proteins were loaded as follows: 20 μg of control cybrids and 30 μg of ΔTA cybrids, 10 μg of control fibroblasts and 30 μg of the SURF1 patient fibroblasts (P3 and P4).

the mitochondrial membrane potential $\Delta\psi_m$, expressed relatively to state 3-FCCP, was very low at state 2 and state 4 (3.1-times and 3.6-times lower in 9205delTA compared with the control cybrids, respectively). This clearly shows that the low content of COX drastically decreases the overall H^+-pumping activity of the respiratory chain. Only a minor decrease in state 2 $\Delta\psi_m$ was observed after the addition of ADP (state 3-ADP), the effect of which was oligomycin-sensitive. In accordance, the respiration in 9205delTA cybrids was only negligibly stimulated by ADP and the rate of respiration at state 3-ADP as well as at state 3-FCCP was very low. The both types of measurements excluded the possibility that alterations in ATP synthase structure would induce an enhanced proton leak.

In further experiments, we used cybrid cell lines with a varying 9205delTA mutation load and investigated how the mutation load affects the function of mitochondrial OXPHOS. We performed combined analysis of respiration by oxygraphic measurements of digitonin-permeabilized cells and of ATP production by estimating the ATP content in the course of coupled respiration with succinate as substrate. The rate of ADP-stimulated oxygen consumption was determined as the oligomycin-sensitive respiration in the presence of an excess of ADP (1.25 mM). Samples were collected during respiration measurements and content of the generated ATP was analysed by a coupled luciferase assay. In the same experiment, we also determined the activity of COX as the KCN-sensitive respiration induced by ascorbate + TMPD in the presence of antimycin A. In 9205delTA cybrid cell lines, both the oligomycin-sensitive ADP-stimulated respiration (Figure 7A) and ATP production (Figure 7B) were maintained at the control levels up to circa 90 % heteroplasmy. Both parameters decreased rapidly beyond this threshold. As shown in Figure 7C, COX activity displayed an analogous dependence on the mutation load. In 9205delTA homoplasmic cell line, the ADP-stimulated oligomycin-sensitive respiration, ATP production and COX activity were reduced to 10 %, 27 % and 16 % of the control values, respectively.

Altogether, these attempts to correlate the OXPHOS function, COX and ATP synthase activities as well as the primary changes in the F_o-a and Cox3 subunits with the 9205delTA mutation load revealed a highly similar threshold dependence. This implies that the energetic function of the mitochondrial OXPHOS could be proportionally related to the available quantity of these subunits. Figure 8 demonstrates that this was indeed the case as a near-linear relationship was observed between the content of the F_o-a and Cox3 subunits and the measured functional parameters: ADP-stimulated respiration, ATP synthesis and COX activity.

DISCUSSION

In the present study, we investigated a unique model of mitochondrial dysfunction based on selective down-regulation of biosynthesis of two OXPHOS subunits encoded by the mtDNA *MT-ATP6* and *MT-CO3* genes due to the altered processing and maturation of their mRNAs, caused by the mtDNA 9205delTA microdeletion.

The 9205delTA mutation has so far been found in only two cases that differed markedly in biochemical and clinical phenotypes, although both showed a nearly homoplasmic mutation load [3,7]. This could suggest the involvement of a nuclear-encoded factor that would take part in posttranscriptional regulation of F_o-a/Atp6 biosynthesis and thus modulate the presentation of homoplasmic mutation [7]. However, it is relatively difficult to rule out that in the "homoplasmic" cases, there are not trace amounts of wild-type mtDNA present. Indeed, after extended cultivation and numerous passages of fibroblasts from P1 (with a milder presentation) the presence of increased and detectable level of wild-type mtDNA became apparent. This suggests that at least P1 was not 100 % homoplasmic for the 9205delTA mutation. The distinct phenotypic presentation of the two cases thus could results from differences in the mutation load with a critical threshold for disease manifestation present at a very high heteroplasmy level. To unravel the biochemical consequences of the mtDNA 9205delTA microdeletion, we prepared a panel of cybrid cell lines with variable heteroplasmy ranging from 52 % to 100 % and investigated the structure and function of ATP synthase and COX at different heteroplasmy levels.

The first important finding of these studies was that the homoplasmic mtDNA 9205delTA microdeletion leads to down-regulation of the content of both F_o-a and Cox3 subunits to less than 20 % and 10 %, respectively, relative to the control. The

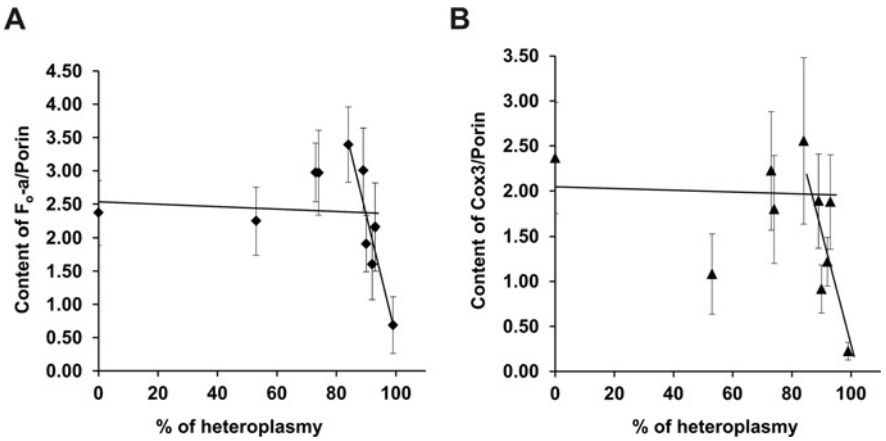

Figure 5 Dependence of subunit F_o-a and Cox3 content on the 9205delTA mutation load

Specific content of (**A**) F_o-a and (**B**) Cox3 subunits was determined in mitochondria of control and 9205delTA cybrid clones by SDS-PAGE and WB, normalized to the content of porin and plotted against the 9205delTA mutation load expressed as a percentage. Data are the means ± S.E.M. for three experiments.

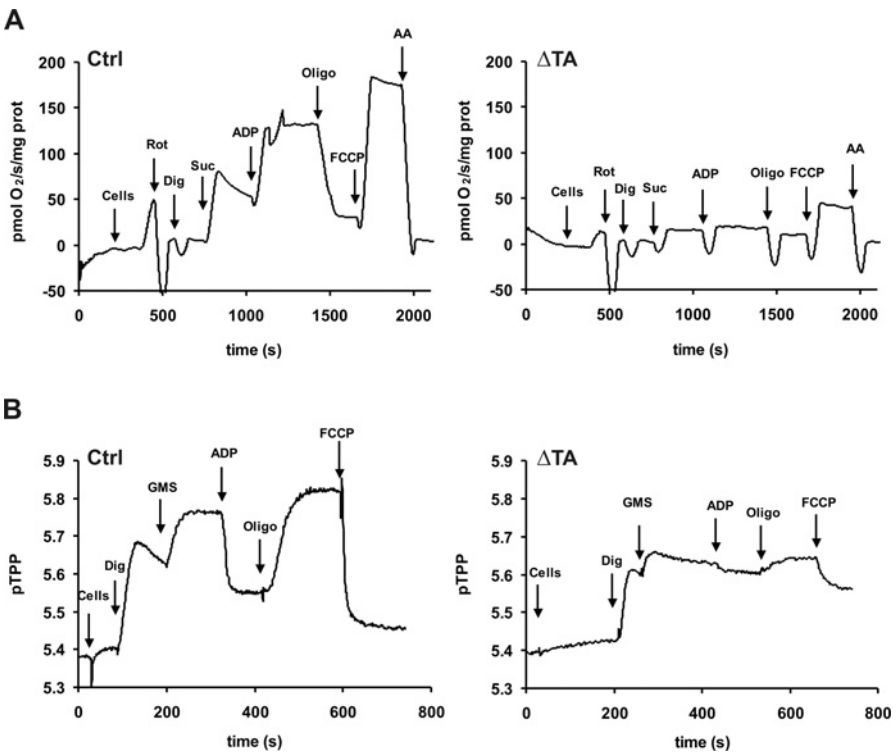

Figure 6 Respiration and mitochondrial membrane potential analysis in control and 9205delTA homoplasmic cybrid mitochondria

(**A**) Respiration and (**B**) TPP^+ measurement of $\Delta\psi_m$ were performed in control (Ctrl) and 9205delTA homoplasmic (ΔTA) cybrids permeabilized with digitonin (Dig) using glutamate (G), malate (M), succinate (Suc, S), ADP, oligomycin (Oligo), FCCP and antimycin A (AA) as indicated.

previously observed insufficient maturation of the *MT-ATP6* and *MT-CO3* mRNAs originating from the polycistronic primary transcript (*MT-ATP8*/*MT-ATP6*/*MT-CO3*) [7] thus decreases the efficacy of their translation to a very low level. Here we show that all the successive changes in the biogenesis and function of OXPHOS complexes cIV and cV are caused by the lack of these two proteins.

The manifestation of the 9205delTA microdeletion in the cybrid cell lines displayed a non-linear dependence on the mutation load and exerted a threshold effect at about 90 % heteroplasmy. This dependence was observed at several levels – the content of subunits F_o-a and Cox3, the content and activity of COX, as well as OXPHOS function measured as coupled respiration and ATP synthesis. Apparently, the non-linear threshold character of the dependence of structural-functional consequences of the 9205delTA mutation originates at the gene–protein level, due to post-transcriptional events affecting the amount of translated subunits F_o-a and Cox3. The 9205delTA microdeletion thus behaves similarly to missense mutations of *MT-ATP6* although the underlying mechanism is mRNA processing and maturation.

From the bioenergetics point of view, it is difficult to conclude which enzyme deficiency is more critical for the disease progression. There was no real difference in threshold effects

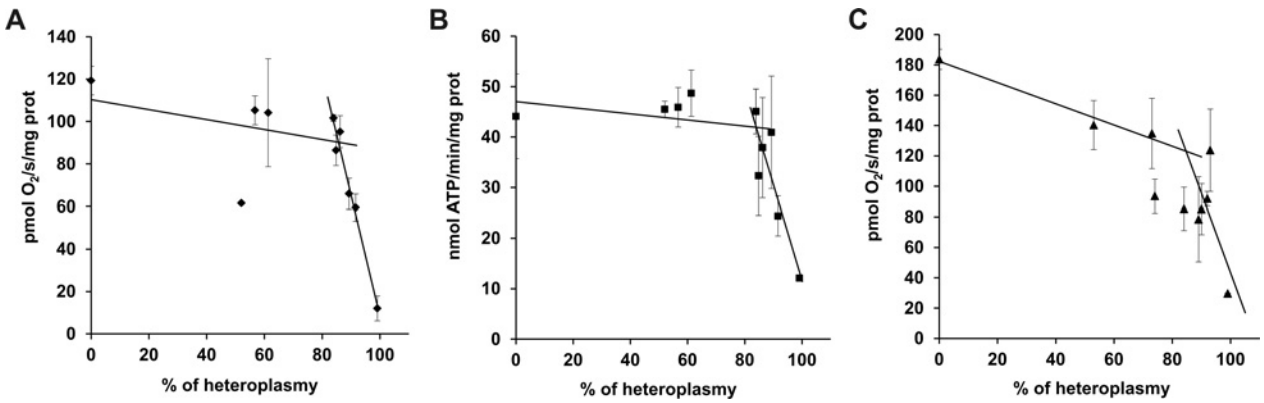

Figure 7 Dependence of ADP-stimulated respiration and ATP synthase and cytochrome *c* oxidase activities on the 9205delTA mutation load

In cybrid cells permeabilized with digitonin, (**A**) ADP-stimulated, oligomycin-sensitive respiration with succinate was measured by oxygraphy, (**B**) ATP production was measured by luciferase assay and (**C**) cytochrome *c* oxidase activity was measured as antimycin A + TMPD + ascorbate oxygen consumption sensitive to KCN. All three parameters were expressed per mg of protein and plotted against the 9205delTA mutation load expressed as a percentage. Data are the means ± S.E.M. for three experiments.

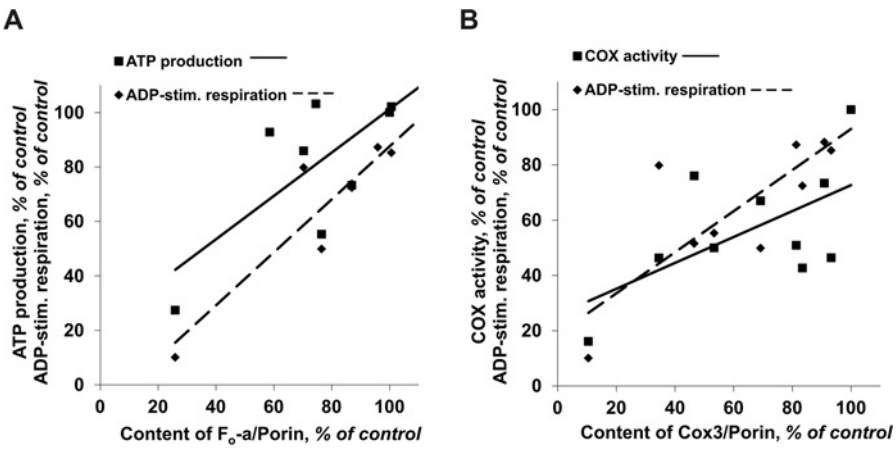

Figure 8 Correlations among 9205delTA-dependent variables

ADP-stimulated respiration, ATP synthesis and cytochrome *c* oxidase activity were plotted against the content of (**A**) F_o-a or (**B**) Cox3 subunits, as indicated, using data from Figures 5 and 7. All values are expressed as a percentage of control.

of COX activity, ADP-stimulated respiration and ATP synthesis. However, the measurements of mitochondrial membrane potential indicated that respiration-dependent proton translocation is severely affected by 9205delTA homoplasmy despite the fact that about 15–20 % of assembled COX and COX activity was preserved. This would imply that the deficiency of COX might be primary and more critical for the overall mitochondrial energy provision.

Our analysis of subunits and assembly forms of cIV and cV revealed, in accordance with our previous studies [7], that for COX, the lack of Cox3 limits the amount of the matured enzyme, but not its structure, while in the case of ATP synthase it is the quality of the enzyme, which is changed – lack of F_o-a results in the production of incomplete, labile and non-functional enzyme.

COX consists of 13 subunits. The three largest mtDNA-encoded Cox1, Cox2 and Cox3 form the catalytic core, the ten small regulatory subunits (Cox4, Cox5a, Cox5b, Cox6a, Cox6b, Cox6c, Cox7a, Cox7b, Cox7c and Cox8) are encoded in the nuclear genome. COX assembly is a stepwise process, which proceeds through several intermediates (S1–S4) [24]. Cox1 represents the first intermediate S1 which progresses to Cox1–Cox4–Cox5a sub-assembly. Subsequently, Cox2 joins this intermediate S2. The process continues with the formation of intermediate S3 after the addition of Cox3 and most of the other remaining subunits. The COX holoenzyme formation (S4) is then completed by the addition of Cox7a/b and Cox6a to S3 [24–27].

At least 14 different heteroplasmic and/or homoplasmic mutations in the *MT-CO3* gene have been reported (www.mitomap.org); and in most cases the decrease in COX activity associates with the defect in COX biogenesis. The clinical outcomes are variable, from optic neuropathies, through Alzheimer's disease, rhabdomyolysis, mitochondrial encephalopathies and myopathies with lactic acidosis, to Leigh or Leigh-like syndromes (LS, LLS). Analysis of affected families in accordance with the studies of the cybrid cell lines revealed that the severity of several *MT-CO3* mutations is heteroplasmy-dependent [28–31]. Interestingly, an improvement in clinical presentation in the case of the 9379G>A mutation was connected with a pronounced decrease in the mutation load [32].

As with many other mtDNA-encoded proteins, most of the *MT-CO3* mutations are single base pair transitions that change highly conserved amino acid residues. Predominantly, they are proposed to affect the interaction of Cox3 with Cox1, or they create a premature stop codon [33,34]. Another type of mutation

is a single base pair insertion or deletion [35,36] leading to the synthesis of truncated Cox3 protein. In addition, a 15-bp deletion, 9480del15, that removes five amino acids (two of them highly conserved) in the third transmembrane region of Cox3 protein was described [29]. This caused pronounced down-regulation of Cox3 steady state levels, similar to the frameshift mutation 9537insC leading to the incomplete Cox3 protein of only 110 amino acids [36]. In 9480del15 cells, Cox3 was translated but was highly unstable. In 9537insC cybrids, the mature *MT-CO3* mRNA was present but was not translated, while in our case of 9205delTA, the low Cox3 content was due to the altered splicing and maturation of the *MT-CO3* transcript. These Cox3-lacking cell lines displayed a pronounced decrease in the content of Cox1 and Cox2 but not of Cox4. No change in Cox5a was found in 9537insC and our 9205delTA cybrids (Figure 2) or in 9952G>A muscle [33] while Cox6c subunit content was significantly reduced in 9205delTA cybrids (Figure 2) and 9952G>A muscle. When Tiranti et al. [36] investigated COX assembly intermediates in 9537insC cybrids, they found the majority of Cox1 in S1 (free Cox1), but significant amounts of Cox1 were also associated with Cox2-containing intermediates depicted as S3 and S2a, both larger than canonical S2. In 9205delTA assembly intermediates (Figure 4), we also found most Cox1 as S1 and little as S3, but there was no indication of S2a, which appears to be specific for 9537insC cells and may reflect the presence of low levels of truncated Cox3. However, we have not observed any accumulated S2 in 9205delTA cells either (Figure 4), which may indicate that these intermediates are quickly degraded, if the COX biogenesis is stalled between S2 and S3. The relative accumulation of free small subunits Cox4 and Cox5a which we observed in our model (Figure 2) was repeatedly described also in other types of COX deficiencies, e.g. *SURF1* or *SCO1* mutations [37], and stems from their relative resistance to degradation.

ATP synthase complex is composed of 16 different subunits organized into membrane-extrinsic F_1 catalytic part (F_1-α, F_1-β, F_1-γ, F_1-δ and F_1-ε subunits) and membrane-embedded F_o part (F_o-a, F_o-c, F_o-e, F_o-f, F_o-g, A6L, F_o-b, F_o-d, F_6 and OSCP) that are connected by two stalks [38]. Small regulatory subunit IF_1 binds to F_1 at low pH and prevents the enzyme from undergoing a switch to hydrolytic mode and ATP hydrolysis. The formation of ATP synthase from individual subunits is a stepwise procedure, expected to proceed via assembly of several modules, starting with an independent formation of F_1 and oligomer of F_o-c subunits [39,40]. Afterwards the F_1 is attached to the membrane-embedded c-ring and the subunits of peripheral arm and of the membranous subcomplex are added. In the last stage the enzyme structure is completed by incorporation of the two mtDNA-encoded subunits, F_o-a and A6L [22].

Over 20 mutations in the mtDNA *MT-ATP6* gene have been reported, all of them single base pair missense mutations. They have been associated with variable brain, heart and muscle disorders, but also with autism, multiple sclerosis, optic neuropathy and diabetes in combination with other mtDNA mutations (www.mitomap.org). The most common are 8993 T>G and T>C mutations manifesting as early-onset maternally inherited Leigh syndrome (MILS) or milder neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP) [41–43]. T>G mutations are clinically and biochemically more deleterious, T>C transitions are less frequent and rather late-onset. Similar features were described for the second most common transitions at nt 9176, T>G and T>C associated with LS or familial bilateral striatal necrosis [44,45]. The 9176T>C mutation was also found in the patients with Charcot-Marie-Tooth hereditary neuropathy [46] or the late-onset hereditary spastic paraplegia [47]. Other rare *MT-ATP6* mutations (9185T>C, 9191T>C, 8851T>C, 8989G>C, 8839G>C, 8597T>C) present as LS, LLS, NARP or cardiomyopathy [48–54].

Distinct phenotypes of different *MT-ATP6* mutations are related to the mutation load, but with variable relationships between heteroplasmy and phenotypic presentation. The asymptomatic family members often have a mutation load lower than the affected patients; however, the implicated threshold mutation level for the disease manifestation varies. The best example of phenotypic dependence on the mutation load represents 8993T>G transition – the severity of the disease increases with the mutation load and a milder NARP manifests at lower heteroplasmy (around 70 %) than early-onset devastating MILS (around 90 %). In some cases the severity of symptoms in 8993 patients was found to be heteroplasmy-dependent but without a distinct threshold level for the disease manifestation [55–57] or even with a linear correlation between the mutation load and biochemical parameters [58]. As discussed above, the biochemical defects and the severity of the 9205delTA disease appear to be also heteroplasmy-dependent and point to a steep decline in mitochondrial energy provision above the threshold close to mutation homoplasmy. Interestingly, the healthy mother of the second patient had 85 % heteroplasmy in the blood and 92 % heteroplasmy in fibroblasts [7]. Considering the results obtained in the cybrid cells, the threshold of 9205delTA mutation occurs above 90 % heteroplasmy.

The pathogenicity of *MT-ATP6* mutations is usually given by decreased synthesis of ATP due to defective translocation of the protons across the membrane in 9176T>G mutation [59], or by inefficient coupling between proton translocation and synthesis of ATP in the 8993T>G, 8993T>C, 9035T>C, 9176T>C and 8839G>C mutations [53,57–61]. In the 8993T>C, 9035T>C and 9176T>C mutations, the ATP synthesis is not that severely affected and increased production of ROS (reactive oxygen species) can also contribute to the proposed pathogenic mechanism [47,57,60]. In the 9205delTA mutation, severe reduction in the production of ATP is given by the lack of subunit F_o-a, making the F_o proton channel unable to translocate protons as the reduction in ATP synthesis is accompanied with decreased ADP-stimulated respiration and almost no effect of ADP on mitochondrial membrane potential.

From the structural point of view, the insufficient production of F_o-a subunit resulted in the formation of several BNE-resolved F_1-containing complexes which were smaller than ATP synthase monomer. On the other hand the total amount of various intermediates from F_1 up was normal or even increased. The size of these subcomplexes (460, 380 and 350 kDa), their relative abundance and involvement of F_o subunits closely resembled such complexes found in cells with *MT-ATP6* mutations, ρ° cells, cells upon mtDNA depletion or inhibition of mitochondrial protein synthesis [18,19,21,62–65], where they represent breakdown products of fully assembled ATP synthase complex rather than assembly intermediates. As demonstrated by our hrCNE1 analysis, ATP synthase devoid of subunit F_o-a had a size only ~60 kDa smaller than the control enzyme and was detected as the only form of the 9205delTA enzyme when Coomassie Blue G was omitted. Our data thus provide clear evidence that, in the absence of F_o-a, the almost complete F_1F_o-ATP synthase complex can be quantitatively formed.

F_o-a-deficient cells represent a valuable model for a better understanding of the assembly of mitochondrial ATP synthase structure as well as its function. F_o-a/Atp6 has been implicated as the last subunit incorporating into the enzyme complex during biosynthesis of both the eukaryotic and the prokaryotic enzyme. Our data suggest that ATP synthase lacking F_o-a is assembled and incorporated into the membrane. This is evident from the hrCNE1 experiments, where we could resolve fully assembled (albeit

without F_o-a) enzyme. However, this complex becomes unstable and dissociates when exposed to Coomassie Blue G. After the dye binds to the proteins, it introduces negative charge which apparently breaks down some fragile inter-subunit interactions which keep the F_1c rotor structure connected with the external stalk of the enzyme in the absence of F_o-a. After Coomassie Blue G binding, the major form of F_o-a-deficient enzyme had molecular mass of approximately 460 kDa and contained F_1 subunits and subunit F_o-c, but not subunits F_o-d and OSCP.

The F_o-a-deficient ATP synthase was unable to synthesize ATP but did not leak the protons as both the respiration and mitochondrial membrane potential were affected by FCCP. When an analogous model of bacterial ATP synthase lacking subunit F_o-a was investigated [66], the enzyme complex was found to be rather stable. It could be isolated upon solubilization with Triton X-100 and deoxycholate, incorporated into liposomes and the isolated F_o lacking F_o-a could be reconstituted with F_1. The bacterial enzyme lacking F_o-a was also not proton leaky and, as expected, unable to synthesize ATP as the proton channel was inactive. The absence of F_o-a in the bacterial enzyme further decreased/prevented ATP-hydrolytic activity, indicating that altered F_o structure, possibly the anomalous interaction between c-ring subunits and subunits F_o-b, prevented the rotor rotation. In contrast, the F_o-a-deficient 9205delTA enzyme retained its hydrolytic activity [7], suggesting that c-ring rotation was possible and not hampered, possibly reflecting differences in structure between bacterial and mammalian mitochondrial F_o.

AUTHOR CONTRIBUTION

Josef Houštěk and Kateřina Hejzlarová conceived and designed the experiments. Vilma Kaplanová and Kateřina Hejzlarová prepared cybrid cell lines and analysed mtDNA. Kateřina Hejzlarová and Nikola Kovářová performed electrophoretic experiments, Kateřina Hejzlarová, Pavel Ješina, Hana Nůsková and Zdeněk Drahota performed oxygraphic and spectrophotometric measurements. Kateřina Hejzlarová, Sara Seneca, Tomáš Mráček and Josef Houštěk analysed the data and wrote the paper.

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