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Separate Summary of the PhD Thesis

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

Genetické a funkční příčiny mitochondriálních chorob vyvolaných defekty ATP syntázy

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Contents

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.

1. Introduction

1.1 Mitochondrial ATP synthase

1.1.1 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*, *F6*, *OSCP* and *IF1*) organized into membrane-extrinsic F_1 catalytic part and membrane-embedded F_o part that are connected by two stalks [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*) [2-4]. In mammals, only two ATP synthase subunits of F_o sector - 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. 1). 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 [5]. 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, Atp23 and Atp25) are involved in the F_o biogenesis [6-11]; however, none of them exists in mammals reflecting differences in structure of mitochondrial genes and expression of mtDNA-encoded subunits.

Assembly of the yeast F_1 part depends on three additional factors, Atp11, Atp12 and Fmc1 [6, 12, 13]. Only the first two have their homologues in humans [14] interacting with the subunits β and α, respectively, during the $\alpha_3\beta_3$ hexamer formation (Fig. 1). Recently new ATP synthase ancillary factor was described [15, 16]. Transmembrane protein 70 (TMEM70), specific for higher eukaryotes, is suggested to be involved in the early stages of ATP synthase assembly [17].
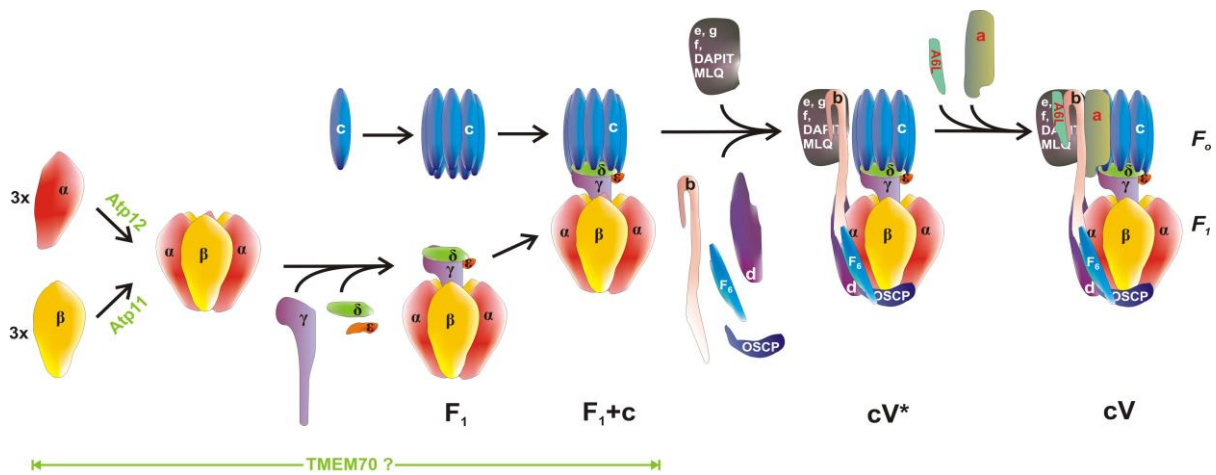
Figure 1. Mammalian ATP synthase assembly. *ATP synthase assembly starts with the independent formation of F_1 part (composed of subunits α, β, γ, δ and ε) and subunit c-oligomer, that are in the next step connected together to form F_1+c intermediate. Assembly then follows with the subsequent addition 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 $α_3β_3$ 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 [18].*

1.1.2 Function of F_1F_o ATP synthase

ATP synthase operates as a unique molecular motor utilizing 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 submitted to the *c*-ring. 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. 2). As γ rotates inside the $α_3β_3$ hexamer it provides energy for ATP synthesis by a rotary catalysis explained by a "binding change mechanism" originally proposed by P. Boyer [19]. 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. 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 utilized. The most efficient is thus mammalian enzyme containing 8 subunits, while 10–15 are found in yeast and bacterial enzymes [1].
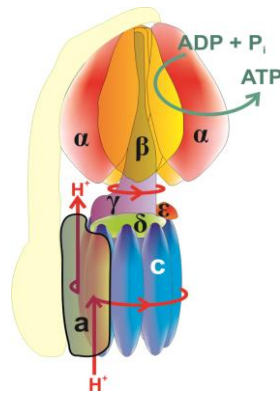
Figure 2. Production of ATP by mitochondrial ATP synthase. *Protons at the IMS side of the membrane are bound to subunit a and submitted to subunit c. Binding of protons powers rotation of c-ring, that causes rotation of subunit γ. As γ rotate inside the hexamer it causes conformational changes of subunit β, required for interaction of ADP and P_i to create ATP.*

1.2 Biogenesis of OXPHOS system

OXPHOS biogenesis is remarkably complex process requiring cooperation of many factors, controlling not only the assembly of the individual complexes, that is quite complicated itself and still not fully understood, but also the coordination of the both genome expression, modulation by extrinsic signals etc. Many of the processes where extensively studied in the lower eukaryotes, especially in *Saccharomyces cerevisiae*. 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.

1.2.1 Regulation of expression of mitochondrial proteins

Deep 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 react to actual conditions. The activity of OXPHOS system is controlled via regulation of both nuclear and mitochondrial genes expression. The synthesis of mtDNA encoded proteins can be regulated at various levels. The mtDNA expression can be indirectly influenced by the expression of proteins involved in mtDNA replication, such as Twinkle helicase [20]. Similarly, mtDNA transcription can be controlled by regulation of activity of the respective mtDNA transcription factors, mainly the POLRMT and TEFM [21], or mitochondrial topoisomerase I [22].

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. Stability and degradation of mitochondrial mRNAs transcribed from the heavy strand is partially regulated via polyadenylation/deadenylation of their 3´ends. Two proteins have been described as the main regulators of this process, mitochondrial poly(A) polymerase mtPAP [23, 24], and 2´phosphodiesterase PDE12 [25]. However, 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. LRPPRC (leucine-rich pentatricopeptide repeat-containing) protein was found together with SLIRP protein to form a complex that is involved in the regulation of stability of all mature mitochondrial mRNAs [26]. 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, usually signed as assembly factors of cytochrome *c* oxidase [27-29]. 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 most of 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 [30]. 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 [31].

1.2.2 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 fundamental step in the biogenesis of OXPHOS apparatus. In yeast the mtDNA encoded subunits are synthesized on the membrane-bound ribosomes, and then inserted into the membrane via several proteins, of them the best characterised is the Oxa1 [32]. Oxa1L, human homolog of the yeast Oxa1, is suggested to bind to the

mitoribosomes as well [33]. CRIF1 is the next mitochondrial protein, which was described to be associated with large mitoribosomal subunit [34].

Recently so called MITRAC complexes were described in humans, containing mtDNA encoded COX subunits and specific factors for Cox1 assembly and translation, and Tim21 protein, involved in the transport and insertion of proteins from cytosol across/to the inner mitochondrial membrane. [35]. 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.

1.3 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 muscles. OXPHOS 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), 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 characterized. Several hundreds of them have been described to date (MITOMAP inventory, www.mitomap.org, [36]) and their detection and screening has become rather routine task [37]. However, as according to current estimates, mtDNA mutations are responsible for only ~25 % of mitochondrial diseases, the majority originates from mutations in nuclear genes. Their identification and characterization thus 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 [38] including numerous novel factors of mitochondrial biogenesis (e.g. TMEM70 [15, 16], C12orf65 [39, 40] C20orf7 [41], TACO1 [27], RMND1 [42], SDHAF1 [43], SDHAF2 [44]). Nevertheless, most of the disease-causing genes still remain to be identified

among more than thousand nuclear genes contributing to the mitochondrial proteome (www.mitocarta.com [45]).

1.3.1 Mitochondrial DNA mutations

Mutations in mtDNA 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 the 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. The most pathogenic mtDNA mutations are usually heteroplasmic, homoplasmy is rather found in case of mutations with very mild phenotypes not affecting the key life functions.

1.3.2 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

(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. The most common are mutations *m.8993T>G* (p.L156R) and *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). 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, more severe T>G transition then T>C, were described for *m.9176T>G* (p.L217R) and *m.9176T>C* (p.L217P) mutations. Other rare *MT-ATP6* mutations mostly present as Leigh or Leigh-like syndrome, NARP or cardio-myopathy.

Distinct phenotypes of different *MT-ATP6* mutations are related to the mutation load, but with variable relationships between heteroplasmy and phenotypic presentation emerging as a threshold for the disease progression. However, some cases with very low heteroplasmy suggest that the threshold effect is not always linked with mtDNA mutations. Even the results of some studies on cybrid cell lines with different heteroplasmy are not consistent with the threshold effect in *MT-ATP6* mutations.

Subunit *a* is necessary for the proton translocation and thus for ATP synthesis, and is thought to stabilize ATP synthase complex. Mutations in *MT-ATP6* usually affect the efficiency of proton translocation leading to the reduced production of ATP [46-51]. In some cases may be also increased ROS production [46, 49, 52]. As shown by native electrophoresis, the stability of ATP synthase complex is affected by *MT-ATP6* mutations as well. In fibroblasts with 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 [53-56]. 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 [5].

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*) removing the STOP codon of *MT-ATP6* gene and altering the splicing site for processing of the polycistronic MT-ATP8/MT-ATP6/MT-CO3 transcript. The first case with *m.9205delTA* mutation presented with a relatively mild phenotype – seizures with several episodes of transient lactic acidosis [57], and insignificant biochemical changes [58, 59].

Table 1: Single *MT-ATP8/6* and *MT-ATP6* mutations associated with isolated ATP synthase disorders.

Gene	Mutation	Clinical phenotype	References
MT-ATP8/6	m.8528T>C missense (p.W55R; p.M1T)	Infantile cardio-myopathy	[60]
	m.8529G>A nonsense; silent (p.W55X; p.M1M)	Apical HCMP	[61]
MT-ATP6	m.8597T>C missense (p.I24T)	LS	[62]
	m.8668T>C missense (p.W48R)	LHON	[63]
	m.8719G>A nonsense (p.G65X)	Mitochondrial disease suspected	[64]
	m.8836A>G missense (p.M104V)	LHON	[65]
	m.8839G>C missense (p.A105P)	NARP	[50]
	m.8851T>C missense (p.W109R)	BSN, LS, ataxia	[66, 67]
	m.8950G>A missense (p.V142I)	LHON	[68]
	m.8969G>A missense (p.S148N)	MLASA	[69]
	m.8989G>C missense (p.A155P)	NARP	[70]
	m.8993T>G missense (p.L156R)	NARP, LS	[51, 54, 71-78]
	m.8993T>C missense (p.L156P)	NARP, LS, ataxia	[51, 72, 76, 79-82]
	m.9035T>C missense (p.L170P)	Ataxia	[49]
	m.9101T>C missense (p.L222P)	LHON	[83]
	m.9176T>G missense (p.L217R)	LS	[84, 85]
	m.9176T>C missense (p.L217P)	LS, CMT, BSN, Hereditary spastic paraplegia	[51, 52, 86-89]
	m.9185T>C missense (p.L220P)	LS, CMT, Motor neuron syndrome, ataxia	[90-94]
	m.9191T>C missense (p.L222P)	LS	[92]
	m.9205delTA frameshift; splicing (p.X227M)	Encephalo-myopathy, LA	[57, 95]

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. *MT-ATP6* mutations associated with human pathologies in combination with other mtDNA mutation are not included.

The second case of *m.9205delTA* mutation was a child with severe encephalopathy and hyperlactacidemia [95]. In accordance with the fatal clinical course, fibroblasts of this patient showed a pronounced alteration of ATP synthase structure and a low activity and 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.

1.3.3 Nuclear DNA mutations leading to the mitochondrial dysfunction

Nuclear DNA mutations are frequent cause of mitochondrial metabolism disorders. 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 [96]. 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" [97].

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 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-sulphur cluster assembly (BOLA3, ISCU) or coenzyme Q biosynthesis (CoQ2, CoQ4, CoQ9). Mitochondrial disease can be caused yet 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).

1.3.4 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

In 2004 De Meirleir described one patient with severe neonatal encephalopathy who harboured missense mutation in Atp12 assembly factor essential for incorporation of α subunit into F_1-ATPase structure [98]. 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. 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. With the help of a yeast model, it was later shown that this mutation affects the solubility of Atp12 protein which tends to aggregate [99].

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 [16].

TMEM70 was first described in 2006 by Calvo as a gene coding for potentially mitochondrial protein [100]. Since TMEM70 patients have very low levels of fully assembled ATP synthase and accumulate some F_1 containing subassemblies, TMEM70 was recognized as a new biogenetic factor of ATP synthase [16]. However, the properties and mechanism of TMEM70 function remained to be elucidated.

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	[101]
ATP5E	*c.35A>G* missense (p.Y12C)	Neonatal respiratory distress, LA, 3-MGA, severe peripheral neuropathy, exercise intolerance	[102]
ATPAF2	*c.280T>A* missense (p.W94R)	3-MGA, LA, neonatal encephalopathy, dysmorphism	[98]
TMEM70	*c.317−2A>G* splicing	IUGR, LA, HA, EOH, FD, HCMP, 3-MGA, cataract, encephalopathy, FTT, PMR, persistent PAH	[16, 103-110]
	c.317−2A>G/c.118_119insGT frameshift (p.S40CfsX11)	LA, 3-MGA, HA, HCMP, FD, PMR,	[16, 107, 110, 111]
	c.317−2A>G/c.494G>A missense (p.G165D)	LA, 3-MGA, HA, HCMP, Reye-like syndrome, exercise intolerance	[112, 113]
	c.336T>A nonsense (p.Y112X)	IUGR, LA, HA, HCMP, FD, PMR	[107, 114]
	c.316+1G>T splicing	IUGR, Encephalopathy, HCMP, EOH, LA, FD	[107, 114]
	c.238C>T nonsense (p.R80X)	IUGR, EOH, LA, 3-MGA, HA, HCMP, multiorgan failure, dysmorphism,	[107, 109, 114]
	c.578_579delCA frameshift (p.N198X)	IUGR, EOH, LA, 3-MGA, FD, cataract, Encephalopathy, HCMP, PMR	[107, 114]
	c.211−450_317−568del (2290bp deletion) frameshift	IUGR, HCMP, LA, 3-MGA, PMR	[105]
	g.2436−3789 in-frame deletion (1353bp)	IUGR, LA, HA, HCMP, PMR, ptosis	[115]
	c.317−2A>G/c.628A>C missense (p.T210P)	HCMP, LA, 3-MGA, HA, resistent or persistent PAH, WPW	[104, 107, 108]
	c.535C>T missense (p.Y179H)	IUGR, LA, EOH, FD, HCMP, bilateral cataract, PMR, HA	[107, 116]
	c.317−2A>G/c.349_352del frameshift (p.I117A, p.224X?)	IUGR, LA, PMR, HCMP, EOH, dysmorphism, HA	[109]
	c.317−2A>G/c.783A>G frameshift (p.X261Wext17)	IUGR, LA, PMR, HCMP, dysmorphism	[109]
	c.701A>C missense (p.H234P)	IUGR, LA, 3-MGA, PMR, HCMP, HA, EOH, dysmorphism, leuko-encephalopathy, persistent PAH	[108, 109]
	c.317−2A>G/c.251delC	Hypoglycemic seizures, epilepsy	[107]
	c.317−2A>G/c.470T>A	n.a.	[107]
	c.359delC	n.a.	[107]

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.

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).

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 described in 48 patients [16, 103-110] and other cases are known but have not been officially 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, mostly in the first months of life. On the other hand some patients can survive significantly longer (up to 18 years). If the patient survives the critical postnatal period, the metabolic problems and cardiac disorders may improve [110].

Several other homozygous mutations may result in the putative aberrant TMEM70 protein: *c.316+1G>T* splicing site mutation, *g.2436–3789* in-frame deletion, frameshift *c.578_579delCA* deletion, nonsense *c.336T>A* mutation, *c.211–450_317–568del* frameshift mutation and nonsense mutation *c.238C>T*. For references see Table 2.

The common *c.317–2A>G* mutation can also be found as compound heterozygous in combination with *c.118_119insGT* or *c.349_352delC* frameshift mutations or missense mutations *c.494G>A*, *c.628A>G* or *c.783A>G*.

Only two homozygous missense mutations were described so far: *c.535C>T* mutation, changes the highly conserved tyrosine to a histidine at position 179 at the beginning of the C-terminus while *c.701A>C* mutation replaces conserved histidine 234 with proline. Both patients presented with cardio-myopathy, hypotonia and metabolic crisis, and with less frequent arterial hypertension.

Although the changes in the quantity and structure of TMEM70 protein were not addressed in most of the case reports, 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 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 all known missense mutations affect the C-terminal region of the protein.

Mutations in structural subunits of ATP synthase α and ε

The first "direct hit" nuclear mutation affecting structural subunit of ATP synthase was found in 22 years old patient. Early-onset lactic acidosis and 3-MGA without cardiac involvement was 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 [102]. In patient fibroblasts an insufficient capacity of ATP synthase was observed. 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 ATP synthase 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 [117] a similar phenotype was found. Changes in ATP synthase structure and function due to the mutation and downregulation of ε subunit thus 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 presenting with severe neonatal encephalopathy [101]. 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.

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. ATP synthase disorders thus can be caused by mutations in mtDNA or in nuclear genes.

The thesis was focussed on (i) elucidation of 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, and (ii) further characterization of basic mechanisms and components ATP synthase biogenesis, de-novo synthesis and enzyme assembly with the help of derived 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 affecting expression of ATP6 subunit of ATP synthase and subunit Cox3 of cytochrome *c* oxidase.

3. Summary of the results

This thesis consists of 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 publication in which nuclear genetic defects of mitochondrial ATP synthase are summarised. In the last and the most recent publication 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, <u>Kateřina Hejzlarová,</u> Wolfgang Sperl, Jiří Zeman, Josef Houštěk and Stanislav Kmoch. *Nature Genetics*, 2008, 40:1288–1290. **IF = 30.259**

The first publication desribes 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 recognized 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 uniquely 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 domain. 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, yet 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, <u>Kateřina Hejzlarová</u>, 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 providing system. As expected, individual ATP synthase subunits and fully assembled ATP synthase complexes were drastically reduced to 82–89 % of control. In contrast, complex III was significantly increased about 22–53 % and complex IV about 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*, <u>Kateřina Hejzlarová</u>*, 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**

* Equal contribution

Although the number of patients with different *TMEM70* mutations further increases, the proper mechanism of its function remains unclear. In this study we proceeded with the characterisation of TMEM70 protein structure, which could bring more light into 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 the fluorescence of GFP, under conditions when the reagent can reach the intermembrane space or 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**

In this review article, genetic bases of isolated defects of ATP synthase 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 interest is focused on the nuclear DNA mutations. There are only three other nDNA encoded proteins besides TMEM70 that associate with mitochondrial diseases 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 pictures and the severity of the disease in the individual patients are different, there are some phenotypes more frequent than others. These are metabolic distress represented by hyperammonemia, lactid 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.** <u>Kateřina Hejzlarová,</u> 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**

In the last publication the consequences of mtDNA *m.9205delTA* mutation in *MT-ATP8/MT-ATP6/MT-CO3* gene was studied. This very rare mutation was found in two unrelated patients with very distinct severity of the disease course. 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 results 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 localized 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 involved in the mechanism of TMEM70 function, the protein may operate 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.
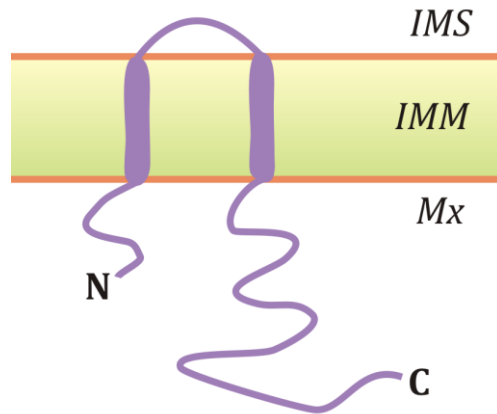
3. Distinct phenotypes of two patients with mtDNA *m.9205delTA* mutation affecting synthesis of subunits *a* and Cox3 and thus the function of ATP synthase and COX are explained by different mutation load, with steep decline of structural and functional changes close to mutation homoplasmy. Threshold 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 characterization 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. Mechanismus 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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* Equal contribution

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