

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 encephalomyopathies. 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_0 -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_0 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_0 moiety. The F_0 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_0 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).

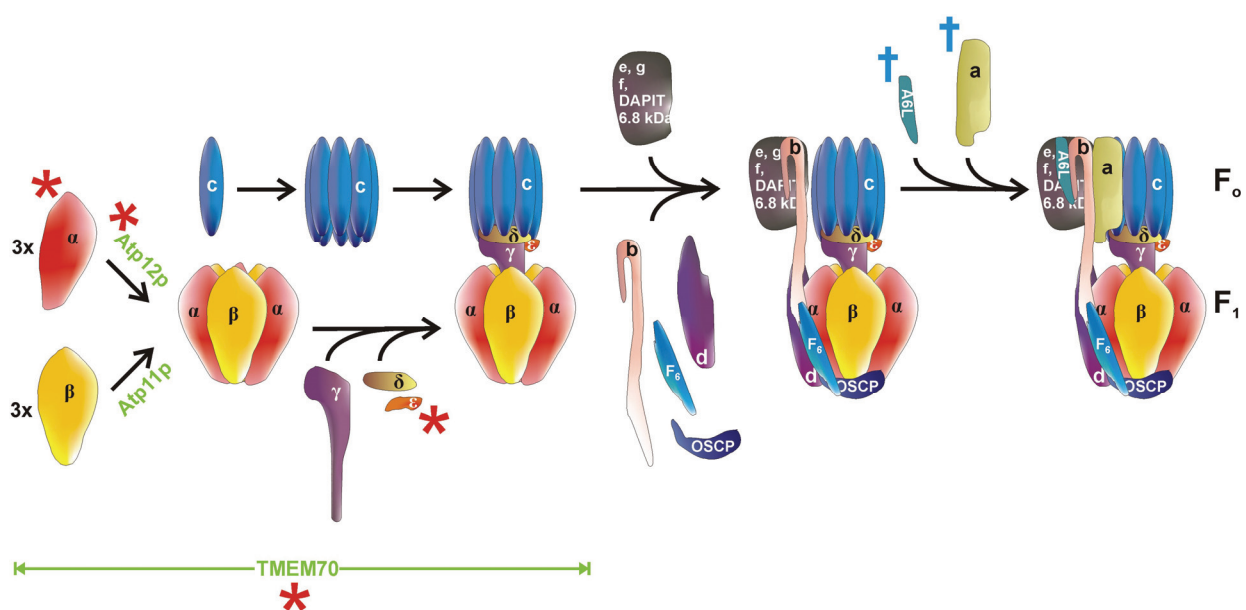


Fig. 1. Assembly scheme of mammalian ATP synthase. ATP synthase assembly starts with the $\alpha_3\beta_3$ hexamer formation, then central stalk subunits γ , δ and ϵ are added. The newly formed F_1 part is connected with the c-ring (F_1+c intermediate) and the assembly follows with subsequent addition of the F_0 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 F₁ and oligomer of c-subunits. After the F₁ attaches to the membrane-embedded c-ring, the subunits of peripheral arm (consisting of subunits b, d, F₆ 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 F₀ 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 F₁ 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* F₀ 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 F₀ 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_0 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 F₁-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 F₁ containing subcomplexes was discovered. Significantly reduced content of individual ATP synthase subunits suggested that F₁ 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 F₁ 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.

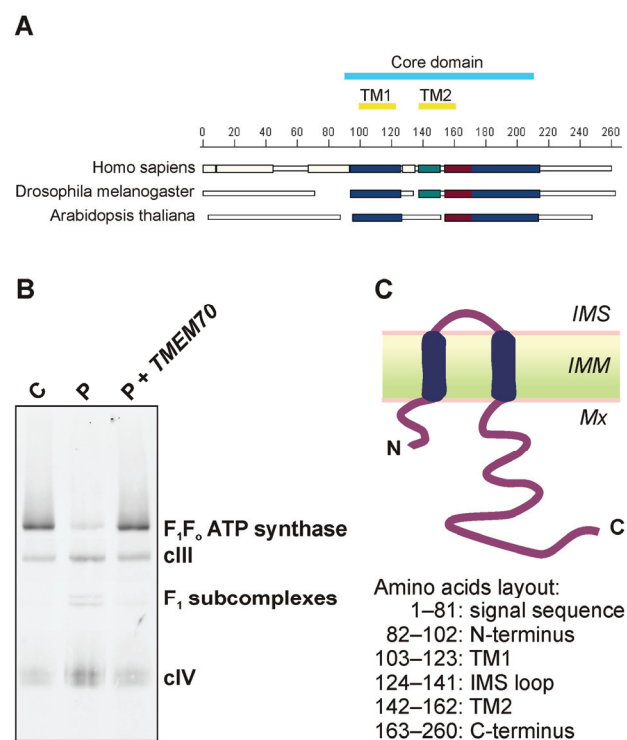


Fig. 2. Structure of TMEM70 protein and *TMEM70* complementation of ATP synthase deficiency. **A.** Sequence alignment of TMEM70 protein. Color of the boxes indicates the level of homology between different organisms (red, blue, green, and white; from the most homologous to the least, respectively). TM1, TM2 denote putative transmembrane regions. Core domain represents central, the most homologous part of the protein. **B.** Decrease of assembled ATP synthase (F₁F₀ ATP synthase) and accumulation of F₁ 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 F₁ 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	<i>c.985C>T</i> missense (p.R329C) (<i>c.-49+418C>T</i> substitution)	Severe neonatal encephalopathy	(Jonckheere <i>et al.</i> 2013)
ATP5E	<i>c.35A>G</i> missense (p.Y12C)	Neonatal respiratory distress, LA, 3-MGA, severe peripheral neuropathy, exercise intolerance	(Mayr <i>et al.</i> 2010)
ATPAF2 (ATP12)	<i>c.280T>A</i> missense (p.W94R)	3-MGA, LA, neonatal encephalopathy, dysmorphism	(De Meirleir <i>et al.</i> 2004)
TMEM70	<i>c.317-2A>G</i> splicing	IUGR, neonatal LA, EOH, FD, HCMP, 3-MGA, cataract, encephalopathy, FTT, PMR	(Cizkova <i>et al.</i> 2008, Wortmann <i>et al.</i> 2009, Tort <i>et al.</i> 2011, Toraco <i>et al.</i> 2012, Stojanovic and Doronjski 2013)
	<i>c.317-2A>G/c.118_119insGT</i> frameshift <i>c.317-2A>G/c.494G>A</i> missense (p.G165D)	LA, 3-MGA, HCMP, FD, PMR LA, 3-MGA, HCMP, Reye-like syndrome, exercise intolerance	(Cizkova <i>et al.</i> 2008, Cameron <i>et al.</i> 2011) (Shchelochkov <i>et al.</i> 2010)
	<i>c.336T>A</i> frameshift (p.Y112X) <i>c.316+1G>T</i> splicing <i>c.238C>T</i> frameshift (p.R80X) <i>c.578_579delCA</i> frameshift (p.N198X)	IUGR, LA, HCMP, FD, PMR IUGR, encephalopathy, HCMP, EOH, LA, FD IUGR, EOH, LA, 3-MGA, multiorgan failure IUGR, EOH, LA, 3-MGA, FD, cataract, encephalopathy, HCMP, PMR	(Spiegel <i>et al.</i> 2011) (Spiegel <i>et al.</i> 2011) (Spiegel <i>et al.</i> 2011) (Spiegel <i>et al.</i> 2011)
	<i>c.211-450_317-568del</i> (2290bp deletion) frameshift <i>g.2436-3789</i> in-frame deletion (1353bp) <i>c.317-2A>G/c.628A>C</i> missense (p.T210P)	IUGR, HCMP, LA, 3-MGA, PMR IUGR, LA, HCMP, PMR, ptosis HCMP, LA, 3-MGA, arterial pulmonary hypertension, WPW	(Tort <i>et al.</i> 2011)
	<i>c.535C>T</i> missense (p.Y179H)	IUGR, LA, EOH, FD, HCMP, bilateral cataract, PMR	(Jonckheere <i>et al.</i> 2011) (Toraco <i>et al.</i> 2012) (Atay <i>et al.</i> 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 threonine 210 to proline (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 (Mattiuzzi *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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