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Mitochondrial nucleic acid binding proteins associated with diseases

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1. ABSTRACT

Mammalian mitochondrial DNA (mtDNA) exists in structures called nucleoids, which correspond to the configuration of nuclear DNA. Mitochondrial transcription factor A (TFAM), first cloned as an mtDNA transcription factor, is critical for packaging and maintaining mtDNA. To investigate functional aspects of TFAM, we identified many RNA-binding proteins as candidate TFAM interactors, including ERAL1 and p32. In this review, we first describe the functions of TFAM, replication proteins such as polymerase γ and Twinkle, and mitochondrial RNA binding proteins. We describe the role of mitochondrial nucleic acid binding proteins within the mitochondrial matrix and two oxidative phosphorylation-related proteins within the mitochondrial intermembrane space. We then discuss how mitochondrial dysfunction is related to several diseases, including mitochondrial respiratory disease, Miller syndrome and cancer. We also describe p32 knockout mice, which are embryonic lethal and exhibit respiratory chain defects. Miller syndrome is a recessive disorder characterized by postaxial acrofacial dysostosis and caused by a mutation in *DHODH*. Finally, we explain that p32 and mitochondrial creatine kinase may be novel markers for the progression of prostate cancer.

2. INTRODUCTION

Mitochondria are responsible for the generation of ATP through oxidative phosphorylation (OXPHOS) and also play vital roles in beta-oxidation, Ca^{2+} buffering, apoptosis, and reactive oxygen species (ROS) production (1). The circular 16.5. kb human mtDNA molecule encodes two rRNAs, 22 tRNAs and 13 proteins of the OXPHOS system. Because ATP production depends on the respiratory chain, maintenance of the mitochondrial genome is critical to normal health. The two rRNAs and 22 tRNAs, used for constructing mitochondrial translational machineries, are also essential for synthesis of the proteins encoded by mtDNA (2, 3). Synthesis of these 13 proteins is carried out with a specialized translation apparatus located in the mitochondrial matrix: the mammalian mitoribosome. This is a 55S particle composed of small (28S) and large (39S) subunits and contains many more proteins and RNAs than the bacterial 70S ribosome (4).

Mitochondrial dysfunction caused by damage and mutagenesis of mtDNA and deregulation of its expression has been increasingly implicated in human disease, aging and age-related pathology. TFAM, cloned as a transcription factor for mtDNA (5), is essential for mtDNA maintenance. It may be a primary factor in the packaging of mtDNA into nucleoids (6). Nucleoids also include RNA and RNA: DNA hybrids and are associated with proteins involved in replication, RNA processing and mitochondrial ribosome biogenesis. A direct link between mitochondrial transcription and translation was demonstrated in mammalian cells; therefore,

mitochondrial DNA/RNA binding proteins are essential for mitochondrial maintenance and homeostasis. In this review, we first discuss TFAM, mtDNA replication proteins and also TFAM associated proteins, which are involved in translation within mitochondria. We then discuss mitochondrial dysfunction involved in cancer development, with an emphasis on cancer metabolism, which places demands on mitochondrial function.

3. ROLES OF MITOCHONDRIAL NUCLEIC ACID BINDING PROTEINS IN DISEASE

3.1. TFAM

TFAM is a nucleus-encoded protein that binds two major promoters of mtDNA and promotes mtDNA transcription (5). TFAM also binds to branched DNA structures with high affinity, probably because of its general properties as a high mobility group (HMG) protein family member (6). It is present at a ratio of about 1000 TFAM molecules per mtDNA molecule (7). This suggests that TFAM is essential for mtDNA packaging and maintenance and also plays a crucial role in its transcription. Expression of exogenous TFAM leads to increased levels of mtDNA, whereas mtDNA content is gradually decreased by siRNA-mediated TFAM suppression (8, 9).

In crystal structure analyses, TFAM binds to heavy chain specific promoter 1 (HSP1) and to nonspecific DNA. In both cases, TFAM similarly distorted the DNA into a U-turn. Moreover, the crystal structures revealed dimerization of DNA-bound TFAM. This dimerization enabled DNA bending and appeared to be important for DNA compaction. Ngo *et al* proposed that TFAM dimerization enhances mitochondrial DNA compaction by promoting DNA looping (10, 11).

Reduction of TFAM levels leads to reduced heart function and accelerated aging (12, 13). Moreover, depletion of mtDNA and reduction of TFAM-dependent mtDNA expression are believed to be involved in infantile mitochondrial myopathy, Parkinson's disease and Alzheimer's disease (14). The tissue-specific regulation of mitochondrial biogenesis in aging and calorie restriction highlights the importance of modulating TFAM–mtDNA interactions (15). Specific inactivation of *Tfam* in mouse skeletal muscle results in myopathy with ragged-red fibers, and a progressive decrease of OXPHOS function (16). Deletion of *Tfam* in mouse pancreatic β-cells leads to diabetes at 5 weeks and severe mtDNA depletion and OXPHOS deficiency at 7 to 9 weeks, leading to decreased insulin release and reduced β-cell mass (17). These results suggest that TFAM is essential for mtDNA maintenance in mammalian cells.

Recently, transgenic mice were generated that over-expressed human TFAM under control of the beta-actin promoter (TFAM Tg). These mice show an increased mtDNA copy number in the myocardium and their hearts were protected from mitochondrial dysfunction (18). In another report, over-expression of TFAM or Twinkle increased mtDNA copy number and facilitated cardioprotection associated with limited mitochondrial oxidative stress (19). Furthermore, TFAM Tg mice exhibit prominent amelioration of age-associated effects, including accumulation of lipid peroxidation products and a decline in complex I and IV activities in the brain. In aged TFAM Tg mice, deficits in motor learning memory, working memory and hippocampal long-term potentiation are also significantly less than in wild-type mice (20). Over-expression of TFAM ameliorates the delayed neuronal death caused by transient forebrain ischemia in mice (21).

Oxidative stress-induced mitochondrial dysfunction and mtDNA damage in peripheral neurons are considered to be important in the development of diabetic neuropathy. TFAM Tg mice are resistant to diabetic neuropathy and, compared with wild-type mice, show a reduced mtDNA copy number when induced with diabetes. These findings suggest that TFAM activation is a potential therapeutic strategy for peripheral neuropathy (22). Experiments in TFAM Tg mice demonstrated that TFAM might maintain mtDNA integrity and protect it from oxidative stress.

3.2. POLG

POLG (mitochondrial DNA polymerase γ), Twinkle (a DNA helicase), mtSSB (mitochondrial single-stranded DNA-binding protein) are essential for mitochondrial replication processes. Twinkle unwinds double-stranded DNA into single-stranded DNA (ssDNA) and then POLG synthesizes DNA using ssDNA. mtSSB binds the ssDNA, protecting it from nucleases (23, 24). During replication, the POLG complex comprises a catalytic subunit (POLG) and an accessory subunit, POLG2, which anchors POLG to mtDNA. POLG contains the DNA polymerase active site and the 3′-5′ exonuclease for proofreading, whereas POLG 2 facilitates DNA synthesis by improving ground state nucleotide binding (25).

More than 200 mutations have been reported in the POLG gene. Mutations S305R and P1073L are associated with childhood Alpers syndrome and Y955C is associated with progressive external ophthalmoplegia (PEO) (26). Meanwhile, POLG D181A induces an accumulation of mtDNA point mutations and large-scale deletions (27). Homozygous *Polg* knockout mice die *in utero* due to an almost complete absence of mtDNA, indicating that DNA-polymerase activity is essential for mtDNA replication during embryonic development (28). Transgenic mice expressing the human POLG Y955C mutant die prematurely with massive cardiomegaly (26). These mutations affect DNA-polymerase activity by causing stalling.

Mutations in POLG2, which are not as predominant as mutations in POLG, have been described in patients with autosomal dominant progressive external ophthalmoplegia (adPEO) (29). However, homozygous deletion of *Polg2* in mice results in embryonic lethality with loss of mtDNA and mtDNA-encoded proteins (30).

3.3. Twinkle

Twinkle is a mitochondrial hexameric helicase belonging to the RecA superfamily that forms, along with POLG and mtSSB, the minimal mtDNA replisome (24). Mutations in the Twinkle gene are associated with adPEO (31), mtDNA depletion syndrome (MDS) (32) and with recessively inherited infantile onset spinocerebellar ataxia (IOSCA) (33). The common feature of all these diseases is the presence of mtDNA depletion or deletions caused by an alteration of the replication machinery.

Homozygous depletion of Twinkle in mice causes embryonic lethality indicating an essential role in embryonic development (34). Two transgenic mouse lines over-expressing different mutant forms of Twinkle (Twinkle A360T and Twinkledup353–365) have been developed. Both mutant mice develop the same pathological phenotype in adulthood. Multiple deletions, mtDNA depletion and COX-negative cells are also observed in the brain of 18-month-old animals (35).

3.4. ERAL1

The mammalian 55S mitoribosome differs substantially from its 70S and 80S counterparts found in prokaryotes and the eukaryotic cytosol, respectively (36, 37). Protein synthesis is performed by the mitoribosome; the formation of functional small and large ribosomal subunits is necessary for mitochondrial translation and might be involved in mitochondrial biogenesis, rRNA folding and RNA modification. We previously found that ERAL1, the human ortholog of Era (*E. coli* Ras-like protein), associates with mitochondrial TFAM (8). We also observed that ERAL1 localizes to the mitochondrial matrix and associates with mitoribosomal components, including 12S rRNA. RNAi-mediated knockdown of ERAL1 decreases mitochondrial translation, causes redistribution of ribosomal small subunits and reduces the levels of 12S rRNA (38). Dennerlein *et al*. (39) demonstrated ERAL1 binding *in vivo* to the rRNA component of the small mitoribosomal subunit and mapped the ERAL1-binding site to a 33-nucleotide section delineating the 3′ terminal stem–loop region of the 12S rRNA. This loop contains two adenine residues that are dimethylated on mitoribosome maturation. These two reports suggested that ERAL1 is localized in the small subunit of the mitochondrial ribosome, is an important constituent of this subunit and is also involved in cell viability. siRNA-mediated knockdown of ERAL1 inhibits mitochondrial protein synthesis and promotes ROS generation, leading to autophagic vacuolization in HeLa cells. Upregulation of tumor protein p53 is required for this autophagy induction (40).

We reported that ERAL1-interacting proteins are associated with human mitochondrial nucleoids (38). These results raised the possibility that, also in higher eukaryotes, mitoribosomal proteins and their associated factors are coupled with transcription *via* transcription-related factors, such as TFAM. Recently, Wang and Bogenhagen reported EF-Tu, LRP130, ATAD3 and DHX30 as TFAM- and mtSSB-associated proteins (41). Thus, nucleoids harbor proteins related to translation in addition to those involved in transcription, replication and mtDNA maintenance.

3.5. LRPPRC and RNaseP

LRPPRC (LRP130), a yeast translational activator of Pet309 (42), co-immunoprecipitates with TFAM and ERAL1. LRPPRC contains PPR motifs that consist of degenerate sequences of 35 amino acids and form antiparallel alpha helices. PPR motif proteins are thought to be involved in post-translational mRNA metabolism, especially in mitochondria (43). Mutations in LRPPRC were described in French-Canadian individuals with Leigh syndrome harboring an isolated COX deficiency (44-46). LRPPRC knockdown in mammalian cells leads to an imbalance between mitochondrial-encoded and nuclear-encoded subunits of complex IV and this imbalance triggers the mitochondrial unfolded protein response (UPRmt). Mitochondrial proteostasis is not only critical in pathophysiology but also during aging, as proteotoxic stress has been shown to increase with age. It has been speculated that the coordination of these two mitochondrial stress responses plays a more global role in mitochondrial proteostasis (47). SLIRP, a stem-loop RNA-binding protein, was found to be completely dependent on LRPPRC for its stability; however, reduced levels of LRPPRC persist in the absence of SLIRP *in vivo*. *Slirp* knockout mice are apparently healthy and display only a minor weight loss, despite a 50–70 percent reduction in their steady-state levels of mtDNA-encoded mRNAs. SLIRP is required for proper association of mRNAs with the mitochondrial ribosome and efficient translation (48).

It is generally accepted that tRNAs are excised from long polycistronic primary transcripts to generate mature mRNAs and rRNAs; this is called the tRNA punctuation model (49-51). This processing of tRNAs requires at least two endonuclease activities, RNase P and tRNase Z, which cleave the 5′ and 3′ termini, respectively. The mitochondrial RNase P comprises only three proteins (MRPP1–3) that form the enzyme complex. MRPP1 (RG9MTD1) is homologous to a tRNA m^1G methyltransferase responsible for modification of eukaryotic tRNAs (52, 53). SDR5C1 (MRPP2) is an amino and fatty acid dehydrogenase/reductase, apparently with a second function as a component of human mitochondrial RNase P. Missense mutations in SDR5C1 cause a disease characterized by progressive neurodegeneration and cardiomyopathy, called HSD10

disease. Structural and functional alterations of SDR5C1 are proposed to impair mitochondrial RNA processing and modification, leading to the mitochondrial dysfunction observed in HSD10 patients (54, 55). The third member of the mitochondrial RNase P complex, MRPP3, contains a PPR domain often found in mitochondrial RNA-binding proteins. In crystal structure analysis, MRPP3 exhibits an auto-inhibitory conformation in which metal ions essential for catalysis are excluded from the active site (56).

3.6. RNA binding protein p32

The p32 (C1QBP/gC1qR/HABP1) protein is closely localized with nucleoids and is abundant in the mitochondrial matrix. The p32 protein was first isolated from a membrane preparation of Raji cells and was co-purified with the pre-mRNA splicing factor SF2/ASF in human HeLa cells (57-59). It is a doughnut-shaped trimer primarily localized in mitochondria (60-63). Human p32 interacts with a variety of molecules, suggesting that p32 may be a multifunctional chaperone protein (64). The p32 protein is also a novel target for viral protein ICP34.5. of herpes simplex virus type 1 and facilitates viral nuclear egress (65).

An important question is what is the primary function of p32 in the mitochondrial matrix? Compared with wild-type, p32-knockdown cells exhibit reduced synthesis of the mtDNA-encoded OXPHOS proteins and were less tumorigenic *in vivo* (66). These investigators suggested that p32 is involved in mitochondrial translation and that p32 depletion impairs mitochondrial respiratory activity. Similarly, *mam33*, the *Saccharomyces cerevisiae* homolog of the human *p32* gene, localizes to the mitochondrial matrix. Disruption of the *mam33* gene causes growth retardation and impairment of mitochondrial ATP synthesis (57). Taken together, this evidence suggests that both yeast mam33 and human p32 play important roles in maintaining mitochondrial OXPHOS.

It was also proposed that p32 provides a link to autophagy (67, 68). Silencing p32 profoundly impairs starvation-induced autophagic flux and clearance of damaged mitochondria caused by a mitochondrial uncoupler. These findings support a cytoprotective role for p32 under starvation conditions by regulation of ULK1 stability, and reveal a crucial involvement of the p32-ULK1-autophagy axis in coordinating the stress response, cell survival and mitochondrial homeostasis (69). Mammalian RNase H1 is implicated in mitochondrial DNA replication and RNA processing and is required for embryonic development. The p32 protein binds specifically to human RNase H1, but not to human RNase H2. The p32-RNase H1 complex physically interacts with mitochondrial DNA and pre-rRNA, suggesting that p32 might enhance the enzymatic efficiency of human RNase H1 (70).

4. MITOCHONDRIAL DYSFUNCTION AND DISEASE

4.1. Knockout mice for *p32* and *C1qbp*

We established *p32* knockout mice to identify the function of p32 in mitochondria. The p32 deficient mice exhibit mid-gestation lethality associated with a severe developmental defect of the embryo. Primary embryonic fibroblasts isolated from p32-knockout embryos have a severely dysfunctional mitochondrial respiratory chain, caused by highly impaired mitochondrial protein synthesis (71). Recombinant p32 binds RNA, not DNA, and endogenous p32 interacts with all mitochondrial mRNA species *in vivo*. The RNA-binding ability of p32 has been well correlated with mitochondrial translation. Based on co-immunoprecipitation experiments, p32 also associates with the mitochondrial ribosome and various RNA binding proteins. We found that p32 is required for functional mitoribosome formation to synthesize proteins within mitochondria (71) (Figure 1).

Recently *p32* and *C1QBP* mutations were found in a patient with mitochondrial respiratory disease; combined respiratory chain deficiencies (I, III, and IV). This patient harbored compound heterozygous variants in *p32* and *C1QBP* genes. Though there was no direct evidence supporting the pathogenesis of the compound heterozygous variants in the patient, the investigators suspected the *p32*/*C1QBP* mutations as causing the mitochondrial respiratory chain deficiency (72). Thus we have proposed a new mechanism for mitochondrial translation, with p32 working as an RNA and protein chaperone (Figure 1). p32 also makes critical contributions to the morphology of mitochondria and the ER under normal conditions, as well as being important for cellular metabolism and various stress responses (73).

4.2. DHODH and miller syndrome

The enzyme dihydroorotate dehydrogenase (DHODH) catalyzes the fourth step in the *de novo* biosynthesis of pyrimidine by converting dihydroorotate (DHO) to orotate (74, 75). DHODH is also the only enzyme of this pyrimidine biosynthesis pathway that is located on the inner membrane of mitochondria; all the others are located in the cytosol. DHODH relies on ubiquinone to form a functional link between the mitochondrial respiratory chain and pyrimidine biosynthesis. (Figure 2).

Miller syndrome is a recessive inherited disorder characterized by postaxial acrofacial dysostosis (76, 77). It is caused by dysfunction of the *DHODH* gene (78, 79). We investigated the consequences of three missense mutations of *DHODH*, G202A, R346W and R135C, all previously identified in patients with Miller syndrome. The three mutant proteins retained the proper mitochondrial localization. We found that the G202A and R346W mutations cause deficient protein stability. The R135C mutation does not affect stability but impairs substrate-induced enzymatic

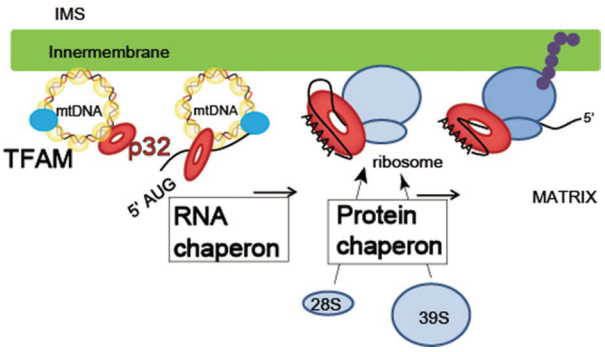

Figure 1. p32 acts as RNA and protein chaperone. TFAM and p32 are shown as a closed blue circle and a red ring, respectively. Endogenous p32 interacts with all mitochondrial mRNA species *in vivo* and is closely associated with the mitoribosome. Translation of mitochondrial mRNAs is performed by mitoribosomes, which sediment as 55S particles and dissociate into 28S and 39S subunits. Thus, we propose that p32 is required for functional mitoribosome formation to synthesize proteins within mitochondria.

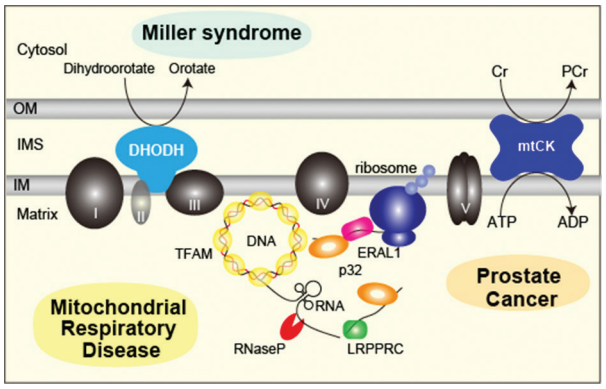

Figure 2. Mitochondrial DNA/RNA binding proteins and related diseases. TFAM is shown as a closed hexagon. As much mtDNA exists as can be bound up with TFAM, which means that TFAM titrates the mtDNA copy number. The existence of an inner membrane protein integrated with mitochondrial DNA has been speculated. mtDNA is almost fully transcribed on both strands, producing polycistronic RNA units that are co-transcriptionally processed. tRNAs require endonuclease activity of RNase P, and mature into three classes: mt-rRNAs, mt-tRNAs, and mt-mRNAs. Several mitochondrial proteins (including LRPPRC and p32) are capable of binding mt-mRNA. ERAL1 is localized in the small subunit of the mitoribosome and is an important constituent of this structure. p32 is also associated with the mitoribosome and regulates mitochondrial translation. mtCK and DHODH are localized in the intermembrane space and are physically or functionally associated with OXPHOS proteins.

activity, suggesting that impairment of DHODH activity is linked to the Miller syndrome phenotype (80, 81). We also observed that *DHODH* knockdown induces cell growth retardation by G2/M cell cycle arrest. DHODH depletion partially inhibits respiratory chain complex III activity and increases generation of ROS. DHODH physically interacts with respiratory complexes II and III. Considering that pyrimidine deficiency alone does not induce craniofacial dysmorphism, the *DHODH* mutations may contribute to the Miller syndrome in part through somehow altering mitochondrial function (82).

4.3. Involvement of p32 and mtCK in cancer

The Warburg effect represents a prominent metabolic characteristic of malignant cells. A phenomenon first described by Otto Warburg, the Warburg effect is a metabolic phenotype characterized by a shift from OXPHOS to aerobic glycolysis as the main source of ATP production (83). There are several reports on the correlation between p32 expression and cancer progression. Chen *et al*. reported that p32 might be an independent predictive factor for breast cancer prognosis and that its upregulation might play an important role in metastasis of breast cancer (84) (Figure 2).

We found that p32 is highly expressed in prostate tumor samples and its expression level correlates significantly with the Gleason score, pathological stage and relapse (85). We also observed that p32 is over-expressed in several prostate cancer cell lines and that siRNA-mediated p32 knockdown inhibits the growth of prostate cancer, but not non-cancerous, cells (85). Ghosh *et al*. reported that p32 accumulates in inflammatory subsquamous tissue during tumor initiation and is over-expressed during tumor progression in papillomatous and acanthotic tissues (86). A possible tumor suppressor role for p32 was initially proposed, but its over-expression was reported in tissue samples from several cancers, including breast cancer (84, 87). Fogal *et al*. demonstrated that cells with p32 knocked down produce smaller tumors than control cells, suggesting that p32 is involved in tumorigenesis *in vivo*. The p32 gene is a direct transcriptional target of Myc and high levels of Myc in malignant brain cancers correlate with high p32 expression. Loss of p32 in glutamine addicted glioma cells induces resistance to glutamine deprivation and imparts sensitivity to glucose withdrawal. These findings suggest that Myc promotes p32 expression, which is required to maintain sufficient respiratory capacity to sustain glutamine metabolism in Myc transformed cells (88). These observations also suggest that high expression of p32 might play a key role in tumorigenesis in several cancers.

Creatine kinase (CK) isoenzymes catalyze the reversible transfer of phosphate groups from phosphocreatine to ADP, to yield ATP and creatine. There are two forms of mitochondrial CKs (MtCKs), sarcomeric MtCK (sMtCK) and ubiquitous MtCK (uMtCK) (89). uMtCK is co-expressed with cytosolic brain-type subunits in many cells and tissues with high energy demand (90) and is a central controller of cellular energy homeostasis (91). Notably, over-expression of MtCK has been reported in tumors (92, 93). We found that MtCK expression is significantly lower in high Gleason grade carcinomas compared with normal prostate or low-grade carcinomas. We also observed that uMtCK expression is almost absent in highly malignant cell lines. Moreover, in low uMtCK expressing cell lines, glycolytic ATP production is increased, whereas mitochondrial ATP production is decreased. This suggests that uMtCK is down-regulated

as prostate cancer progresses, in correlation with a metabolic switch in ATP usage (94).

It is now recognized that the Warburg effect represents a prominent metabolic characteristic of malignant cells. Though Warburg speculated that mitochondrial respiration is decreased in cancer cells, mitochondrial activity is essential for cancer cell survival. In fact, cancer uses a number of essential metabolic processes, such as the oxygen-dependent ATP production, redox regulation and biosynthetic reactions. Moreover, mitochondria are involved in the regulation of hypoxic adaptation, a process that must be triggered in any solid neoplasia to overcome the initial steps of tumorigenesis regulated by hypoxia-inducible factor 1α (95). Mitochondria also participate in many essential and fundamental metabolic pathways in addition to respiration (96).

5. SUMMARY AND FUTURE PERSPECTIVES

There have been many recent breakthroughs in the identification of factors required for human mitochondrial gene expression and translation. mtDNA decline and mitochondrial defects are now well known to be associated with a variety of diseases, including neurodegenerative diseases, inherited abnormalities, diabetes mellitus and cancer, as well as with normal aging. We propose that the protection of mtDNA integrity in somatic cells is very important for maintaining proper cellular function. TFAM is a key factor for mtDNA maintenance and, with further knowledge of its mechanisms of action it may eventually be possible to use this protein to develop novel strategies for the treatment of such diseases.

Understanding the mechanisms of regulation of mitochondrial RNA processing and translation is also very important. In our own research, we have focused on the mitochondrial nucleic acid binding proteins, p32, ERAL1, LRPPRC and RNaseP, which associate with TFAM. Here, we have discussed these mitochondrial proteins and relevant mitochondrial diseases, including cancer, in which the mitochondria are central to energy homeostasis.

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