

Institute of Physiology of the Czech Academy of Sciences

Department of Membrane Transport Biophysics

Faculty of Science, Charles University in Prague

Department of Physiology



**Quantitative aspects of mitochondrial genome in cancer
and diabetes**

**Kvantitativní aspekty mitochondriálního genomu v
diabetu a rakovině**

PhD Thesis

Mgr. Lukáš Alán

Supervisor: RNDr. Petr Ježek, DrSc

Prague 2015

Statement of authorship

I hereby declare that I completed this PhD thesis independently, except where explicitly indicated otherwise, and that this manuscript has not been submitted for application to any other academical degree. It documents my own work, carried out under the supervision of RNDr. Petr Ježek, DrSc. My participation is specified at the end of the comments to each paper.

In Prague

M.Sc. Lukáš Alán

Statement of co-authors

I certify that Lukáš Alán substantially contributed to the formation of the papers used as a basis of this thesis, and that his participation specified at the end of the comments to each paper is correct.

In Prague

RNDr. Petr Ježek, DrSc.

Acknowledgement

I would like to express my sincere thanks to my supervisor RNDr. Petr Ježek, DrSc., for the scientific and financial support throughout my PhD studies, to all the friends and colleagues from the Department of Membrane Transport Biophysics for their help and goodwill and the collaborators and co-authors of our publications as well. Special thanks go to Ing. Jaroslav Zelenka PhD for his great thoughts, enthusiastic approach and fruitful scientific discussions.

I would like to thank also to my family, friends and mainly to my wife Petra, for her never ending support.

Abstract

Mitochondria are cellular powerhouses and physiological regulators with many features resembling their prokaryotic ancestors. They maintain their own mitochondrial DNA (mtDNA) encoding 13 inner mitochondrial membrane proteins of oxidative phosphorylation machinery, 22 transfer RNAs and two ribosomal RNAs. Healthy mitochondria in normal cells form a dynamic network consisting of highly interconnected tubules. The mitochondrial disorders are very heterogeneous and difficult to diagnose and cure. It is due to combining products of two genomes, the complexity of mitochondrial structure and due to insufficiency of current state of knowledge.

To understand the mitochondrial nucleic acid species distribution, we have developed and established new techniques to visualize mitochondrial network, nucleoids and different RNA species together with qPCR techniques for monitoring the mitochondrial intactness. We determined nucleoid distribution and mtDNA amount following rotenone mediated respiratory inhibition or following degeneration of the electrical component of the protonmotive force by valinomycin treatment. Native mitochondria were mostly tubular with average nucleoid spacing $1.1 \pm 0.2 \mu\text{m}$ which we termed as a nucleoid code. Subsequently induced fission resulted in mitochondrial network fragmentation and each mitochondrial fragment contained several nucleoids. Only rarely the fragments contained one nucleoid and barely were empty. It indicates that mitochondrial fission is nucleoid-centric implicating existence of factor connecting nucleoids with fission machinery. As a model of mitochondrial pathology, we employed a model of type 2 diabetes, the Goto Kakizaki rats, where we measured amount of mtDNA in pancreatic β -cells as well as respiration of pancreatic islets. We observed a dramatic decrease in the amount of mtDNA in beta cells of ageing rats and also in livers and heart muscle. Moreover Goto Kakizaki pancreatic islets displayed slightly impaired response to glucose in comparison with Wistar rats. Our findings support the hypothesis, that mitochondria play a key role in the development and progression of type 2 diabetes. Finally, we have developed techniques for monitoring several mitochondrial nucleic acid species for both fluorescence *in situ* or *in vivo* hybridization (FISH or FIVH). Our developed *in situ* and *in vivo* fluorescent hybridization showed mitochondrial mRNAs localization pattern in cancer cells and also pattern of mitochondrial non-coding RNAs. The 5S rRNA enables import of desired small RNA sequence into mitochondria. Both techniques have potential to find an application in diagnostics and in the future can be upgraded for importing larger RNA sequences to compensate point mutations in mtDNA genes or inhibit translation of mutated mRNA.

In conclusion we have established and tested several techniques for monitoring mitochondrial nucleic acid species, which can be potentially used in clinical diagnostics.

List of original publications used for PhD thesis:

A: Alán L, Zelenka J, Ježek J, Dlasková A, Ježek P., Fluorescent in situ hybridization of mitochondrial DNA and RNA.

Acta Biochim Pol. 2010;57(4):403-8. IP: 1,234 (2010)

B: Zelenka J, Alán L, Jabůrek M, Ježek P., Import of desired nucleic acid sequences using addressing motif of mitochondrial ribosomal 5S-rRNA for fluorescent in vivo hybridization of mitochondrial DNA and RNA.

J Bioenerg Biomembr. 2014 Apr;46(2):147-56. IP: 2,7 (2013)

C: Tauber J, Dlasková A, Šantorová J, Smolková K, Alán L, Špaček T, Plecitá-Hlavatá L, Jabůrek M, Ježek P. Distribution of mitochondrial nucleoids upon mitochondrial network fragmentation and network reintegration in HEPG2 cells.

Int J Biochem Cell Biol. 2013 Mar;45(3):593-603. IP: 4,24 (2013)

D: Alán L, Špaček T, Zelenka J, Tauber J, Berková Z, Zacharovová K, Saudek F, Ježek P., Assessment of mitochondrial DNA as an indicator of islet quality: an example in Goto Kakizaki rats.

Transplant Proc. 2011 Nov;43(9):3281-4. IP: 1,005 (2011)

The above papers are included in a full form in this PhD thesis. For the complete list of my published articles, see the List of publications.

Abbreviation list

AP - Apurinic/apyrimidinic site in DNA

ATP, ADP - Adenosine triphosphate, Adenosine diphosphate

BER – Base excision repair

CJ – Cristae junctions

ClpX - Caseinolytic protease X homolog

COSCOFA - Conventional strand-coupled Okazaki fragment associating replication model

CPEO - Chronic progressive external ophthalmoplegia

CS – Contact sites

DAPI - 4',6-diamidin-2-fenylindol

FADH₂ - Flavin adenine dinucleotide (reduced form)

FISH – Fluorescent *in situ* hybridization

FIVH – Fluorescent *in vivo* hybridization

GTP, GDP – Guanosine triphosphate, Guanosine diphosphate

HIF – Hypoxia induced factor

HSP1, HSP2, LSP – Heavy strand promoter 1 and 2, light strand promoter

HSP70 – Heat shock protein 70

IBM – Inner boundary membrane

KSS - Kearns-Sayer syndrome

LHON - Leber's hereditary optic atrophy

MAM – Mitochondria associated membranes

MELAS - Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes

MERRF - Myoclonic epilepsy and ragged red fibers

MICOS – Mitochondrial contact sites

MILS - Maternally inherited Leigh syndrome

MINOS - Mitochondrial inner-membrane organizing system

MITOS - Mitochondrial organizing structure

MMR – Mismatch repair

MPP - Mitochondrial-processing protease

mPTP – Mitochondrial permeability transition pore

mtDNA, ssDNA, dsDNA – mitochondrial, single strand, double strand Deoxyribonucleic acid

mTERF - Mitochondrial transcription termination factor

mtSSB – Mitochondrial single stranded binding protein

NADH - Nicotinamide adenine dinucleotide (reduced form)

NARP - Neuropathy, ataxia, and retinitis pigmentosa syndrome

OXPHOS – Oxidative phosphorylation system

PBS - Phosphate buffered saline

PD – Parkinson disease

PolG – Polymerase gamma

qPCR – Quantitative polymerase chain reaction

RITOLS - Ribonucleotide incorporation throughout the lagging strand

ROS, NOS, RONS – Reactive oxygen and nitrogen species

SIM – Structured Illumination Microscopy

SNARE - SNAP (Soluble NSF attachment protein) receptor

STED - Stimulated Emission Depletion Microscopy

TFAM, TFBM1, TFBM2 – Mitochondrial transcription factor A, B1 or B2

TIM, TOM - Translocase of the inner or outer membrane

TMRE - Tetramethylrhodamine, ethyl ester

mRNA, ncRNA, rRNA, tRNA – Messenger, non – coding, ribosomal or transfer RNA

.

1 Contents

1 Contents.....	1
2 Review of literature	4
2.1 Fundamentals of mitochondrial biology.....	4
2.2 Mitochondrial genetics	5
2.2.1 Composition of mitochondrial nucleoids	6
2.2.2 Nucleoid dynamics	8
2.2.3 Mitochondrial biogenesis	9
2.3. Mitochondrial DNA replication, transcription and repair	9
2.3.1 Proteins involved in mtDNA replication.....	10
2.3.2 Mechanisms of mtDNA replication.....	10
2.3.3 mtDNA copy number	12
2.3.4 mtDNA transcription.....	14
2.3.5 Mitochondrial translation	15
2.3.6 mtDNA repair.....	16
2.3.7 RNA import into mitochondria	18
2.4 Mitochondrial dynamics.....	19
2.4.1 Mitochondrial fusion	20
2.4.2 Mitochondrial fission.....	21
2.4.3 Mitochondrial cristae morphology	23
2.4.4 Mitophagy	24
2.4.5 Mitochondrial – ER crosstalk.....	26
2.5 Mitochondrial pathologies.....	28
2.5.1 Oxidative stress	28
2.5.2 MtDNA mutations	29
2.5.4 Mitochondrial disorders with nuclear origin	31
2.5.3 MtDNA haplogroups and prevalence for disorders.....	32
2.5.5 Aging.....	33

3. Materials and methods.....	35
3.1 Fluorescent protein constructs and fusion protein expression.....	35
3.2 Lentiviral and baculoviral particle production	35
3.3 Molecular beacon design.....	36
3.4 Fluorescent <i>in vivo</i> hybridization (FIVH) probe design.....	37
3.5 Nucleic acid transfections	38
3.6 Cell cultures.....	38
3.7 Immunocytochemistry and cell staining.....	39
3.8 Fluorescence <i>in situ</i> hybridization.....	39
3.9 Confocal microscopy.....	40
3.10 Structured illumination microscopy (SIM).....	40
3.11 Image analysis.....	40
3.12 Pancreatic islet isolation.....	40
3.13 β -Cell isolation	41
3.14 DNA isolation from tissues and cell lines	41
3.15 Mitochondrial copy number	42
3.16 Import verification by qPCR	43
3.17 Agarose electrophoresis and Southern blots.....	43
3.18 Islet respiration measurements	44
3.19 Cellular ATP content.....	44
3.20 Buffers	44
3.21 Instruments	45
4. Aims of the thesis	46
5. Results of selected publications.....	47
5.1 Publication A: Fluorescent <i>in situ</i> hybridization of mitochondrial DNA and RNA.....	47
5.2 Publication B: Import of desired nucleic acid sequences using addressing motif of mitochondrial ribosomal 5S-rRNA for fluorescent <i>in vivo</i> hybridization of mitochondrial DNA and RNA.....	51
5.3 Publication C: Distribution of mitochondrial nucleoids upon mitochondrial network fragmentation and network reintegration in HEPG2 cells.....	55

5.4 Publication D: Assessment of mitochondrial DNA as an indicator of islet quality: an example in Goto Kakizaki rats.....	60
6. Discussion	63
7. Conclusions	66
8. List of all my publications.....	67
9. Reference list.....	68
10. Publications enclosed in full.....	82

2 Review of literature

2.1 Fundamentals of mitochondrial biology

First ancestors of mitochondria appeared approximately two billions years ago. The alphaproteobacterium similar to Rickettsia family with complete oxidative phosphorylation system (OXPHOS) was engulfed by a eukaryotic precursor. Whether the eukaryotic precursor was formed before endosymbiosis (archezoan scenario) or after endosymbiosis during mutual evolution remains to be elucidated (Gray, 2012). Since the single bacterial cell can produce only a limited amount of ATP to maintain the whole genome and integrity of cell wall, they can grow only till a limited size. On the contrary, by acquiring more oxidative bacteria and transferring their genes into the host DNA new eukaryote cells gained a great advantage against contemporary bacteria and archaea. Transferring most of genes to the host nucleus led to the savings in energy and also to tighter integration of protomitochondria and eukaryotic cell (Gray, 2012). Nevertheless, the gene transfer was stopped at the number 13 in mammals. Curiously, this is the minimal number of the OXPHOS polypeptide genes which left in the mtDNA between all fungi and animals. It includes seven subunits of mitochondrial complex I, cytochrome b from complex III, three proteins from complex IV and two subunits of complex V (ATP-synthase). These 13 mitochondrially encoded proteins create an integrated electrical circuit, in which each other polypeptides must be functionally compatible. Such an arrangement prevents any possible leakage of electrons from the electron transport chain and represents the functional unit, which has to be inherited together (Wallace, 2013). Therefore, these 13 remaining proteins can be still found in mtDNA (Wallace, 2013). To further fix the advantage of the functional unit inheritance, higher organisms have developed only uniparental inheritance, which is maternal in case of mammals (Wallace, 2007).

Thus mitochondria are semiautonomous organelles owing DNA and surrounded with two membranes. The outer membrane is permeable for molecules up to 10kDa. Larger proteins must be transported throughout the translocase complexes of the outer and inner membrane (TOM and TIM). The inner membrane is highly folded and forms structures called cristae. Complicated system of membranes is forming many isolated environments, which are used for creating local gradients of electrochemical gradient. Therefore, mitochondria stand in the center of all energetic processes. Pyruvate as an endproduct of glycolysis gets into the mitochondria via pyruvate dehydrogenase and then enters tricarboxylic acid cycle as acetyl-CoA. Acetyl-CoA is also the final product of mitochondrial β -oxidation, together with $\text{NADH} + \text{H}^+$ and FADH_2 , which represents the entry into the respiratory chain. $\text{NADH} + \text{H}^+$ donate two electrons to the Complex I. FADH_2 -containing enzymes such as Complex II transfer electrons to ubiquinone. Complex I also reduces ubiquinone and together with Complex III and IV transfers protons to the intermembrane, mostly intercrystal space. Electrons

from the respiratory chain terminate on complex IV, where they are passed on O₂ to produce H₂O. The resulting electrochemical gradient ($\Delta P = \Delta \Psi + \Delta \mu^{H^+}$) established on the inner mitochondrial membrane is used for various processes, such as production of ATP by the ATP-synthase, ATP, phosphate, metabolite and ion transport, protein import into mitochondrion, Ca²⁺ import, generation of heat, regulation of pH, etc (Wallace, 2007).

Moreover Complexes I and III and several mitochondrial enzymes generate reactive oxygen species (ROS), notably superoxide transformed into H₂O₂ by the matrix MnSOD and the intermembrane space CuZnSOD. The downstream radicals formed can damage lipids, proteins and mtDNA. However, superoxide and H₂O₂ are also important in mitochondrial redox signaling, e.g. in initiating hypoxia inducible factor (HIF) response. It has been hypothesized, that when the mitochondrial damage overcomes a certain threshold mitochondrial permeability transition pore (mtPTP, yet of unknown origin) is activated initiating the programmed cell death (apoptosis). After activation, mtPTP opens a pore between mitochondrial matrix and cytosol and depletes mitochondrial membrane potential. Upon apoptosis, which can be induced by other ways, Cytochrome *c* is released. Presence of the Cytochrome *c* in cytosol leads to the formation of complexes initiating the apoptosis (Wallace, 2007).

2.2 Mitochondrial genetics

Almost 50 years ago, two laboratories independently observed mtDNA by electron microscopy (Nass, 1966; Van Bruggen et al., 1966). They showed 5µm long DNA in mitochondria isolated from mouse or chick cells. Several years later, Satoh and Kuroiwa observed mtDNA under the fluorescent microscope, using longer time of staining with DAPI, usually used only for the nuclear staining (Satoh and Kuroiwa, 1991). Ten years later, mitochondrial nucleoids complexes of mtDNA with maintenance and replication and transcription proteins, were visualized by specific *in situ* protein colocalization by Twinkle helicase with mtDNA (Spelbrink et al., 2001).

Mitochondrial genetics is a mixture of bacterial bacteriophage and eukaryote genetics. For example, mitochondrial polymerase gamma shares exonuclease active site motifs with *Escherichia coli* DNA polymerase I and bacteriophage T7 DNA polymerase (Luo and Kaguni, 2005). Also mitochondrial helicase Twinkle shows many structural similarities with bacteriophage T7 gene 4 primase helicase (Szczytny et al., 2013). Moreover mitochondrial ribosomes are very similar to prokaryotic ribosomes and both are targets for some kinds of antibiotics, such as chloramphenicol, tetracyclines and linezolid (Leach et al., 2007). The mitochondrial small and large ribosomal RNAs are minimalized versions of prokaryotic rRNA. And almost half of the mammalian mitochondrial ribosomal proteins have recognizable bacterial homologs (Christian and Spremulli, 2012).

In humans mtDNA contains 13 OXPHOS polypeptides, 22 tRNAs, 12S rRNA and 16S rRNA. Nuclear genome maintains approximately 2000 genes for all mitochondrial functions, such as the rest of the OXPHOS genes, enzymes required for mitochondrial biogenesis and maintenance and all metabolic pathways. mtDNA is double stranded DNA 16.6 kbps long sequence with different weight of their single stranded chains. Based on sedimentation coefficient we are discriminating heavy and light chain of mtDNA. All of the rRNA polypeptide genes except of ND6 are located on heavy strand, thus their mRNA sequence is same as light strand sequence of mtDNA.

Mitochondrial genetic code is a bit different from the so called, universal genetic code. In mammals, UGA code for tryptophan instead of termination codon. AUA, AUC and AUU are reserved for initiation codons and AGA with AGG are termination codons instead of encoding arginine. Moreover, the simplification in mitochondrial genetic system allow for using only 22 tRNAs (Fernández-Silva et al., 2003).

2.2.1 Composition of mitochondrial nucleoids

Mitochondrial nucleoids are ribonucleoprotein complexes. The first discovered and most abundant proteins of mitochondrial nucleoids are mitochondrial single strand binding protein (mtSSB) and mitochondrial transcription factor A (TFAM) (Rajala et al., 2014). Since mitochondria lack histones, these two proteins have to substitute their role. The histone proteins bind to DNA in salt resistant manner. The only mitochondrial protein having ability to bind DNA independently on substantial ionic strength is mtSSB (Curth et al., 1994). This protein forms a homo-tetramer (Oliveira and Kaguni, 2010) and enhances mtDNA replication up to 20 times by interacting with mitochondrial polymerase gamma (PolG) and with mitochondrial helicase Twinkle (Farr et al., 1999). Except of being a part of mtDNA replication fork, mtSSB was found also to support the single strand DNA in D-loop formation (Mignotte et al., 1985) and also in base excision repair (Wollen Steen et al., 2012).

The most common nucleoid protein is TFAM. It was described to bind to double stranded DNA in dimers making a U-turn on DNA (Hallberg and Larsson, 2011; Ngo et al., 2011). TFAM has also an important role in transcription initiation, probably thanks to the ability to bend DNA and thus enable annealing of other transcription factors. The TFAM binding stoichiometry varies between 16 to 300 bps depending on the method used for quantification (Bogenhagen, 2012). Data can be found in Table 1. Based on the crystal structure, it was described that TFAM binds to 22 base pairs in the heavy strand promoter, indicating that the TFAM binding ability depends on the sequence (Ngo et al., 2011; Rubio-Cosials et al., 2011). Therefore, there must be two forms of the TFAM, the first one involved in the D-loop formation, inducing transcription and primer formation for mtDNA replication and the

second one, which probably forms dimers and participate in nucleoid compaction (Bogenhagen, 2012).

Table 1. Measurements of the TFAM/mtDNA and mtDNA copy number in mammalian cells. Adopted from (Bogenhagen, 2012)

Species	Cell	mtDNA/cell	TFAM/mtDNA	Reference
Human	HeLa	1000	1700	* (Takamatsu et al., 2002)
	HeLa	5000	50.3	** (Cotney et al., 2007)
	Fibroblast	2721	1000	(Kukat et al., 2011)
Mouse	Kidney	nc	977	(Ekstrand et al., 2004)
	Liver	nc	1480***	(Park et al., 2007)

* Also reported 3000 +/- 1000 mtSSB/mtDNA.

** Also reported 6 mtRNA Pol/mtDNA, 2.7 TFB1M and 8.6 TFB2M/mtDNA.

*** Calculated from reported one TFAM occurrence each 10–12 bp; also noted one TERF2/260 bp, about 63/mtDNA.

nc Not calculated

Further biochemical studies based on paraformaldehyde crosslinking of mitochondrial nucleoids or native purified nucleoids by TFAM tag revealed the existence of tightly and loosely coupled proteins (Bogenhagen et al., 2008; Wang and Bogenhagen, 2006). In all types of purification the core nucleoid proteins involved in mtDNA metabolism and maintenance were found, such as mtSSB, TFAM, both subunits of PolG, mtRNA polymerase, DNA helicase Twinkle, mitochondrial transcription factor B1 and B2 (TFB1M, TFB2M), SUV3-like helicase, mitochondrial transcription termination factor (mTERF), mitochondrial topoisomerase I, Lon protease, caseinolytic protease X homolog (ClpX), heat shock protein HSP70, HSP 60, HSP40 (DNAJ3), intermembrane space chaperon prohibitin, RNA stabilization protein LRPPRC, RNA helicases DDX28 and DHX30 (Bogenhagen et al., 2008).

Another class of proteins was found only in one type of isolation indicating more loose connection with nucleoids. Here numerous proteins from mitochondrial ribosomes belong, which are

probably assembling in the close vicinity to nucleoids, lipid metabolic enzymes such as HADHA (enoyl-CoA hydratase), HADHB (acetyl-CoA acyltransferase) or ATAD3 a member of AAA⁺ family of ATPases. Moreover cytoskeleton proteins such as actin and vimentin are associated with nucleoid (Wang and Bogenhagen, 2006). Nucleoids prepared from rat liver by high salt lysis method showed incidence of non-muscle myosin heavy chain and β -actin in nucleoids indicating that mitochondrial movement or nucleoid segregation can be driven by cytoskeleton from cytosol (Reyes et al., 2011).

2.2.2 Nucleoid dynamics

Nucleoids probably undergo divisions (which has not been described yet) and must be distributed equally in the mitochondrial network. We have estimated that nucleoid spacing is approximately 1 μ m (Tauber et al., 2013). So nucleoids after division must be distributed to reach 1 μ m distance. It is probably achieved by movement of cytoskeleton which was reported to be associated with nucleoids (Bogenhagen, 2012). Moreover, the nucleoids are part of the mitochondrial network and such as they also move in the direction of a mitochondrial motion. Nevertheless, longitudinal movement within the mitochondrial tubules is hindered by the rich cristae (Kopek et al., 2012).

Mitochondrial nucleoids form large clusters under certain conditions. The clustering has been described at many situations. First, down-regulation of Dynamin related protein 1 (Drp1), which participates in mitochondrial fission, led to mtDNA accumulation in bulkier parts of mitochondrial (Ban-Ishihara et al., 2013). Drp1 was also described as a neighbour of mtDNA marking a site for mitochondrial fragmentation around nucleoids (Iborra et al., 2004). Nucleoid aggregates are quite common in experiments manipulating nucleoid proteins. Thus, overexpression of Twinkle or TFAM or their co-expression led to the increased mtDNA copy number in vivo in mouse tissues. Moreover, the elevated amount of mtDNA in Twinkle- and TFAM- overexpressing mice were accompanied with formation of large nucleoid clusters (Ylikallio et al., 2010). Twinkle is naturally forming hexameric structures and some mutations in this protein are associated with the autosomal dominant progressive external ophthalmoplegia. These Twinkle mutations are associated with mtDNA replication stalling, accumulation of replication intermediates and impaired nucleoid segregation (Goffart et al., 2009). Nucleoid aggregates were found even in cells overexpressing Polg β (Di Re et al., 2009). Interestingly, down-regulation of TFAM or ClpX protease led also to cluster formation (Kasashima et al., 2012, 2011). All of these studies have a common feature in disturbance of nucleoid protein homeostasis.

Moreover some DNA intercalators contribute to the impaired nucleoid segregation. Between these intercalators belong Topoisomerase II inhibitors such as Doxorubicine widely used as anticancer drug and Ethidium bromide, which is widely used for depleting mtDNA (Ashley and Poulton, 2009). Finally, giant nucleoids were observed in heteroplasmic cybrid cell lines forced to OXPHOS by

ketogenic treatment. The cybrids improved levels of heteroplasmy from 13% to approximately 22% after 5 days of growth on ketogenic bodies. In this case, nucleoid aggregates contained preferentially wild type (wt) mtDNA (Santra et al., 2004).

2.2.3 Mitochondrial biogenesis

Since the mitochondrial nucleoids are formed as layers forming the core centre containing mtDNA replication and transcription proteins and the second layer with loosely associated different proteins and lipid biogenesis proteins, it has been postulated that nucleoids might serve as mitochondrial centres for biogenesis (Bogenhagen et al., 2008). This notion was further supported by SILAC pulse-chase experiments with newly synthesized ribosomal proteins and their co-purification with nucleoids (Bogenhagen et al., 2014) or by the existence of specific foci near nucleoids, in which the mitochondrial rRNA is processed by methyltransferases before becoming part of mitochondrial ribosome (Lee et al., 2013).

2.3. Mitochondrial DNA replication, transcription and repair

The genetic organization of mtDNA is extremely compact. Polypeptide, tRNA and rRNA genes are smaller than the cytosolic and prokaryotic homologs (Schneider and Ebert, 2004). The spacing between individual genes is only a few bases and the polypeptide genes are without introns. Most of the mitochondrial genes coding for polypeptides are surrounded by tRNA genes (Fig1), which plays an important role in processing of mitochondrial transcripts. Some of the protein sequences even overlap. In humans, ATPase 6 and 8 subunits share 46 nucleotides and ND4 with ND4L subunits have seven nucleotides in common. In many cases the termination codons are added by post-transcriptional polyadenylation (Ojala et al., 1981). There are two non-coding regions (NCR). The main NCR is approximately 1.1 kbp long located between tRNA^{Phe} and tRNA^{Pro}. It contains replication origin for heavy strand and promoters for transcription from both light and heavy strands. The mtDNA replication starts by synthesis of RNA primer followed by synthesis of 7S DNA. Not all 7S DNAs continue in mtDNA replication. Many of them are stalled and degraded. 7S DNAs form a triple DNA structure called D-loop. 7S DNA has between 550 to 620 nucleotides and can be dissociated from mtDNA by heating at 80°C (Brown et al., 1978). The second NCR region can be found between tRNA^{Asn} and tRNA^{Cys} and contains a replication origin for the light chain.

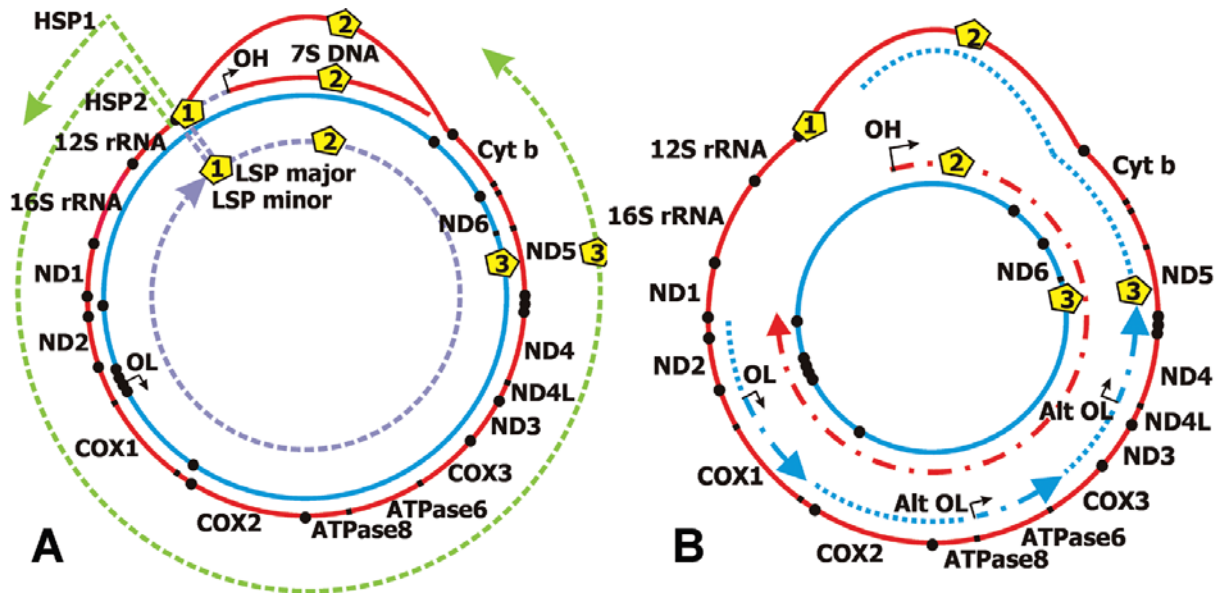


Figure 1. Transcription (A) and replication (B) schema of mtDNA. Adopted from (Alán et al., 2010). The transcription schema illustrates the heavy (red) and light (blue) strands of ds circular mtDNA, where borders between the genes are indicated as black squares or as black dots representing tRNA genes. Two possible polycistronic RNAs derived from each mtDNA strand are depicted as dashed lines indicating a direction of the transcription. Schema B represents direction of mtDNA replication as indicated by dot-dashed lines. The blue dashed lines represent RITOLS structures.

2.3.1 Proteins involved in mtDNA replication

The least portion of mitochondrial proteins necessary to synthesize 16kbp-long DNA must contain TFAM, mtSSB, DNA PolG and Twinkle (Korhonen et al., 2004). Practically, there is more proteins involved in mtDNA replication like mitochondrial RNA polymerase (POLRMT), RNase H1, DNA ligase III and topoisomerases (Holt and Reyes, 2012). Interestingly, Twinkle, PolG and POLRMT share a homology with T7 bacteriophage enzymes.

2.3.2 Mechanisms of mtDNA replication

Replication of mtDNA differs from the chromosomal one in many ways. Firstly, the mtDNA is circular molecule without telomeres. In contrast to many replication origins of chromosomes, mtDNA has two. Probably for each strand there is one replication origin. To date, three different models of mtDNA replication have been described. The first model was proposed in 1972 and was

called strand-displacement model (Robberson et al., 1972). This model was based on the two replication origins, and was derived from CsCl gradient centrifugation in combination with electron microscopy. The authors have observed several types of replication intermediates and were able to put them together in time scale from the replication start to the end. They clearly demonstrated the existence of new double strand (ds) DNA with an indigenous displaced single stranded (ss) DNA. After replicating approximately one third of the mitochondrial genome, a new dsDNA appeared indicating the beginning of the new replication and the second replication origin (Robberson et al., 1972). Almost 30 years later Holt and colleagues have found RNA/DNA hybrids as a result of the ribonucleotides incorporation into the light strand. These ribonucleotide-rich sequences were sensitive to RNase H and were subsequently converted to DNA (Yang et al., 2002). Discovery of RNA/DNA hybrid sequences led to formulation of a new model for mtDNA replication based on RITOLS (Ribonucleotide Incorporation Throughout the Lagging Strand) (Yasukawa et al., 2006). Later, it has been proposed that those RITOLS participate in both protecting ssDNA and forming multiple RNA primers for synthesis of lagging strand, thus creating strand-coupled model of replication (Holt, 2009). This was also supported by the existence of Okazaki fragment maturation proteins in mitochondria (Holt, 2009). Replication forks for all mtDNA replication models are shown in Fig2.

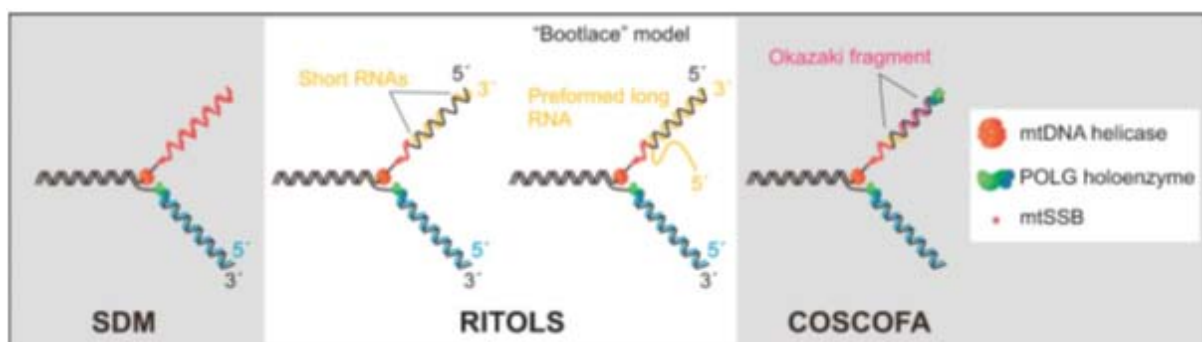


Figure 2. Replication forks for mtDNA replication models adopted from (Pohjoismäki and Goffart, 2011). The black helixes represent old mtDNA, while the blue helix is newly synthesized mtDNA fragment. The incorporation of long or short RNAs on lagging DNA strand is marked as yellow in RITOLS and COSCOFA models. The COSCOFA model contain purple helix representing an Okazaki fragment of the newly synthesized mtDNA.

Two types of mtDNA replication are showed in Figure 3. In A) the RITOLS type of mtDNA replication and so called COSCOFA (conventional strand-coupled Okazaki fragment associating) model.

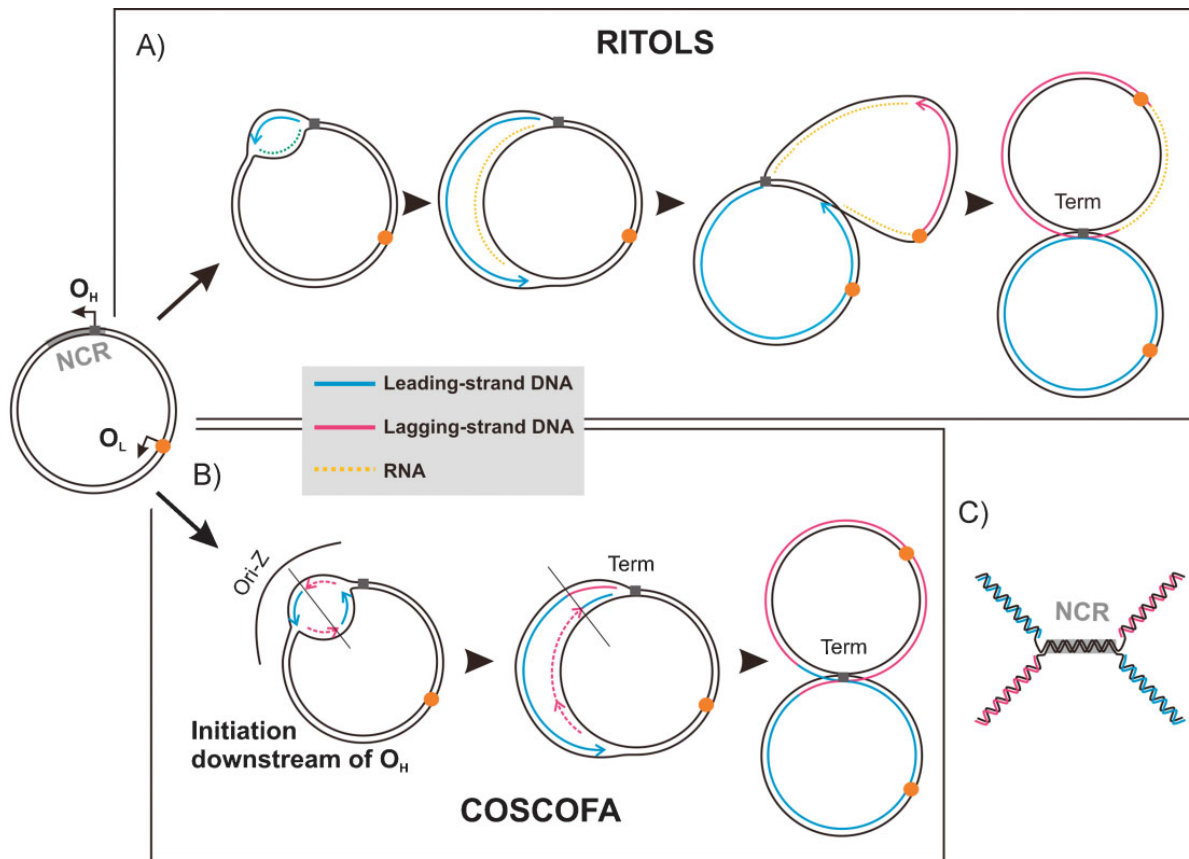


Figure 3. Replication of mtDNA RITOLS (A) and COSCOFA (B) models of replication. Adopted from (Pohjoismäki and Goffart, 2011). Old mtDNA is depicted in black colour, while the newly synthesized mtDNA is shown in the case of leading strand in blue and in the case of lagging strand in pink. The yellow lines represent RNA incorporation during lagging strand mtDNA synthesis. The potential split of the newly synthesized two mtDNA molecules (C).

2.3.3 mtDNA copy number

Amount of mtDNA per a single cell together (mtDNA copy number) with a number of mtDNA in a single nucleoid became a source of controversies in the field. There were several methods developed to quantify mtDNA in the cells such as southern blotting, quantitative PCR or flow cytometry (Bogenhagen and Clayton, 1974; Brown et al., 2011; Cavelier et al., 2000; Iborra et al., 2004; Kukut et al., 2011; Legros et al., 2004; Satoh and Kuroiwa, 1991). Differences in the methods probably led to the large inconsistency in the results displaying very often difference in one order of magnitude for a given cell type (Table 2). The results vary between 500 and 13000 copies of mtDNA per one cell. Similar large spread can be found in estimate of mtDNA per a single nucleoid. Again, different techniques displayed results between 1.4 and 10 mtDNA molecules per nucleoid (Table 2). It was hypothesized that the assessment of the exact number of mtDNA per cell or per nucleoid is not

crucial and that more important is whether the products of mtDNA could be equally distributed throughout the mitochondrial network (Bogenhagen, 2012). We have estimated the average distance between functional nucleoids as 1 μ m per one nucleoid (Tauber et al., 2013). Moreover, even for nucleoids with more mtDNA molecules, one has to admit the result of critical experiment demonstrating that nucleoids do not exchange mtDNA molecules between each other (Gilkerson et al., 2008).

Table 2. mtDNA copy number and nucleoid number from different cell lines. Adopted from (Bogenhagen, 2012)

MtDNA/cell	Species and Cell	MtDNA/nucleoid	Reference
8800	Human HeLa		(Bogenhagen and Clayton, 1974)
800	Human Fibro		(Robin and Wong, 1988)
500	Human A2780	1.4	(Sato and Kuroiwa, 1991)
7200	Human HeLa		(Shmookler Reis and Goldstein, 1983)
7200	Human KB		(Shmookler Reis and Goldstein, 1983)
2933	Human Fibroblast		(Shmookler Reis and Goldstein, 1983)
5200	Human fibroblast		(Shmookler Reis and Goldstein, 1983)
1632	Human Male Fibro	2.3	(Legros et al., 2004)
1961	Human Fem Fibro	2.4	(Legros et al., 2004)
2637	Human HeLa	5.7	(Legros et al., 2004)
4126	Human 143B	7.5	(Legros et al., 2004)
3500	Human ECV304	7.3	(Iborra et al., 2004)
2721	Human Fibroblast	1.4	(Kukat et al., 2011)
12900	Human Fibroblast		(Tang et al., 2000)
821	Mouse 3T3	3	(Brown et al., 2011)
1100	Mouse L		(Bogenhagen and Clayton, 1974)
720	Mouse LA9		(Robin and Wong, 1988)

Variations in mtDNA copy number may result in several mitochondrial diseases. High mtDNA in mice overexpressing TFAM and/or Twinkle led to decrease in the mt-mRNAs levels, age-related accumulation of mtDNA deletions and respiratory chain defects (Ylikallio et al., 2010). Moreover increased mtDNA levels were described in human aging (Barrientos et al., 1997). On the contrary mtDNA depletion was found in infantile neurogenetic disorders (Macmillan and Shoubridge, 1996), multiple sclerosis (Blokhin et al., 2008), renal cell carcinoma (Xing et al., 2008), liver disease (Morten et al., 2007), type 2 diabetes (Choi et al., 2001), cardiomyopathy (Bai and Wong, 2005) and breast cancer (Yu et al., 2007).

Amount of mtDNA varies during human ontogenesis (Fig4) and also differs in tissues (St. John, 2014). Changes in the mtDNA copy number are also utilized in mtDNA purification and clonal expansion (bottle-neck effect) during oogenesis as described later. However, it is not clear how mtDNA copy number is regulated. We still do not know, if there is a synergy between degradation and biosynthesis of mtDNA (Clay Montier et al., 2009).

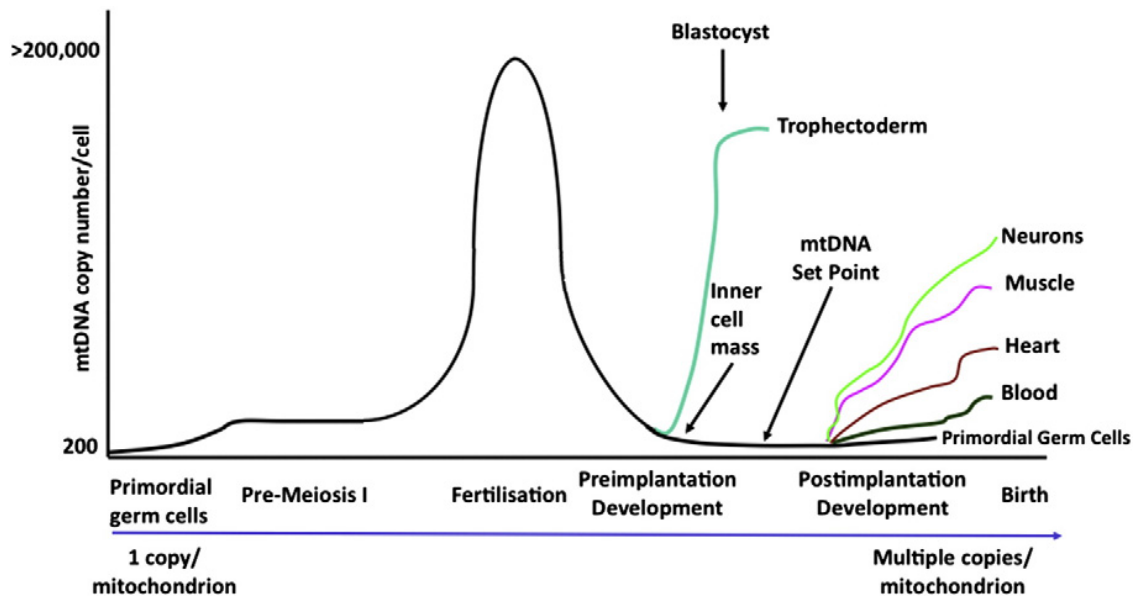


Figure 4. mtDNA copy number during the prenatal development. Adopted from (St. John, 2014)

2.3.4 mtDNA transcription

Mitochondrial transcription starts from three major transcriptional promoters. The two are from heavy strand – HSP1 and HSP2 (heavy strand promoter) and the third one is LSP (light strand promoter). Transcription machinery consists of POLRMT, TFAM, TFB2M and many regulatory factors such as mitochondrial transcription elongation factor (TEFM), mTERFs and mitochondrial ribosomal protein L12 (MRPL12) (Rorbach and Minczuk, 2012).

Each mitochondrial RNA (mtRNA) molecule arises from the polycistronic transcript and is separated according to the tRNA punctuation model. Based on this model, tRNA molecules form a secondary cloverleaf structure enabling them to be self-cleaved from the polycistronic mtRNA (Ojala et al., 1981). However, not all mt-mRNAs are surrounded by tRNAs and in this case, the self-cleaving processes are supported by mitochondrial RNase P. In contrast, this enzyme is a hetero-multimeric enzyme consisting of three different protein subunits, probably existing without the RNA subunit

(Holzmann et al., 2008). The 3'-ends of mt-tRNAs are cleaved by ELAC2 (elaC homologue 2), which has been proposed to have similar activity as endonuclease RNase Z (Rossmanith, 2011). The last step in mt-tRNA maturation is addition of a CCA trinucleotide to the 3'-end by ATP(CTP):tRNA nucleotidyltransferase (Nagaike et al., 2001).

Additional modifications are usually required in mt-mRNA post-transcriptional life. The most important post-transcriptional modification is polyadenylation. More than half of the mitochondrial mRNA terminates by U or UA. Thus polyadenylation, which adds poly (A) tail, forms the termination codon in these mRNAs (Anderson et al., 1981). Eukaryotic poly (A) signal has a major role in the exporting of mRNA from nucleus and promotes initiation of translation in the cytosol. Bacteria and chloroplasts use poly (A) signal for RNA degradation. On the contrary, mitochondrial polyadenylation pattern from various organisms does not show any common features. For example, the yeast mitochondrial transcripts has an AU-rich dodecamer sequence at the 3'-end instead of poly (A) stretches (Butow et al., 1989). In human mitochondria, ND6 mRNA is not polyadenylated, while the rest of the mRNAs contain approximately 45 nucleotides (Rorbach and Minczuk, 2012). The role of possible mitochondrial proteins synthesizing poly (A) such as poly (A) polymerase (PAP) or polynucleotide polymerase (PNP) is not completely clear as well as the contribution of mitochondrial poly(A) tails in half-life of mt-mRNAs (Rorbach and Minczuk, 2012).

Post-transcriptional modifications of ribosomal RNA occur in the vicinity of mitochondrial nucleoids (Lee et al., 2013). In contrast to bacterial or human cytoplasmic rRNAs, which contain 33 and over 200 modifications respectively, human mitochondrial rRNAs possess nine modified bases. They have no methylated ribose residues, rather they carry dimethylation. Five dimethylated ribonucleotides were found in small ribosomal subunit 12S rRNA and four in large subunit 16S rRNA (Rorbach and Minczuk, 2012). Up to now, only the two methylation factors were found, TFB1M (Seidel-Rogol et al., 2003) and NSUN4 (Cámara et al., 2011).

2.3.5 Mitochondrial translation

Mammalian ribosomes sediment as 55 S aggregates, which consist of large 39 S subunit and 28S subunit (O'Brien, 1971). These ribosomes have 2.7 MDa and contain only two rRNA species. They also contain very low rRNA mass (27%) in comparison with bacterial (65%) or cytosolic (55%) ribosomes (Christian and Spremulli, 2012). Moreover, there is also a low amount of mitochondrial ribosomes in comparison with the amount of cytosolic ribosomes (Bogenhagen, 2012).

Mitochondrially encoded proteins are translated from nine monocistronic and two bicistronic mRNAs (Anderson et al., 1981). Since many of mt-mRNAs contain an initiation codon at or very

close to the 5' end, they lack the canonical Shine-Dalgarno sequence. Mitochondrial protein biosynthesis has four phases, initiation, elongation, termination and ribosome recycling. In initiation phase, the mitochondrial initiation factor IF3 interacts with 55 S ribosome and helps to loosening interactions between the two subunits. The small subunit together with IF3 interacts with mRNA, IF2 and fMet-tRNA. If there is a start codon, IF2 promotes binding of fMet-tRNA to the initiation codon, forming stable translation initiation complex. The large subunit joins the initiation complex and IF2 hydrolyzes GTP to GDP and the initiation factors are released from the ribosome (Christian and Spremulli, 2012).

The next step in mitochondrial translation is elongation. Elongation factor Tu (EF-Tu) binds to aminoacyl-tRNA and together enters the A-site of the ribosome, which led to EF-Tu GTP hydrolysis. The ribosome itself catalyses peptide bond formation and the EF-Tu is released to matrix. Mitochondrial elongation factor G1 (EF-G1) catalyses removal of the deacylated tRNA from the P-site and move of the peptidyl-tRNA from the A-site to the P-site (Christian and Spremulli, 2012). Cryo-EM of the mitochondrial ribosomes did not find the E-site similar to prokaryotic (Sharma et al., 2003).

Mitochondrial translation is stopped by two stop-codons UAA and UAG. Two others mRNAs terminates with AGA and AGG. To date, there is no evidence of any type of releasing factor for these reading frames, rather one base back frame shift movement in the A site occur leading to recognition of the standard UAG stop codon (Temperley et al., 2010). Mitochondrial stop codons are recognized by a release factor mtRF1a, which triggers the GTP hydrolysis together with hydrolysis of the peptidyl-tRNA bound. The nascent polypeptide becomes free and mtRRF1 binds together with mtRRF2 to the A-side of ribosome and promotes ribosomal subunit dissociation and release of the deacylated tRNA and the mRNA (Christian and Spremulli, 2012).

There is a lot of evidence that mitochondrial ribosomes are associated with the mitochondrial inner membrane and that the protein synthesis is coupled with co-translational insertion into the inner membrane (Christian and Spremulli, 2012). The most studied candidate for ribosome-membrane interactions is Oxa1p. This integral membrane protein was described to possess 100 aminoacids on the C-terminal, which are located to the matrix and interact with mitochondrial ribosomes (Jia et al., 2003; Szyrach et al., 2003).

2.3.6 mtDNA repair

Mitochondrial reparation system is based on several mechanisms. One approach is focused on molecular mechanisms such as base pair excision or recombination. On the other hand, evolution had developed another mechanism which is called mitochondrial bottle-neck effect. It takes place during

female primordial germ cells maturation. The primordial germ cells are going through ~ 20 mitotic cell divisions producing several million proto-oocytes. During this process primordial cells contain only limiting amount of mtDNA, which is dispersed by genetic drift into predominantly normal or mutant variants (Jenuith et al., 1996). The proto-oocytes with most severe mitochondrial defects are eliminated by the intra-ovarian selection system and normal oocytes are ovulated. Unfortunately, mechanism by which the defective primordial cells are recognized is still unknown (Wallace, 2008).

In addition, mitochondria possess repair pathways correcting mistaken or damaged nucleotides. The most studied and major mitochondrial repair pathway is the base excision repair (BER). This pathway removes small DNA lesions induced by oxidation, alkylation and spontaneous hydrolysis. The BER starts by recognition of a damaged base by DNA glycosylases. Monofunctional DNA glycosylases hydrolyze *N*-glycosidic bond, leaving an apurinic/apyrimidinic (AP) site in DNA. There are two glycosylases described, uracil-DNA glycosylase 1 (UDG1) (Anderson and Friedberg, 1980) and homolog of *Escherichia coli* MutY glycosylase (MYH) (Ohtsubo, 2000). The resulting abasic site is removed by AP endonuclease 1 (APE1). The alternative pathway acts through bifunctional DNA glycosylases, which possess an intrinsic AP-lyase activity and cleave *N*-glycosidic bond together with β -elimination or β,δ -elimination resulting on the AP site (Dodson and Lloyd, 2002). For this pathway, there are four bifunctional glycosylases described. Two of them has β -lyase activity, such as the 8-oxoguanine DNA glycosylase (OGG1) (Takao et al., 1998) and *E. coli* endonuclease III-like 1 (NTHL1) (Karahalil et al., 2003). The other two are β,δ -lyases, Nei *E. coli* endonuclease VIII-like-1 and -2 (NEIL1 and NEIL2) (Hu et al., 2005; Mandal et al., 2012). The next step is 3' processing by polynucleotide kinase 3'-phosphatase, which generate OH group on the 3'-end of single stranded DNA (Xu et al., 2003). In case of short patch BER, the resulting gap enables annealing of PolG and synthesis of the missing nucleotide together with sealing the nick by DNA ligase III α (Bogenhagen, 1999). On the other hand, according to the lately discovered mechanism of long patch BER, mitochondrial PolG synthesizes a six to nine long flap, where the two nucleases FEN1 and DNA2 (Szczyzny et al., 2008). Both nucleases cut the DNA flap and DNA is again sealed by the DNA ligase III α (Fig5).

Another reparation pathway is so called mismatch repair (MMR) pathway, which is searching for base-base mismatches such as G:T and G:G errors. Since the repair is random and introduces mutations, it has been supposed that MMR is more involved in the repair of small loops rather than mismatches (Mason and Lightowlers, 2003). The general repair mechanism consists of localization of mismatch or loop, followed by excision and DNA synthesis finished by ligation (Kazak et al., 2012). The set of proteins participating in the MMR pathway is not fully understood. It is suggested that the Y-box binding protein 1 (YB1) is localized to both nucleus and mitochondria and in mitochondria triggers the recruitment of MMR complex (de Souza-Pinto et al., 2009). The following steps are unknown, but it is speculated that they involve PolG and other proteins participating in BER.

Mitochondria are able to repair also double stranded breaks (DSBs), which can be fixed by both homologous recombination or non-homologous end joining (Coffey et al., 1999; Thyagarajan et al., 1996). The potential candidate for DSB repairs in mammalian mitochondria is Rad51, which is responsible for homologous recombination in nucleus and recently was described also in mitochondria (Sage et al., 2010).

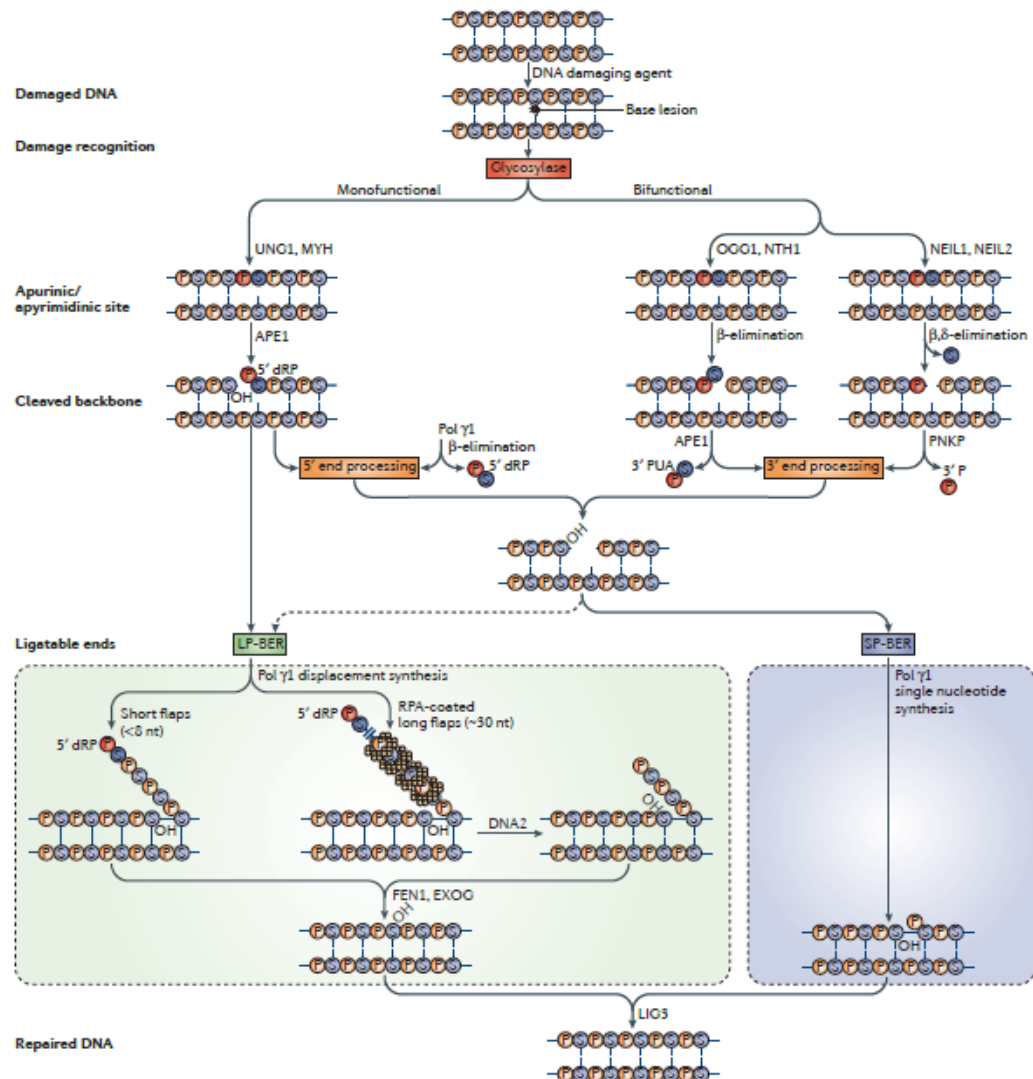


Figure 5. Mitochondrial base excision repair. Adopted from (Kazak et al., 2012). The schema represents mtDNA base excision repair mechanism for both monofunctional and bifunctional type of glycosylases and mechanisms for long (green) and short (blue) patch base excision repair.

2.3.7 RNA import into mitochondria

Import of proteins into mitochondria is very well established (Chacinska et al., 2009). On the contrary, knowledge of mitochondrial RNA import is based on limited amount of articles. Almost

every organism with mitochondria imports tRNAs and aminoacyl-tRNA synthetases (Alfonzo and Söll, 2009). Mammalian mitochondria are able to import several different tRNAs both *in vitro* and *in vivo* (Kolesnikova et al., 2004). Moreover such an imported tRNAs are properly aminoacylated and then participate in mitochondrial translation (Kolesnikova et al., 2004). The existence of other RNAs in mitochondria such as RNA subunit of RNase P or MRP RNase are still under great discussion and evidence for both must be taken into account (Holzmann et al., 2008; Puranam and Attardi, 2001). Many controversies have been found also during investigation small non-coding (nc) RNAs. While the first group in 2006 identified six ncRNAs transcribed from D-loop and antisense orientation to mitochondrial mRNAs ND4 and ND6 with no ncRNAs from nucleus (Lung et al., 2006), another group in 2011 reported mitochondrial import of 13 small ncRNAs all originating from the nucleus (Bandiera et al., 2011). Moreover they also proved existence of Argonaut 2 in mitochondria and its co-precipitation with mitochondrial transcript COX3 (Bandiera et al., 2011). Thus the question was opened for the existence of mitochondrial RNA interference and possible down-regulation of specific mitochondrial proteins. Additionally, long ncRNAs were also found in mouse mitochondria. First 121 nucleotides long RNA and 2374 nucleotides long RNA, both expressed in human proliferating cells (Villegas et al., 2007).

Natural RNA import into mitochondria requires at least two factors. The first one is the mitochondrial enzyme rhodanase, which was described to be essential for import of 5S rRNA (Smirnov et al., 2010), while the second is the mitochondrial polynucleotide phosphorylase (PNPASE), which facilitates import of RNAs for RNaseP, MRP and 5S rRNA (Wang et al., 2010).

2.4 Mitochondrial dynamics

It is now 100 years since the first mitochondrial movement and fission were observed by light microscopy (Lewis and Lewis, 1915). Then in the age of electron microscopy, mitochondria were used to be observed as a sausage or bean shaped organelles surrounded by the cytoplasm. The amount of mitochondria was very often counted as a marker of mitochondria quantity. Later, with the development of new visualization techniques, with the phase contrast and fluorescent microscopy, the mitochondria were again shown to ceaselessly move, divide and fuse (Bereiter-Hahn and Vöth, 1994). Nowadays, it has been proposed that mitochondria form a network, which undergoes fission and fusion and which can be completely fragmented under stress conditions (Plecitá-Hlavatá et al., 2008). Moreover, mitochondrial fission and fusion is now considered to be fundamental for cell survival, health and disease, which will be discussed below.

2.4.1 Mitochondrial fusion

Mitochondrial fusion consists of two membranes merging and is followed by mixing of the membranes, intermembrane space and matrix. The most important and frequently studied proteins of mitochondrial fusion machinery are mitofusin1 and 2 (Mfn1 and Mfn2) and Opa1 (according the optic atrophy disorders). All of the three proteins belong to dynamin protein family and contain the functional GTPase domain and two transmembrane domains with both N' and C' terminals oriented out of the mitochondrial matrix (Fig 6). The Opa1, which is an inner membrane protein, contain also GTPase effector domain important for mediating the intra- and inter-molecular interactions, required for self-assembly and oligomerization-dependent activity. Furthermore, both mitofusins contain heptad repeats (HR) domains which are potentially responsible for antiparallel coiled-coil interactions and thus for approximation of two mitochondrial outer membranes (Koshiba et al., 2004). Based on similarities between HR domains of SNARE proteins and HR domains of mitofusins, it has been postulated how the opposite mitochondrial membrane are fused together. This model suggests mitofusin dimerization and pulling the membranes in SNARE-like mechanism to 100 Å proximity (Koshiba et al., 2004). Simultaneously, Opa1 was described to interact with Mfn1 and this collaboration triggers mitochondrial fusion (Cipolat et al., 2004). Because of differential RNA splicing and protein processing, Opa1 has several isoforms divided into two groups, short and long. Both isoforms are important for mitochondrial fusion (Song et al., 2007), but under the stress conditions the long forms of Opa1 appears to be sufficient to enhance mitochondrial fusion (Tondera et al., 2009). Mitochondrial fusion is very often connected with merging of both the inner and the outer membranes. However, fusion of the inner membrane is dependent on membrane potential and in case of mutations or loss of membrane potential only the outer membranes can fuse (Olichon et al., 2003).

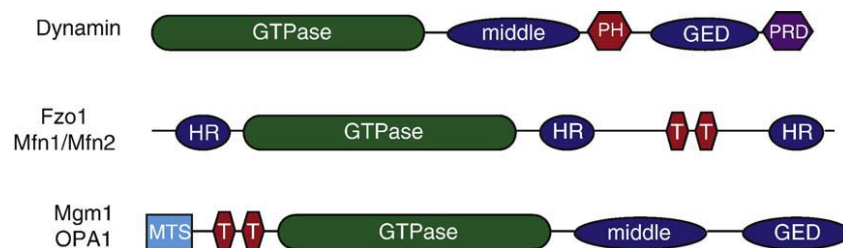


Figure 6. Domain structures of dynamin and the mitochondrial fusion proteins from dynamin family. Adopted from (Hoppins and Nunnari, 2009). The GTPase domain is in green, heptad repeats (HR) domain in blue as well as GTPase effector domains (GED). The pleckstrin homology domain and transmembrane domains are in red. Mitochondrial targeting sequence is shown as light blue in OPA1 gene.

Opa1 protein has also other functions. Only 8% of localized Opa1 proteins by immunogold staining and electron microscopy showed localization into the cristae rim structures, which are proposed to be centers for potential fusion. The rest of the localization pattern was inside cristae. This indicates other functions of Opa1, such as maintaining cristae morphology like mitofilin and ATP synthase oligomerization (Rabl et al., 2009) or involvement in mtDNA replication and distribution (Elachouri et al., 2011; Frezza et al., 2006).

Regulation of mitochondrial fusion is mostly based on ubiquitination and proteolysis. Mitochondrial fusion proteins from the outer membrane are degraded prominently by the ubiquitin mediated degradation and proteins from the inner membrane are cleaved by mitochondrial proteolytic enzymes at a select few sites (van der Bliek et al., 2013). In humans, mitochondrial fusion proteins are eliminated by PTEN-induced putative kinase 1 (Pink1) – Parkin mediated ubiquitin degradation during mitophagy (Youle and Narendra, 2011). The exact mechanism of mitochondrial removal is discussed below. Mitochondrial inner membrane mitodynamain Opa1 has three cleavage sites (S1, S2 and S3) and most of them (S2 and S3) are used for post-translational modification giving rise several Opa1 isoforms. On the other hand, the S1 cleavage site is present on all isoforms and is responsible for cleavage in situations when mitochondria lose their membrane potential or have a low levels of ATP (Baricault et al., 2007; Ehses et al., 2009). This inducible cleavage is mediated by Oma1, which cleaves all long isoforms Opa1 within few minutes after treatment with CCCP (Ehses et al., 2009).

2.4.2 Mitochondrial fission

Mitochondrial fission is not fully understood process and the key role is played by the dynamin related protein1 (Drp1). The Drp1 is mostly cytosolic and is able to be recruited onto and released off the mitochondrial outer membrane. Human Drp1 possesses the GTPase domain and the GTPase effector domain (Praefcke and McMahon, 2004). There are usually numerous sites, where Drp1 localizes on the mitochondrial network, but only some of them continue to complete the fission. After mitochondrial outer membrane localization, Drp1 assembles into multimeric spirals through a series of interactions and crosstalk of different subunits (Chappie et al., 2010). Once a spiral completes a turn, the contacts between the GTPase domains of neighboring partners trigger GTP hydrolysis (van der Bliek and Payne, 2010). This enzymatic activity drives conformational changes within multimers causing constriction of the spiral, which affects both membranes. Inhibition of expression or dominant negative variants of Drp1 led to the elongated mitochondria that entangle and collapse (Smirnova et al., 2001). Similarly mouse embryonic fibroblasts with knock-out for Drp1 has elongated mitochondria and are even resistant to carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) fragmentation (Wakabayashi et al., 2009).

Once the most of the Drp1 is situated in the cytosol, there is a need for adaptor or receptor proteins which would situate Drp1 onto the surface of the outer mitochondrial membrane. Such a candidate can be Fis1, Mff, MiD49 or MiD51. Moreover, recently it has been shown, that mitochondrial-ER contact sites can mark forthcoming mitochondrial fission (Friedman et al., 2011), but the responsible molecules need to be found.

There is an evidence that Fis1 may serve as a receptor or an adaptor protein for Drp1. Thus overexpression of Fis1 is accompanied with increased mitochondrial fission, and Fis1 was found in co-purification with Drp1 (Yoon et al., 2003). Yoon and colleagues also observed that Fis1 antibody injection results in elongation of mitochondria. Interestingly, the targeted deletion of Fis1 in HeLa cells did not show any significant changes in mitochondrial network (Otera et al., 2010). Thus Drp1 could have more potential partners, which could be tissue and cell specific or could be substituted by other proteins. It seems that Mff could be the other receptor for Drp1. Like Fis1, Mff is a mitochondrial outer membrane protein, down-regulation of which led to the mitochondrial elongation (Otera et al., 2010). Recently two novel proteins MiD49 and MiD51 have been discovered to interact with Drp1. When down-regulated both decrease the Drp1 mitochondrial localization and promote mitochondrial fusion. Unexpectedly, their overexpression causes mitochondrial tubule extension and collapse. It indicates that these two proteins may inhibit Drp1 function on mitochondria (Palmer et al., 2011).

Fusion is very often followed by fission to maintain fission – fusion homeostasis. Subsequent fission happens very often on the same part of the mitochondrial network and within 20 minutes (Twig et al., 2008). This finding is further supported by the discovery, that during mitochondrial fusion the mitochondrial phospholipase PLD produces phosphatidic acid (Choi et al., 2006), which activates mitochondrial Lipin1 (Huang et al., 2011). Lipin1 then produces diacylglycerol, the protein kinase C (PKC) activator. Finally PKC phosphorylates Drp1 at Ser616, which promotes binding of Drp1 to other fission proteins on mitochondrial outer membrane (Taguchi et al., 2007).

Recently, the mitochondrial interactions with endoplasmic reticulum (ER) have been described to have an important role in mitochondrial fission. It has been reported that ER wraps around mitochondrial tubules at *foci*, where the fission will occur (Friedman et al., 2011). These authors also shown, that mitochondria are still tightened on the contacts between mitochondria and ER even in the cells down-regulated for Drp1 or Mff. Moreover, the ER protein INF-2 was found to promote mitochondrial fission by inducing mitochondrial constriction before the Drp1 recruitment onto the mitochondria (Korobova et al., 2013). Mitochondrial constriction was accompanied with actin polymerization, which helps to ER in mitochondrial wrapping and preparing it for fission.

2.4.3 Mitochondrial cristae morphology

The most important features of mitochondria are their membranes. The outer membrane serves as an envelope around the whole mitochondrial network and the inner membrane forms cristae invaginations, creating the three major different compartments: *i*) the matrix space of an infinite "star-fish" topology; *ii*) the separate intracristal spaces between/along the cristae invaginations with the outlets, i.e. cristae junctions (CJs), closed by OPA1 trimers - these multiple separate spaces form the major portion of the intermembrane space; and *iii*) the intermembrane space proximal to the outer membrane. The inner membrane, which is in the closest proximity to the outer membrane, is called the inner boundary membrane (IBM). The narrow connections of cristae with IBM form CJs. The mitochondrial outer membrane is tightly linked with the IBM by contact sites (CS). The space between outer membranes and IBMs is called intermembrane space, a part of the intermembrane space between individual cristae. Mitochondrial CS are characterized first by the TOM/TIM complexes ensuring the protein import into all mitochondrial compartments. Just at the edges of cristae junctions, other protein complexes reside, described as the mitochondrial inner membrane organizing system (MINOS) (Herrmann, 2011) or mitochondrial organizing structure (MitOS) (Hoppins et al., 2011) or mitochondrial contact site (MICOS) (Harner et al., 2011). In budding yeasts *Saccharomyces cerevisiae*, MINOS is composed of at least six subunits: Fcj1, Mio10, Aim13, Aim5, Aim37 and Mio27 (Hoppins et al., 2011). In humans were found homologs for Fcj1 - Mitofilin, for Mio10 - Minos1 and for Aim13 it was CHCHD3 (Alkhaja et al., 2012). The suggested interacting partners and composition of MINOS complexes are shown in Fig7.

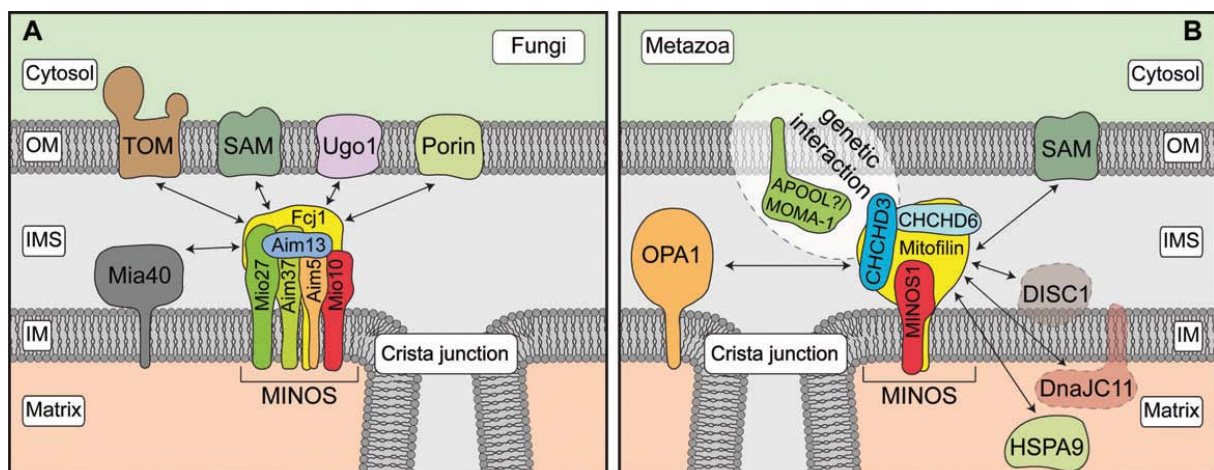


Figure 7. Structure of MINOS complexes in Fungi (A) and in higher eukaryotes (B). Adopted from (Zerbes et al., 2012). The image also shows possible mechanisms of the interactions of the MINOS complex with the outer mitochondrial membrane proteins in Fungi and several potential interacting proteins from the inner and outer mitochondrial membrane in higher eukaryotes (Metazoa).

All of the above mentioned proteins from MINOS complexes are either integral or peripheral proteins of the inner membrane and expose their bulky domains to the intermembrane space. This was confirmed by STED microscopy, which showed mitofilin localization in the inner membrane forming a dull tube (Jans et al., 2013). In human mitochondria, the MINOS complex also co-precipitates with apolipoprotein O-like protein (APOOL) (Weber et al., 2013). APOOL was described as the inner membrane protein with affinity for binding cardiolipin. Down-regulation of APOOL led to reduction in basal oxygen consumption and altered cristae morphology (Weber et al., 2013).

The role of MINOS complexes is to keep the cristae and forming barrier between intracristae lumen and the boundary intermembrane space (Mannella, 2006). Other inner membrane protein complexes, such as supercomplexes of the respiratory chain are specifically localized along the intracristal part of the inner membrane, whereas the ATP-synthase dimers form cristae bending alongside the cristae or at the distal cristae parts opposed to CJs. (Minauro-Sanmiguel et al., 2005). Thus super-complexes of the respiratory chain have been found to be distributed around the middle of cristae with restricted diffusion (Wilkens et al. 2012).

We should also note, that cristae in OPA1-silenced cells are largely depleted (Frezza et al., 2006), whereas in mitofilin knock out cells, the cristae are separated from the CJs and most frequently oriented at the longitudinal tubular axis of the mitochondrial network. It remains to be elucidated, how proteins are imported into such deeply embedded intracristal compartments.

2.4.4 Mitophagy

Mitochondrion as any organelle is subjected to the constant turnover of proteins. Certain proteins exhibit very long half-life, whereas the other, e.g. UCP2, have a short lifetime, hence are subjected to the fast turnover. Also, mitochondria alone are sources of damaging reactive oxygen species and affected biomolecules must be therefore carefully eliminated from the cell. For example, in liver representing centre of detoxification, the entire protein content of mitochondria is replaced within a few days (Miwa et al., 2008). The most efficient way to remove bulky parts of the cell is through a controlled process called autophagy. The autophagy (from the Greek for “self-eating”) can be generally induced during starvation, when the cell needs nutrients. It may also proceed selectively, such as mitochondrial degradation – mitophagy; or peroxisome degradation - pexofagy. The selective mitophagy has been described for example in erythrocyte maturation (Sandoval et al., 2008) or in sperm-derived mitochondrial clearance after oocyte fertilization (Al Rawi et al., 2011).

The mitophagy is controlled directly by the mammalian target of rapamycin (mTOR), which is normally in complex with the autophagy related gene ATG13. Under starvation or stress situations

mTOR-ATG13 interaction is inhibited and ATG13 becomes a part of ATG1 kinase or mammalian homolog ULK1 (Joo et al., 2011). The ULK1 complex activates a class III phosphatidylinositol 3-kinase (PI3K) complex with ATG14, Vps34 and Beclin1. This complex accumulates phosphatidylinositol-triphosphate-rich membranes important for the nucleation of autophagy membrane (Tanida, 2011). Elongation of the membrane is mediated by the two ubiquitin-like conjugation systems. The first one involves a complex of ATG12, ATG5 and ATG16L1 (Ashrafi and Schwarz, 2012). The second conjugation system is the same as the general autophagy system and utilizes the ubiquitin fold-containing protein microtubule-associated protein light chain 3 (LC3). The LC3 has two forms, the first one the inactive cytosolic version, called LC3I, and the second one, LC3II, which possesses the phosphatidyl ethanolamine anchor. The LC3II is activated form localizing to the isolation membranes and is necessary for elongation and closure of the autophagophore (Tanida, 2011). The origin of autophagosomal membrane remains controversial, but the donor role of ER in isolating membranes are mostly accepted (Tooze and Yoshimori, 2010).

The other two key mitophagy players have been found to be mutated in certain types of Parkinson disease (PD). The first one, PTEN-induced putative protein kinase 1 (PINK1) is serine/threonine kinase containing a mitochondrial targeting sequence (Youle and Narendra, 2011). In normal situations, PINK1 is constitutively imported into the intermembrane space, where it is cleaved and degraded by mitochondrial-processing protease (MPP) and the inner membrane presenilin-associated rhomboid-like protease (PARL) (Meissner et al., 2011), as described in the Fig8. In case of damaged mitochondria, PINK1 is not able to be imported through the TIM/TOM import complex and accumulates on the mitochondrial outer membrane in association with the TOM complex (Lazarou et al., 2012). Here, the PINK1 phosphorylates the second protein found to be involved in PD is E3 ubiquitin ligase Parkin (Kitada et al., 1998). The question remains, whether Parkin is phosphorylated directly by PINK1 or other kinase activated by PINK1 (Ashrafi and Schwarz, 2012). The Parkin mediates two types of polyubiquitin chains. On the lysine (K) 48 associated with proteasomal degradation of the substrate and on the lysine K63 associated with autophagic degradation (Lim et al., 2006). A proteomic study in HeLa cells showed proteasome degradation of Miro, Mitofusin, Fis1, Tom 70 and other proteins (Chan et al., 2011).

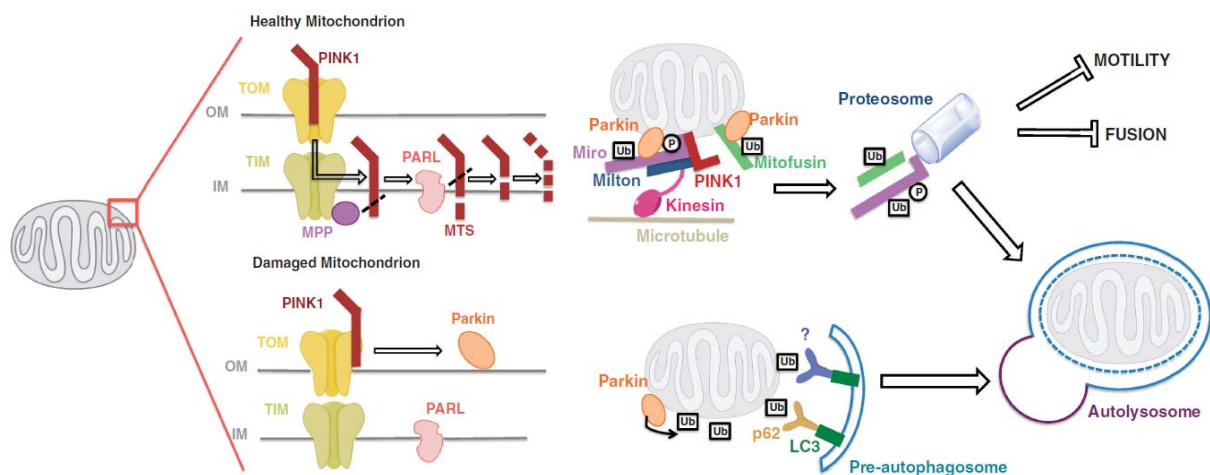


Figure 8. The PINK-Parkin mitophagy pathway. Adopted from (Ashrafi and Schwarz, 2012). Under standard conditions the PINK1 protein enters the mitochondria via TIM-TOM import complex and subsequently is inserted into the inner mitochondrial membrane, where it is degraded by PARL protease. During stress, PINK1 cannot enter the mitochondrion, because of low membrane potential and therefore accumulates on mitochondria, and activates parkin. Activated parkin ubiquitinates mitochondrial outer membrane proteins and thus marks them for degradation.

Moreover, Parkin overexpression has been found to improve a heteroplasmy in cybrid cell lines to higher wild-type/deleted mtDNA ratio (Suen et al., 2010). Also exogenously expressed Parkin-YFP has been shown to increase mitochondrial localization in both Rho0 cells lacking mtDNA and Twinkle mutated cells (Suen et al., 2010). In addition, Eric Schon's group has shown that in cybrid cell lines the Parkin protein levels are suppressed and in order to induce the mitophagy in these cell lines, it is necessary to exogenously increase Parkin levels together with the mTOR inhibitor rapamycin (Gilkerson et al., 2012). Another inducer of mitophagy are treatments with chemical uncoupler CCCP (Chen et al., 2010) and approaches utilizing valinomycin, a K^+ ionophore, and antimycin A, a blocker of complex III (Chan et al., 2011).

2.4.5 Mitochondrial – ER crosstalk

Organelles must somehow interact between each other to maintain their membranes and to sense what happens in the rest of the cell. The vesicle trafficking between ER, Golgi apparatus and plasma membrane is very well known, but until now, it was not observed that mitochondria are recipients of any vesicle from other organelles. The first evidence of mitochondrial proximity to

another organelle was described for ER (Robertson, 1960). A hypothesis concerning the mitochondrial – ER crosstalk was further supported by the finding, that the fraction of ER copurifies with mitochondria in velocity sedimentation experiments (Vance, 1990). The fraction of ER and mitochondrial membranes are rich in lipid biosynthesis proteins. Mitochondria and ER exchange the lipids in their contact sites (Rusiñol et al., 1994) and cooperate in *de novo* aminoglycerophospholipid biosynthesis pathway. Phosphatidylserine (PS) is synthesised on ER and then converted to phosphatidylethanolamine (PE) by mitochondrial enzyme PS decarboxylase (Voelker, 2004). PE is further converted to into phosphatidylcholine (PC) in ER. Taken together, the PC biosynthesis needs cooperation between two organelles through their contact sites.

The second function of ER-mitochondria crosstalk is the Ca^{2+} exchange. Mitochondria contain tricarboxylic acid cycle enzymes, which are Ca^{2+} dependent (Denton, 2009). Mitochondria also prevent Ca^{2+} accumulation in the cytosol, which in high mitochondrial density may open mitochondrial permeabilization transition pore and trigger apoptosis. The Ca^{2+} exchange between these two organelles is mediated by the cooperation between the mitochondrial outer membrane protein voltage dependent anion channel (VDAC) and the inositoltriphosphate receptor (IP_3R) (Baughman et al., 2011).

Except of interaction between VDAC and IP_3R mediated by the cytosolic chaperone glucose regulated protein 75 (GRP75) (Szabadkai et al., 2006), mitochondria can interact with ER through several others proteins such as interactions between ER-bound Mfn2 with mitochondrial Mfn2 and Mfn1 (de Brito and Scorrano, 2008), then interaction between ER b-cell receptor-associated protein 31 (Bap31) and Fis1 (Iwasawa et al., 2011) and the interaction of the protein-tyrosine phosphatase interacting protein 51 (PTPIP51) with the vesicle –associated membrane protein B (VAPB) (De vos et al., 2012). Potential ER-mitochondria interaction proteins are displayed in the fig9.

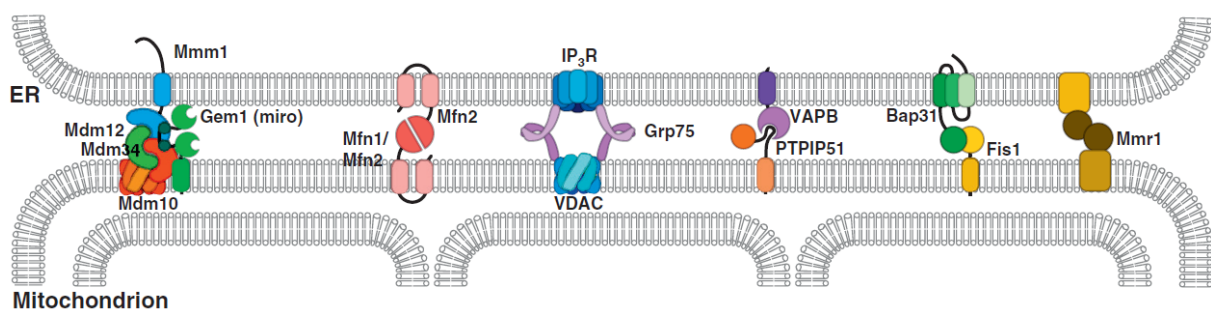


Figure 9. Possible anchoring proteins in ER- mitochondria crosstalk. Adopted from (Kornmann, 2013)

Moreover mitochondria – ER associated membranes were found to be involved in Alzheimer disease since on the contact sites can be found presenilins and γ -secretase activity (Area-Gomez et al., 2009). It was also postulated that increase in ER-mitochondria crosstalk can enhance presenilins and γ -secretase activity which induce amyloid β accumulation and Alzheimer disease progression (Schon and Area-Gomez, 2013).

2.5 Mitochondrial pathologies

2.5.1 Oxidative stress

Oxidative stress has been described to be associated with many pathologies such as atherosclerosis, hypertension, ischemia-reperfusion injury, inflammation, cystic fibrosis, cancer, type-2 diabetes, Parkinson's disease, Alzheimer's disease and other neurodegenerative diseases (Jeřek and Hlavatá, 2005). Oxidative stress is mediated by reactive oxygen or nitrogen species (ROS or RNS). The ROS include superoxide ($O_2^{\bullet -}$), hydroperoxyl radical ($HO_2^{\bullet}$), hydroxyl ($^{\bullet}OH$), carbonate ($CO_3^{\bullet -}$), peroxy ($RO_2^{\bullet}$), alkoxy ($RO^{\bullet}$) and non-radical oxidants such as H_2O_2 , HOCl, fatty acid hydroperoxides (FAOOH), reactive aldehydes, singlet oxygen and others. Likewise, RNS are represented by nitric oxide ($^{\bullet}NO$), nitric dioxide ($^{\bullet}NO_2$), and non-radical peroxyxynitrite ($OONO^{\bullet}$), N_2O_3 , *N*-nitrosamines, *S*-nitrosothiols, nitrosylated fatty acids and others. ROS can be transformed one to another (if ΔG is favourable) by enzymatic and non-enzymatic mechanisms. The ROS is superoxide radical, formed by the mitochondrial electron transport chain, which is spontaneously or enzymatically converted by superoxide dismutase (SOD) to hydrogen peroxide (Fukai and Ushio-Fukai, 2011). In mammals, three SOD isoforms have been described with different metals in reaction centres and different compartmentalization. The mitochondrial matrix form, SOD2, has manganese ion and is therefore called MnSOD (Weisiger and Fridovich, 1973). The Cu, Zn-dependent isoform SOD1 is localized to the mitochondrial intermembrane space and cytosol (Beyer et al., 1991) and finally the SOD3 type of CuZnSOD is secreted to the extracellular space (Marklund, 1984).

The most reactive oxidant from ROS is hydroxyl radical, which attacks DNA bases and has short half-life (Zorov et al., 2014). Hydroxyl radical is generated by various mechanisms, but the most common are from the Haber-Weiss reaction between H_2O_2 and $O_2^{\bullet -}$ and on ferrous reaction centres, such as aconitase.

The most studied mitochondrial sources of ROS are Complex I and III (Esterházy et al., 2008). The ROS production on the complex I increase upon stress or pathological conditions. The ROS formation from complex I has been described in Parkinson's disease (Zoccarato et al., 2005) and hypoxic conditions (Wiesner et al., 1988).

Complex III is an acceptor of electrons from Complex I and II operated by ubiquinol (UQH₂). Ubiquinol is cycling between complexes in so called “Q cycle” (Crofts, 2004). The target site for ubiquinol is Q_o binding site on cytosolic surface of the Complex III, where the first electron is transferred to the Rieske protein and then to cyt *c1* and cyt *c*. It results in a release of two H⁺ ions and formation of UQ^{•-}, which is normally oxidized to UQ by cyt *b_L*. Since oxygen is more soluble in lipid than in water (Thomas et al., 2001), the oxygen dissolved in lipids can interact with UQ^{•-} and gives a rise to superoxide O₂^{•-}. This oxygenation can happen only if the electron transport is slowed by high mitochondrial membrane potential or by other ways (Jeřek and Hlavatá, 2005). The production of ROS from Complex III to mitochondrial matrix has been also described (Fisher and Meunier, 2001). Complex III generates superoxide at hypoxia and thus initiates the proline hydroxylase domain enzyme Fe cofactor oxidation and the resulting HIF1alpha accumulation and resulting transcription upregulation of >100 genes (Chandel, 2010).

It is possible to decrease the ROS production from the electron transport chain by moderate lowering of membrane potential without affecting the ATPase production (Korshunov et al., 1997). Induction of a small proton leak can result in both, increased oxygen consumption and thus lowering the local concentration of oxygen, and lowering of membrane potential, which allows improved reduction states of mitochondrial ROS production sites. Moreover, the existence of mitochondrial uncoupling proteins (UCP) further supports this hypothesis. It has been demonstrated, that inhibition of UCP2 by GDP results in increase of membrane potential and also ROS production (Nègre-Salvayre et al., 1997). The different expression of individual UCPs in different tissues has been described, indicating further importance of lowering of mitochondrial membrane potential (Alán et al., 2009).

There are several other ROS sources in mitochondria such as NADPH-oxidases, monoaminoxidase, p66, α-Glycerophosphate dehydrogenase, electron transfer flavoprotein (ETF) and ETF quinon oxidoreductase and aconitase (Zorov et al., 2014).

2.5.2 MtDNA mutations

The mutation rate of mitochondrial genome is 10 to 17-times faster than of the nuclear genome (Dianov et al., 2001). Since there are more copies of mtDNA occurring in a single cell, the population of mutated and wild-type mtDNA coexist together and their ratio is described as heteroplasmy. The so-called threshold effect is a situation, in which mutated mtDNA reaches a level necessary for disease manifestation. Interestingly, the threshold effect for mtDNA deletions is approximately 70% (Hayashi et al., 1991) and for point mutations in tRNA is 95% (Chomyn et al., 1992). The reason for higher tolerance of tRNA mutations remains unknown but it is speculated that tRNAs due to smaller size can

more easily diffuse throughout the mitochondrial network (Schon et al., 2012). The list of mitochondrial diseases with the best known mtDNA point mutations is described in table 3.

Gene product	Number of mutations	Main disorder
rRNAs		
12S rRNA	5	Deafness
16S rRNA	1	Atypical MELAS
tRNAs		
tRNA Ala	3	Myopathy
tRNA Arg	2	Various
tRNA Asn	5	Myopathy
tRNA Asp	2	Various
tRNA Cys	3	Various
tRNA Gln	3	Various
tRNA Glu	7	Reversible respiratory chain deficiency
tRNA Gly	3	Various
tRNA His	4	Various
tRNA Ile	14	PEO
tRNA Leu(CUN)	8	Myopathy
tRNA Leu(UUR)	23	MELAS
tRNA Lys	14	MERRF
tRNA Met	2	Various
tRNA Phe	14	Myopathy
tRNA Pro	5	Multisystem
tRNA Ser(AGY)	4	Myopathy
tRNA Ser(UCN)	12	Myopathy; deafness
tRNA Thr	2	Various
tRNA Trp	12	Encephalomyopathy
tRNA Tyr	4	Myopathy
tRNA Val	6	Multisystem
Polypeptides		
ATP synthase 6	13	NARP or MILS
ATP synthase 8	2	Various
COX I	10	Various
COX II	8	Various
COX III	6	Myopathy
Cytochrome b	21	Sporadic myopathy
ND1	16	MELAS; LHON
ND2	3	Various
ND3	5	Leigh's syndrome
ND4	5	LHON
ND4L	1	LHON
ND5	12	MELAS
ND6	11	LHON

Table 3. List of mitochondrial DNA mutations in human respiratory chain disorders. Adopted from (Schon et al., 2012).

The clinical syndromes associated with mtDNA mutations are extremely variable and can appear at any stage of life. Generally, mitochondrial diseases can be divided based on the onset in early infancy or in late childhood and adult life. Leigh syndrome belongs in the first group of early manifested diseases belong, which is caused by severe failure of oxidative metabolism and can be caused by mutations in either mitochondrial genome (ATPase 6, tRNA for glycine) or nuclear genome (SURF1 gene) (Finsterer, 2008). Leigh syndrome is progressive neurodegenerative disease affecting brainstem, diencephalon and basal ganglia. Depletion syndrome is associated with decrease of mtDNA, which results in muscle weakness, progressive encephalomyopathy or liver failure. Kearns-

Sayer syndrome (KSS) is characterized by large scale deletions in mtDNA, including so called common deletion, which is 4977 nucleotides long ablation (Krishnan et al., 2008). Patients with KSS very often developed retinis pigmentosa, progressive external opthalmoplegia and other complications such as cerebellar ataxia, cognitive impairment, deafness, cardiomyopathy, complete heart block, short stature, endocrinopathies and dysphagia (Tuppen et al., 2010). Pearson syndrome is very rare disease with large scale deletions in all tissues. It is characterised with sideroblastic anemia with pancytopenia and exocrine pancreatic failure leading to early death.

Among the disorders which develop later in the human life the following disease belong: mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes with seizures (MELAS), chronic progressive external ophthalmoplegia (CPEO), Neuropathy, ataxia, and retinitis pigmentosa (NARP), Leber's hereditary optic atrophy (LHON) and finally myoclonic epilepsy and ragged red fibers (MERRF). Patients with MELAS often present stroke-like episodes with seizures accompanied with intermittent episodes of encephalopathy, vomiting, migraine like headaches, ataxia and cognitive impairment (Tuppen et al., 2010). Over the 80% of patients harbor the m.3243A>G mutation in the mitochondrial tRNA gene for leucine (Goto et al., 1990). CPEO is one of most common mtDNA disorders manifested in adults, characterized by the impaired eye movement and ptosis. The base of CPEO disorder lies in sporadic large-scale single deletions or multiple mtDNA deletions (Van Goethem et al., 2003). Patients with NARP diagnosis harbors a single point mutation in the mitochondrial ATP6 gene and is presented as peripheral neuropathy with other neurological complications including developmental delay, seizures and dementia. The heteroplasmy higher than 95% has an onset in childhood and is called maternally inherited Leigh syndrome (MILS) (Tatuch et al., 1992). Three mtDNA mutations, m.11778G>A in ND4, m.3460G>A in ND1 and m.14484T>C in ND6, are responsible for 95% of LHON cases (Man et al., 2002). LHON is predominantly affecting retinal ganglion cells of the optic nerve. Finally MERRF is a progressive, neurodegenerative disease caused mostly by a point mutation in the mitochondrial tRNA gene for lysine m.8344A>G (Shoffner et al., 1990).

2.5.4 Mitochondrial disorders with nuclear origin

There are approximately 2000 proteins encoded by the nucleus and imported to mitochondria. Mutations in many of them can affect mitochondrial function, dynamic or architecture. An independent set of mutations are mutations in nuclear encoded OXPHOS genes. Mutations in the structural subunits of Complex I and II and in Complex IV assembly factor, SURF1 can cause severe OXPHOS defects and result in the lethal childhood disease, Leigh Syndrome (Zhu et al., 1998). Also mutations in the mtDNA POLG or Twinkle helicase can cause multiple mtDNA deletions and are

associated with the origin of autosomal dominant or autosomal recessive progressive external ophthalmoplegia (Wallace, 2010). Depletion of mtDNA can be manifested in mutations in deoxyguanosine kinase (Mandel et al., 2001) and in mitochondrial thymidine kinase 2 (Saada et al., 2001).

Other mitochondrial pathologies come from mutations in already described fission fusion proteins, mitophagy pathway, mitochondrial biogenesis, mitochondrial enzymes and many others. But also more complex issues have to be taken into account in some age-related diseases such as non-insulin-dependent type 2 diabetes. At present, it is widely accepted that the mitochondria play an important role in type 2 diabetes, but the exact mechanism of insulin resistance origin and linking with mitochondrial abnormalities remains unresolved. The first direct example of mitochondrial involvement in diabetes is mitochondrial diabetes, a rare subform of the disease, which is caused by mutations in mtDNA in pancreatic β -cells (Maechler and Wollheim, 2001). Since the β -cells are poised to adapt insulin secretion to the fluctuations of in blood glucose concentration, thus their mitochondria utilize glucose to ATP. The increased ATP/ADP ratio results in depolarization of plasma membrane by closure of ATP-sensitive K^+ channels (Ashcroft et al., 1994). This allows for opening of voltage-sensitive Ca^{2+} channels and subsequent exocytosis of insulin containing granules (Rorsman, 1997).

In contrast to the rare monogenic mitochondrial diabetes caused by mtDNA mutations, type 2 diabetes is common and polygenic in nature (Busch and Hegele, 2001). This is also case for Goto-Kakizaki (GK) rats, which serve as a model organism for type 2 diabetes. GK rats exhibit down-regulation of Snare family proteins such as Syntaxin1A, Snap25, Vamp2, and nSec1 proteins, all of them prevent exocytosis as well as it has been found in human type 2 diabetes (Östenson and Efendic, 2007). The insulin resistance can be also caused by impaired mitochondrial fitness or increased lipid availability from lipolysis or a high-energy diet (Petersen et al., 2007).

2.5.3 MtDNA haplogroups and prevalence for disorders

Because on the Earth we have many environments, which can be changed quickly humans have developed several types of mtDNA. Our mtDNA cannot recombine with other molecules but has a high mutation rate in comparison with nuclear DNA. It allows mtDNA to generate new mutations each generation by repairing damaged nucleotides after many types of insults, like intoxication or activity of reactive oxygen species (ROS) (Wallace, 2007). If the mutation bring about some advantage, it is quickly fixed as homoplasmic and spread on maternal lineage by rapid segregation (Jenuth et al., 1996). All current human mtDNA evolved from one “mother Eve” mtDNA approximately 200 thousand years ago. During the time as our ancestors migrated around the whole

world, many mtDNA variants evolved and gave rise to mtDNA haplogroups (Fig.10). Those mtDNA variants are often linked with changes in mitochondrial physiology and also with various clinical conditions. Some haplogroups are more prone to milder LHON pathogenic mutations, Alzheimer or Parkinson disease, diabetes, metabolic syndrome and myocardial infarction. Some of them are associated with the decreased risk for developing neurodegenerative diseases, protection against sepsis or metabolic diseases (Wallace, 2008). Moreover, many mtDNA mutations have been found to be associated with increased prevalence in several types of cancer (Wallace, 2005).

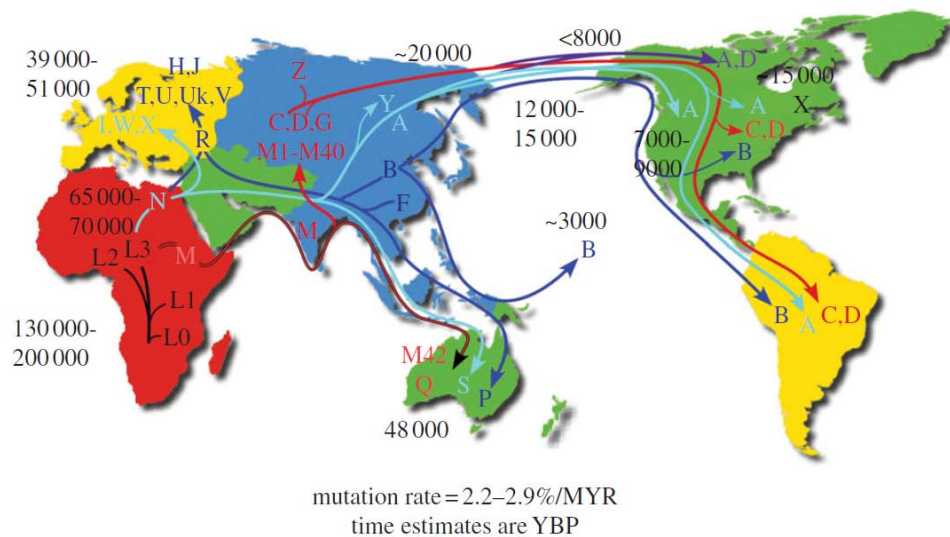


Fig 10. Distribution of mitochondrial haplogroups during human evolution. Adopted from (Wallace, 2007). The mitochondrial “Eve” became from southern part of Africa before 200000 years ago represented as haplogroup L0. During the time, the other mitochondrial haplogroups were formed and spread around whole world.

2.5.5 Aging

Aging is a process accompanied with a structural and functional degradation of cellular components and manifested in many pathological states like cancer, neurodegenerative diseases, metabolic syndrome, sarcopenia, cancer or liver dysfunction (Chung et al., 2009). The first theory on aging appeared almost 60 years ago based on mitochondrial ROS production (Harman, 1956). This was further broadened for the role of mtDNA as a target of ROS and decline in mitochondrial biogenesis in older people (Miquel et al., 1980). The final hypothesis was that the increased ROS production leads to a vicious cycle of increasing levels of mtDNA damage and oxidative stress in the cell (Bandy and Davison, 1990). In contrast to this hypothesis, mice overexpressing PolG without proofreading activity accumulate mtDNA mutations, had impaired respiratory-chain function, shortened life span but the ROS production was unchanged (Hütter et al., 2007). Moreover, overexpression of

mitochondrial antioxidant proteins did not extend life spans and sometimes even led to the life span shortening (Doonan et al., 2008; Zhang et al., 2009). Also supplementation of antioxidants into a diet has no beneficial effect in prevention of age-related diseases and may lead to an increase of mortality in humans (Bjelakovic et al., 2007).

Reduction in nutrient availability termed as caloric restriction extends the life span and improves the health status in many species from yeasts to mammals (Kaeberlein et al., 2007; Weindruch et al., 1986). Additionally, inhibition of insulin signalling or mTOR activity resulted in extended life span (Bonawitz et al., 2007; Holzenberger et al., 2003). All of the studies on caloric restriction or inhibition of mTOR pathway pointed to the increased mitochondrial biogenesis activated through Sirtuin 1 and downstream effector PPAR γ coactivator1- α (PGC-1 α) (López-Lluch et al., 2006). PGC-1 α does not regulate basal mitochondrial biogenesis but rather increase mitochondrial function on demand. Furthermore overexpression of PGC-1 α increases life span in *Drosophila* (Rera et al., 2011). Taken together, organisms are very complicated systems and their aging is caused by more complicated interplay between increased ROS production, mtDNA damage, redox state and impaired biogenesis, rather than by one insult.

3. Materials and methods

If not otherwise specified all chemicals, reagents, plasmids and cells were purchased from Thermo Fisher Scientific, USA.

3.1 Fluorescent protein constructs and fusion protein expression

The vector for the mitochondrial matrix targeting peptide sequence (MTS) conjugated with redox-sensitive GFP (mtRoGFP, a modified EGFP vector of "type 1" containing the MTS of the pyruvate dehydrogenase E1 α subunit) was a kind gift of Dr. Rossignol, Univ. Bordeaux 2, France (Rossignol et al., 2004). The mtRoGFP sequence was amplified by PCR inserted by T4 DNA ligase into the Viral Power system vector, pLenti 6.3/V5-DEST, and ligated by T4 DNA ligase.

The MTS conjugate of fluorescent protein Keima (mtKeima) was prepared using a pRSETB vector containing a tandem dimer Keima sequence, a kind gift from A. Miyawaki (Kogure et al., 2008). The monomeric Keima open reading frame was PCR amplified and inserted into pLenti 6.3/V5-DEST using *Sall* and *MluI* restriction enzymes and T4 ligase. The MTS of cytochrome c oxidase subunit 8, COX8A, was obtained from pcDNA3-mito-EosFPwt (MoBiTec, Germany), amplified using PCR with *EcoRV* restriction site-containing primers, and inserted into the intermediate using T4 ligase so that the ATTATT sequence of the vector was removed and the MTS was fused to the amino-terminal end of the monomeric Keima.

Oligonucleotide expression was made upon the tetracycline inducible promotor from the pENTRH1/TO vector of the BLOCK-iT[®] kit enabling cellular RNA polymerase III to transcribe the RNA oligonucleotide from the vector. The corresponding DNA oligonucleotides with 5'-CACC were inserted into the vector and the resulting construct was transfected into the human embryonic kidney cells, *pre*-disposed for tetracycline-induced expression (T-REx[®]-293 cell line).

3.2 Lentiviral and baculoviral particle production

The pLenti 6.3/V5-DEST-based constructs were overproduced, purified, and used for lentiviral particle production according to the manufacturer instructions. The lentiviral stock was produced in 293LTV cell line, which stably and constitutively expresses the SV40 large T antigen, facilitating optimum lentivirus production. The 293LTV cells were co-transfected by Lipofectamine 2000 and optimized packaging plasmid mix consisting of pLP1, pLP2, and pLP/VSVG, which supplies the helper functions as well as structural and replication proteins required to produce the

lentiviruses. Resulting lentiviral stock was prepared by filtration of 293LTV cultivation medium and concentrated by PEG-it Virus Precipitation Solution (System Biosciences).

Baculoviral particles containing TFAM-EGFP were a kind gift of Dr. Werner Koopman, Radboud University Medical Centre, Nijmegen, Netherlands).

3.3 Molecular beacon design

Standard human mtDNA database was used for mtDNA sequence and probes were designed against regions as illustrate in Fig.X A,B. Thus a molecular beacon ND5 probe (No.3 in Fig.1) for annealing to the L strand (antisense) base pairs (bp) 12925 to 12946 complementary to a portion of the ND5 gene (H strand bp 12337 to 14148) was designed as follows:

5'-Cy3-CGA CGG AAG GGC TAT TTG TTG TGG GTC TCG TCG-BHQ2-3'.

This probe has to anneal also to the mitochondrial polycistronic HSP RNA (Fig.1A) which might be also a part of RITOLS (Fig.1B) and the corresponding ND5 mRNA. Specifically, the ND5 mRNA should not be localized only at mitochondrial nucleoids, but should diffuse all over across the matrix space in its mission to mitochondrial ribosomes. Replicating nucleoids should have twice intensive signal, due to doubling of the L strand.

The two other probes were designed for annealing to the D loop region of the H strand (bp 16024 to 574). The probe No. 2 (Fig.1A), called "7S DNA probe", should anneal to the H strand bp 16109 to 16126, which in fact is a portion of the nascent 7S DNA that is displacing this H strand (Fig.1A,B). Naturally, the probe should anneal also to the 7S DNA. Moreover, the corresponding region of LSP RNA (controlled by the LSP_{major}) should bind this probe as well, either before self-cleavage at the first tRNA gene location or afterwards (Fig.1A). The 7S DNA probe was therefore designed as:

5'-Cy3-ACC GTA GCC ACC ATG AAT ATT GTA CGG T-BHQ2-3'

We suppose that even if such cleaved LSP RNA might diffuse out of mitochondrial nucleoids, its degradation might be faster than that of mRNA. Even faster degradation for the corresponding LSP RNA was assumed in case of the most proximal part of D loop. That is why the third probe, probe No. 1, was designed to anneal to the H strand bp 524 to 547. This is the initially displaced portion of the D loop by the priming RNA portion of LSP RNA, to which this probe has to anneal as well (Fig.1A). This "proximal D-loop probe" or "LSP_{minor} RNA probe" was designed as:

5'-Cy3-ACCTGACACACACCGCTGCTAACCCCATCAGGT-BHQ2-3'.

3.4 Fluorescent *in vivo* hybridization (FIVH) probe design

To achieve import of desired nucleic acid sequences into the mitochondrial matrix, we have employed a defined pathway that facilitates import of 5S rRNA (Magalhães et al., 1998; Rorbach and Minczuk, 2012; A. Smirnov et al., 2008; A. V Smirnov et al., 2008). Although 5S rRNA has not yet been definitively confirmed to be the component of mt ribosomes, it is translocated naturally into mitochondria and even certain mutations of its mitochondrial matrix-addressing motif (MAM) sequence improve such an import (A. Smirnov et al., 2008). We have designed an RNA probe system as a carrier for the desired RNA sequence (principally DNA sequence as well), employing the optimized MAM of 5S rRNA (A. Smirnov et al., 2008), *i.e.*, mutated to its most efficient version (Tab.4.). Thus our fluorescent oligoribonucleotide probes contained the mutated most efficient version of MAM and an exemplar cargo RNA sequence (Tab.4.). In our case, the exemplar cargo was the RNA sequence annealing to a short portion of ND5 mRNA which also has the ability to anneal to the L-strand mtDNA complementary to the H-strand *nd5* mt gene (5'-end 21 base pair sequence) (Zhadanov et al., 2007), as the prototype targeting sequence. A fragment of the human mt genome (NC_012920) (describing the L-strand) corresponding to the base pairs 12 341 to 12 361 was designed to be attached after the MAM in the sequence of the resulting "MAM-L-ND5" RNA probe (Tab.4.). Note that the *nad5* gene is located within the H strand between the base pairs 12 337 to 14 148.

Table 4. Design of *in vivo* hybridization probes

Native 5S rRNA sequence

5'-GTCTACGGCCATACCACCCTGAACGCGCCCGATCTCGTCTGATCTCGGAAGCTAAGCAGGGTCG
GGCCTGGTTAGTACTTGGATGGGAGACCGCCAA GGAATACCGGGTGCTGTAGGCTTT-3'

MAM-L-ND5 & PCR primers

5'-GGCCTGGTTAGTACTTGGATGGG-3'
5'-GGCCTGGTTAGTACTTGGATGGGAGACCGCCAA GGAATACCGGGTG TGGTTATAGTAGTGTGCATG-3'
3'-GGTTCCTTATGGCCACACCA-5'

MAM-000

5'-GGCCTGGTTAGTACTTGGATGGGAGACCGCCAA GGAATACCGGGTG-3'

000-L-ND5

5'-TGGTTATAGTAGTGTGCATG-3'

Scrambled & PCR primers

5'-GGCCGAGCGCAGAAGTGG-3'
5'-GCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCTGCAACTTATCCGCCTCCATCCAGTCTATTAATGC-3'
3'-TTGAATAGGCGGAGGTAGGTC-5'

The custom-synthesized DNA template, containing the 5'-flanked T7 RNA polymerase promoter TAATACGACTCACTATAG was designed to obtain the MAM-L-ND5 ribonucleotide probe (Tab.4.) after *in vitro* transcription with the aid of RiboMax Kit (Promega, USA). After purification, 3' end of the resulting RNA was activated by sodium periodate and subsequently labeled with Alexa Fluor 647 hydrazide or AlexaFluor 488 hydrazide. The resulting probe was purified by LiCl/ethanol precipitation. This approach ensures the defined, pure and correctly folded fluorescent probe. For direct transfections of the constructed fluorescent RNA probes into the cells (Tab.4.), the following probes were used (Tab.4.): "full probe", termed MAM-L-ND5, theoretically capable of binding to the above specified short portion of ND5 mRNA plus to the light-strand mtDNA complementary to the same corresponding portion of the heavy strand *nd5* mt gene. As a second probe, termed MAM-000, was the probe without the ND5 cargo; whereas the third probe was lacking the MAM address, as termed 000-ND5. Finally the fourth probe was designed to contain the scrambled sequence, as termed "Scrambled". Fluorescent probes were transfected into mitochondria of HepG2 cells expressing mitochondrial-matrix-addressed GFP or KEIMA using either Lipofectamine 2000 or DQAsomes (Niazi et al., 2013). Colocalizations of mtGFP with Alexa Fluor® 647 or mt KEIMA with Alexa Fluor® 488 was analyzed by confocal microscopy.

3.5 Nucleic acid transfections

Cells were transfected by Lipofectamine 2000 or Lipofectamine RNAiMax according to manufacturer instructions. Typical transfection of cells on coverslip contained mix of 5uL of Lipofectamine with 250uL of Opti-MEM medium and 3µg of plasmid DNA or 20µM of silencer select siRNA.

3.6 Cell cultures

The human hepatocellular carcinoma HEPG2 cell line (ECACC 85011430) was cultivated at 37°C in humidified air with 5% CO₂ in DMEM medium (contains no glucose) supplemented with 3 mM glutamine, 1 mM sodium pyruvate, 10% (v/v) fetal calf serum (Biochrome), 10 mM HEPES, 100 IU/ml penicillin, and 100 µg/ml streptomycin. The supplemented carbon source was 25 mM glucose (GLC25 cells), 5 mM glucose (GLC5 cells), or 10 mM galactose (OXPHOS cells); for the latter, glucose-free dialyzed fetal calf serum was used (PAA Laboratories, Austria). For confocal

microscopy, HEPG2 cells were cultured for 2 days on glass coverslips coated with poly-L-lysine and transfected or fixed according to experimental design.

3.7 Immunocytochemistry and cell staining

Immunostaining with primary anti-mtSSB or anti-TFAM or alternatively anti-DNA was followed with secondary Alexa-647-, Alexa-568- or Alexa-488-conjugated antibodies. Samples were fixed for 20 minutes with 2.5% paraformaldehyde and 0.025% glutaraldehyde in PBS and washed three times in PBS. All subsequent steps were done in washing buffer containing 0.05% (w/v) Tween 20 (Sigma Aldrich, Germany), 0.05% (w/v) Triton X-100 (Sigma Aldrich) and 0.3 M glycine (Sigma Aldrich). Then samples were blocked with 5% donkey serum for 45 minutes and then incubated for 1 hour with the appropriate primary antibodies. Coverslips were washed three times in washing buffer and incubated with corresponding secondary antibodies for one hour. Last three washes prior to microscopy were done in PBS.

Live cell staining of mitochondria were performed using Tetramethylrhodamine, Ethyl Ester (TMRE) staining of 10 nM final concentration for 5 minutes. Mitochondrial nucleoids were stained by SYTO16 in 20 μ M final concentration for 5 minutes.

3.8 Fluorescence *in situ* hybridization

Standardized protocol for fluorescent *in situ* hybridization of mtDNA or mtRNA was adjusted as follows: Cells were fixed in 0.3% glutaraldehyde in PBS for 20 min at 37°C, followed by two washes with 1 mg/ml NaBH₃ (Sigma Aldrich) in PBS for 15 min to reduce unbound glutaraldehyde molecules. Nuclease treatment then followed. Partial treatment with RNase A, removing single strand RNA (ssRNA) was performed for two hours in PBS at 37°C. To degrade RNA from RNA/DNA hybrids we used RNase H digestion for 30 min at 37°C. DNase I treatment, which digests double strand DNA (dsDNA), proceeded for 30 min at 37°C. DNase I and RNase H treatment were performed in a reaction buffer prepared according to the manufacturer's protocol. Coverslips were pre-hybridized in 50% formamide (Sigma Aldrich) with salmon sperm DNA and 0.2% Tween in twice concentrated saline sodium citrate (Sigma Aldrich) ("2xSSC") buffer for 5 min at 72°C. 100 ng of molecular beacon in 50% formamide with 0.2% Tween in 2xSSC buffer were add on the coverslip and hybridized overnight at 37°C. Coverslips were then washed in 0.2% Tween with 50% formamide in 2xSSC for 5 min at 42°C, followed by three washes in 0.2% Tween in 2x SSC.

3.9 Confocal microscopy

An inverted confocal fluorescent Leica TCS SP2 AOBS microscope was used with a PL APO 100×/ 1.40-0.70 oil immersion objective (a pinhole of 1 Airy unit). SYTO16, RoGFP, EGFP, and tdKEIMA Alexa 488 were excited at 488nm with a 20 mW argon laser, whereas tetramethylrhodamine, ethyl ester (TMRE), and Alexa-568–conjugated antibodies were excited at 543 nm with a 1.2-mW HeNe laser. Alexa-647–conjugated antibodies were excited at 633 nm by a 10 mW HeNe laser. Live cells were imaged in combination with thermostable sample chamber set to 37°C supplied with 5% CO₂, thus mimicking normal cultivation conditions.

3.10 Structured illumination microscopy (SIM)

Custom imaging at the Department of Cell Biology, School of Medicine, Yale University, New Haven, CT, USA, was done by a structured illumination microscope OMX (Applied Precision, Issaquah, WA) with an Olympus 60×, 1.42NA, Plan Apo N oil immersion objective and a CoolSNAP HQ2 camera (Photometrics, Tucson, AZ) and used a 488 nm laser for excitation. Series of 20 to 50 z stacks were taken with a step size of 125 nm, hence the total of z depths was 2.5 to 6.75µm.

3.11 Image analysis

The total number of nucleoids was counted as follows: After basic contrast and brightness adjustment, images or their overlays were manually thresholded, and nucleoids were counted by the “Analyze Particles” function of Image J software (National Institutes of Health, USA). Cell size and tubule/object length were measured after choosing the appropriate scale using the “Measure” function of the software.

3.12 Pancreatic islet isolation

Wistar rats and Goto Kakizaki rats (Taconic, Denmark) were bred, housed, and sacrificed in accordance with the European Guidelines on Laboratory Animal Care and according to the Institute of

Clinical and Experimental Medicine licensing committee approval. GK rats were derived by embryo transfer from Danish (Aarhus) colony established in 1994 from the original Tohoku University (Japan) colony initiated in 1975. The 4-, 6-, and 12-month old Goto Kakizaki rats were taken for this work. PI samples were isolated from one Wistar rat and always from two Goto Kakizaki rats, weighting over 420 g at 12 months (over 360 g at four months) and exhibiting usually hyperglycemia between 16 to 17 mM glucose and crimped intravenous glucose tolerance test (~15 to 20 mM glucose remained 60 min after glucose intake). Wistar rats were non-diabetic (normo-glycemic and normal intravenous glucose tolerance test), weighting over 600 g at 12 months (over 490 g at four months). PI isolation was performed in cooperation with Institute for Clinical and Experimental Medicine in Prague. Briefly, pancreases were distended by an infusion of 15 ml collagenase (1mg/ml; Sevapharma, Czech Republic) and the islets were separated from exocrine tissue by centrifugation in a Ficoll discontinuous gradient (Sigma, USA). Purified islets were cultivated in RPMI 1640 medium without glucose supplemented with 10% fetal calf serum, 1% penicillin/ streptomycin/ L-glutamine, 1% HEPES, and 5 mM glucose, in a humidified CO₂ incubator at 37°C and 5% CO₂ atmosphere. Islet viability was checked by propidium iodide and acridine orange methods.

3.13 β -Cell isolation

β -cells were isolated after PI treatment with StemPro Accutase, cell dissociation reagent (1 ml per 300 to 1000 islets, while waiting for islet disintegration); and were washed with glucose-containing RPMI 1640 medium. A FACS Vantage SE cell sorter was used to separate cells positive on the Zn²⁺ signal of Newport Green DCF into the alkaline lysis buffer according to Tsuchiya et al (Tsuchiya et al., 2005).

3.14 DNA isolation from tissues and cell lines

Selected Wistar or Goto Kakizaki rat tissues were collected to cryotubes and frozen until needed. Samples were then homogenized by scissor and further homogenized on ultrasonic homogenizer (Cole-Parmer, USA) in lysis buffer. In parallel cell cultures were collected by trypsinization, spun down at 220 g for 8 minutes and resulting pellets were dissolved in 600 μ l of lysis buffer. Next steps were same for both types of samples. Lysates were treated with proteinase K (Sigma Aldrich) for 3 hours and then mixed with phenol-chloroform-isoamyl alcohol (25:24:1) and spun down at 10000x g for 10 minutes. The upper aqueous phase was collected, mixed with chloroform-isoamyl alcohol (24:1) and again spun down. The upper aqueous phase was collected and mixed with 10% of 3 M sodium acetate and one volume of isopropanol. Samples were mixed, stored overnight in

the freezer and then centrifuged 19000x g for 20 mins at 4 °C. Obtained pellets were washed in 70% ethanol again centrifuged, air-dried and dissolved in TE buffer. Dissolved DNA was treated with RNase A and again phenol-chloroform purified. Final RNA free DNA was analyzed on NanoDrop spectrophotometer ND-1000. Samples were diluted to 10ng/ml prior to quantitative PCR (qPCR).

3.15 Mitochondrial copy number

Amount of mtDNA was quantified by qPCR as a ratio of a mitochondrial encoded gene to a nuclear encoded gene. As the mitochondrial gene, ND5 was taken (NC_012920.1 - 12337 to 14148 bp for human, or NC_001665.2 - 11736 to 13565 bp for rat, according to Genbank sequence from National Center for Biotechnology Information – NCBI, USA) as the nuclear encoded gene, UCP2 gene was used (NC_000011.9 for human, or NC_005100.3 for rat). Both qPCR reactions were performed in triplicates on a LightCycler 480 (Roche, Germany) using SYBR Green II Master mix. In case of mtDNA analysis of β -cells the mtDNA copy number was determined by 10 cycles of PCR preamplification for ND5 gene, followed by qPCR with SYBR Green detection. The concentration of nuclear gene prior to the preamplification step was not sufficient to perform qPCR. Due to the pre-amplification step, it was not possible to derive copy number just by dividing amount of amplicon by number of cells and therefore only the relative quantification was used.

PCR primers used for qPCR:

Human ND5 forward primer

5'- CAG TCT GCG CCC TTA CAC AAA A – 3'

Human ND5 reverse primer

5'- TGG ACC CGG AGC ACA TAA ATA – 3'

Human UCP2 exon-intron interface forward primer

5'- GGC AGC TTT GAA GAA CGG GAC – 3'

Human UCP2 exon-intron interface reverse primer

5'- CAC AGG GTT AGG AGG CAG CAA – 3'

Rat ND5 forward primer

5'- GAC TAC TAA TTG CAG CCA CAG – 3'

Rat ND5 reverse primer

5'- GTA GTA GGG CAG AGA CGG GAG – 3'

Rat UCP2 exon-intron interface forward primer

5'- AGA ACG GGA CAC CTT TAG AGA – 3'

Rat UCP2 exon-intron interface reverse primer

5'- TGC CCC AGT TTC CAT CAC AC – 3'

Human beta actin forward primer

5′ - GCG GGA AAT CGT GCG TGA CAT – 3′

Human beta actin reverse primer

5′ - GAT GGA GTT GAA GGT AGT TTC – 3′

3.16 Import verification by qPCR

The import of the designed RNA probes into the mitochondrial matrix was tested by reverse transcription qPCR of (RNase washed) mitochondrial fraction isolated from the cells or from whole cell lysate. The import efficiency was taken as the relative amount of amplicon for the MAM-L-ND5 construct. Standardization has been achieved by employing β -actin as the cytosolic marker and the native ND5 mRNA (same as for mtDNA copy number assay) as the mitochondrial matrix marker.

3.17 Agarose electrophoresis and Southern blots

Isolated mitochondria were prepared by homogenization of HepG2 cells with Dounce homogenizer in 180 mM KCl, 5mM K-MOPS, 2 mM K-EGTA pH 7.2. The homogenate was centrifuged at 1,000x g for 10 minutes to pellet the cell debris while mitochondria were harvested at 15,000x g for 15minutes. From the obtained mitochondrial fraction, mtDNA was prepared by alkaline lysis followed by isopropanol precipitation. Resulting mtDNA was separated by agarose electrophoresis (0.4% gel) with GeneRuler DNA ladder. DNA was subsequently blotted to Amersham HyBond N⁺ membrane (GE Healthcare, USA) using semidry blotting equipment TE77XP (Hoefer, USA). DNA was stabilized on the membrane by heating to 80°C for 2 hours. Membrane was then *pre*-hybridized at 42°C for 2 hours in 5× SSC buffer with 50% formamide and 0.2% SDS containing 10 $\mu\text{g}.\text{ml}^{-1}$ of pre-boiled salmon sperm DNA as a blocking agent. Hybridization proceeded in the same buffer with 1 $\mu\text{g}.\text{ml}^{-1}$ of the MAM-L-ND5 probe conjugated with AlexaFluor 488 at 42°C overnight. Membrane was then washed and analyzed. While a direct densitometric detection failed to show any signal, the membrane was cut into defined pieces, boiled in distilled water for 10 minutes and the fluorescence of particular supernatants was measured by spectrofluorimetry (RF-5301-PC, Shimadzu, Japan).

3.18 Islet respiration measurements

Oxygen consumption of one hundred PI used in a chamber was calculated from changes of oxygen concentration, measured by HIOXY probe and NeoFox Phase Measurement system (Ocean Optics, USA), in a 175 micro liter tempered chamber.

3.19 Cellular ATP content

The ATP content during the agent treatments was assayed using the Luciferase ATP assay kit (Roche).

3.20 Buffers

- DNA Lysis buffer
10mM Tris-HCl (pH 8.0)
1mM EDTA
0.1% SDS
- 5x TBE buffer – for DNA electrophoresis
2.34g EDTA
27g Trizma base
13.75g boric acid
500ml distilled water
- 0,01M PBS (Phosphate Buffered Saline)
8g NaCl
2.9g Na₂HPO₄ x 12 H₂O
2g KH₂PO₄
2g KCl
1000 ml distilled water

- Mitochondrial isolation buffer
 - 180mM KCl
 - 5mM K-MOPS
 - 2 mM K-EGTA pH 7.2
- Immunocytochemical washing buffer
 - 0.05% (w/v) Tween 20
 - 0.05% (w/v) Triton X-100
 - 0.3 M glycine
 - PBS

3.21 Instruments

- Laminar flow cabinet: Herasafe HS12/2, Heraeus instruments
- Laminar flow cabinet: Herasafe KS12, Heraeus instruments
- Direct Heat CO₂ incubator: Innova CO-170, New Brunswick Scientific
- Centrifuges:
 - o Hermle Z323K
 - o Eppendorf 5417R
- Horizontal gel electrophoresis apparatus: Hoefer HE33, USA
- Power supply units for electrophoresis: Amersham EPS301
- UV transilluminator: Uvitec – UK
- NeoFox Phase Measurement system: Ocean Optics, USA
- Ultrasonic homogenizer: Cole-Parmer, USA
- Real-time PCR: LightCycler 480, Roche
- UV-Vis Spectrophotometer: Nanodrop ND-1000, Thermo Fisher Scientific
- Spectrofluorimeter RF-5301-PC: Shimadzu, Japan
- Inverted microscopes:
 - o Olympus IX 71
 - o Confocal microscope Leica TSC SP2
- pH-meter: PHM210, MeterLab
- Analytical laboratory scales: AG204, Mettler Toledo
- Other common laboratory equipment: vortex, pipettes, tips, flasks and other glass equipment, incubators, etc.

4. Aims of the thesis

The general goal of the thesis was to develop and established techniques for monitoring mitochondrial nucleic acid species, and proves their specificity and reliability on selected models of diabetes and cancer. The main focus was on visualization techniques for confocal microscopy with the projection for the super-resolution microscopy techniques together with establishing qPCR techniques for monitoring mtDNA and mtRNA levels.

The specific aims were:

- A. To develop *in situ* hybridization of the sequence specific molecular beacons against different mitochondrial RNA species and test the possibilities of nucleic acid digestions for contrasting mitochondrial replication intermediates.
- B. To develop the techniques for import of artificial RNA sequences into mitochondria with stress on visualization potential.
- C. To investigate the distribution of mitochondrial nucleoids upon mitochondrial network fragmentation and following reintegration induced by oxidative stress or by changing mitochondrial membrane potential or by altering mitochondrial fusion machinery.
- D. To investigate changes in the mtDNA amount in β -cells during ageing of diabetic model of Goto Kakizaki rat strain.

5. Results of selected publications

5.1 Publication A: Fluorescent *in situ* hybridization of mitochondrial DNA and RNA

Synopsis of results of the article “**Fluorescent *in situ* hybridization of mitochondrial DNA and RNA**”, by Alán et al., published in Acta Biochimica Polonica 2010.

The objective of this study was to develop sequence specific hybridization of fluorescent probes against different kinds of mtRNA species. The second goal was to investigate possibilities of various types of nuclease treatments and their impact on mtRNA distribution while aiming on visualization of mtDNA replication intermediates.

All the experiments were done on human hepatocellular carcinoma HepG2 cell line. The probes were designed based on molecular beacon schema containing fluorophore and quencher at both antiparallel cohesive ends usually used for qPCR (Santangelo et al., 2005). All the probes were designed according to Revised Cambridge Reference Sequence of the human mtDNA (Andrews et al., 1999). The first probe called ND5 was designed to hybridize with corresponding ND5 mRNA and to ND5 DNA sequence on the L-strand of mtDNA (Fig 11). The ND5 probe has a potential to anneal also to RITOLS replication intermediates. Two other probes were designed to anneal to the D-loop region of H-strand. We called these probes 7S DNA probe and light strand promoter (LSP) minor probe. The 7S DNA probe hybridizes to the L-strand of mtDNA, to the 7S DNA, which represents primer for mtDNA replication and to RNA from the light strand promoter. Finally, the LSP minor probe was designed to hybridize with L-strand of mtDNA and with RNA transcribed only from the minor L-strand promoter. By such a design, we expected the lowest RNA signal in mitochondria.

To test the specificity, we measured fluorescence of our molecular beacons alone or co-incubated with cytosolic or mitochondrial RNA. The signal from molecular beacons incubated with mitochondrial RNA showed significant, nearly 300% increase in signal in comparison to cytosolic RNA (Fig 11). Moreover, the signal from molecular beacon with two times higher concentration of mitochondrial RNA behaved in dose dependent manner (Fig 11).

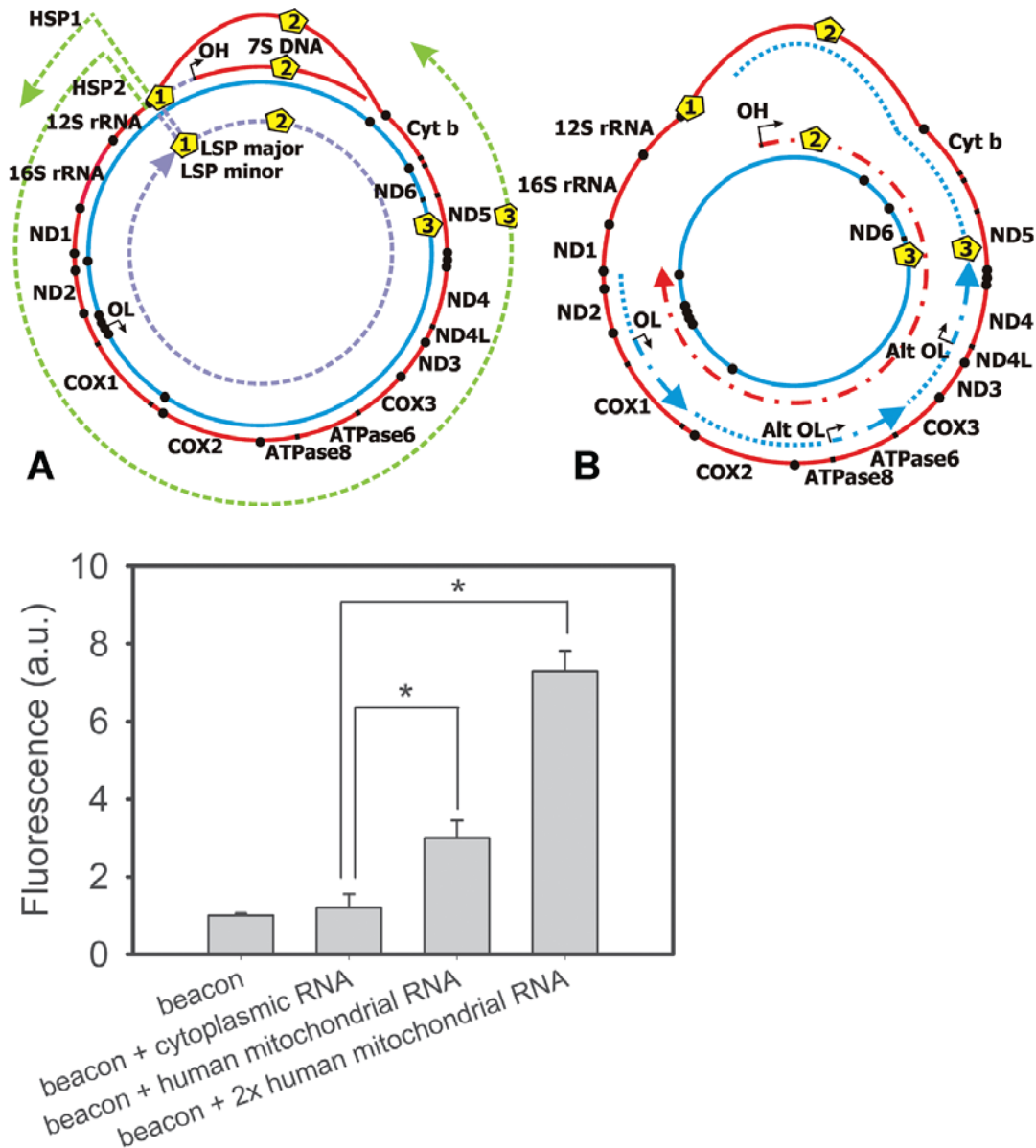


Figure 11. Schema of possible hybridization of the developed probes to mitochondrial DNA and RNA structures and transcription (A) and replication (B). **A)** Transcription schema illustrates the heavy (H, red) and light (L, blue) strands of ds circular mtDNA, where borders between mt genes (their common abbreviations used in bold) are indicated by small black squares and tRNA genes are localized as black dots; and two possible polycystronic RNAs derived from them (HSP RNA green and LSP RNA violet dashed lines) with indicated transcription direction. The displacement (D) loop region contains permanent third strand with OriH ("OH"). The possible hybridization positions of molecular beacon probes are denoted by numbers in yellow pentagons: 1) the proximal D loop probe, termed also LSP_{minor} RNA probe; 2) the 7S DNA probe; and 3) the ND5 probe. **B)** Replication schema uses the same graphics as A) plus RITOLS are illustrated by the blue dashed line, while the nascent H and L strand by the red and blue dot-dashed line, respectively. For the strand-coupled bidirectional replication model, alternative origin sites (Alt OL) are designed as the nascent L strand starts to be synthesized at them and replaces protecting RNA, i.e., RITOLS. Theoretically, thus the ND5 probe (No.3) could anneal to the corresponding RITOLS, to nascent L strand plus to the original L strand in a replicating nucleoid.

To further confirm the specificity of our probes we performed fluorescence *in situ* hybridization on cells permanently expressing green fluorescent protein with mitochondrial targeting sequence or transduced with TFAM-EGFP for mitochondrial nucleoid staining. Both approaches confirmed mitochondrial localization of our probes and concentration of the 7S DNA probe in the vicinity of mitochondrial nucleoids (Fig 12).

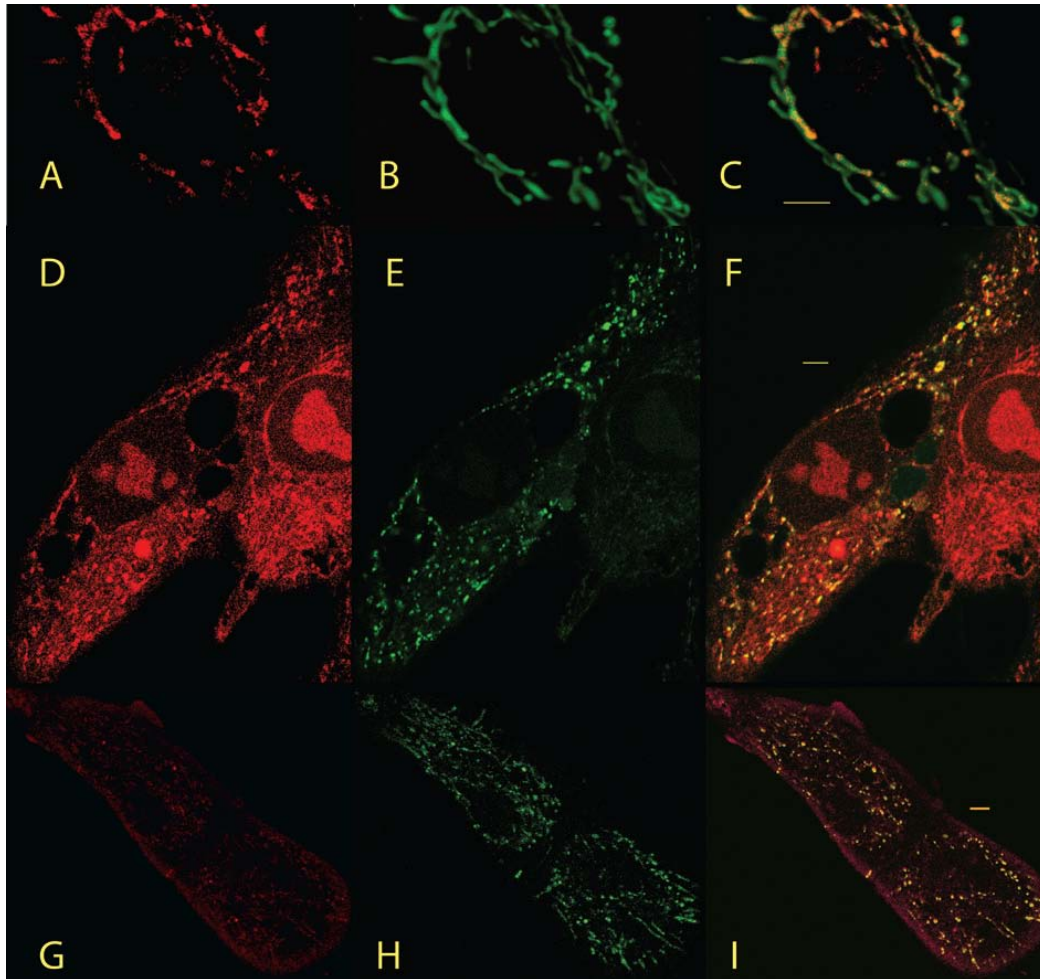


Figure 12. Colocalization of molecular beacon probes with tubules of mitochondrial reticulum and with nucleoids of mtDNA. Confocal images of **A)** ND5 molecular beacon probe; **B)** matrix addressed RoGFP; and **C)** overlay of A plus B illustrate co-localization of ND5 probe with tubules of mitochondrial reticulum. Confocal images of **D)** ND5 probe and **G)** 7S DNA probe show more punctuate character of the 7S DNA probe image and its similarity to the EGFP-TFAM visualized mt nucleoids (**E** and **H**). The overlays show clearly that the ND5 probe hybridizes also to ND5 mRNA spread (plus to HSP RNA diffused out of mt nucleoid) all over the matrix space of mitochondrial reticulum tubules (**F**), whereas the 7S DNA probe is still more concentrated in mt nucleoids (**I**). Scale bars at images represent 5 μ m.

Using three different probes we were able to observe changes in the distribution of corresponding nucleic acids. A comparison of fig 12D vs 12G illustrates the difference between ND5 mRNA and non-coding RNA and DNA corresponding to selected sequences. The ND5 probe was spread around the whole mitochondria (Fig. 12D and Fig. 13A), while both non-coding D-loop probes showed more punctuate pattern of distribution (Fig. 12G, Fig. 13E and Fig. 13I). *Post-fixational* treatment of the cells with RNase A, digesting single strand RNA, resulted into the punctuate character of mitochondrial signal from ND5 probe (Fig. 13B) and to mote clear visualization of spots from D-loop probes (Fig. 13F and Fig 13 J), which probably corresponds to nucleoids. Further treatment with DNase I, digesting DNA, and RNase H, digesting RNA from RNA-DNA hybrids, decreased the signal from mitochondria and increased background signal from D-loop probes (Fig. 13G, H and Fig 13K, L).

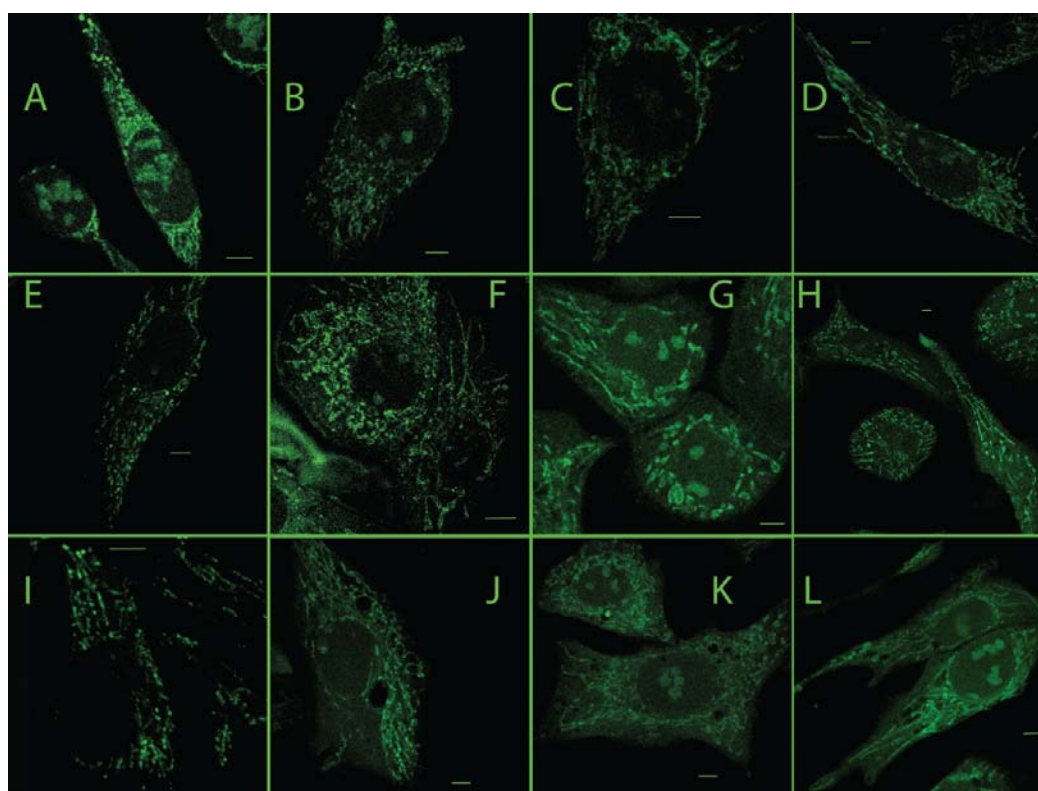


Figure 13. Confocal images of three different beacon probes prior and after treatment with RNase A, or RNase A plus RNase H, and DNase I. Note, unlike in Fig. 2, now the green channel is used for molecular beacon probes. Confocal images are shown for the ND5 probe (A) to D)); the 7S DNA probe (E) to H)); and the proximal D loop probe (annealing also to LSP_{minor} RNA) (I) to L)) in the absence of any further treatment (A), E), I)); after treatment with RNase A, digesting ssRNA (B), F), J)); after treatment by RNase A plus RNase H treatment, when the latter digesting RNA from RNA-DNA hybrids (C), G), K)); and, after treatment with DNase I plus RNase H (D), H), L)). Scale bars at each image represent 5 μ m.

My contributions to this work were the design of the molecular beacon probes, the establishment of the hybridization protocol and the performance of the confocal microscopy.

5.2 Publication B: Import of desired nucleic acid sequences using addressing motif of mitochondrial ribosomal 5S-rRNA for fluorescent *in vivo* hybridization of mitochondrial DNA and RNA

Synopsis of results of the article “**Import of desired nucleic acid sequences using addressing motif of mitochondrial ribosomal 5S-rRNA for fluorescent *in vivo* hybridization of mitochondrial DNA and RNA**”, by Zelenka et al., published in Journal of Bioenergetics and Biomembranes 2014.

The aim of this study was to develop and establish *in vivo* hybridization system to enable direct visualization of the selected nucleic acids entities in mitochondria.

We have tested a mitochondrial import system based on natural mitochondrial ribosomal 5S rRNA (termed MAM). Thus, we have constructed DNA oligonucleotides, containing MAM and exemplar cargo, annealing sequence to a short portion of ND5 mRNA. Such a probe was done by *in vitro* transcription, labeled with Alexa Fluor 488 or Alexa Fluor 647 dye and consequently delivered into the cells by transfection with dequalinium micelles. The verification of import into the mitochondrial matrix of cultured HepG2 cells was provided by confocal microscopy colocalizations. Alternatively, we have reached similar results by employing the same oligonucleotides containing 5'-CACC overhang (substituting T7 promoter) but now subcloned into the pENTRH1/TO vector and transiently transfected in the HepG2 cell line or stably inserted into the human embryonic kidney (HEK) 293 cells predisposed for tetracycline-induced expression (T-Rex).

We have first attempted to employ sole native 5S rRNA import pathway in HEK 293 cells containing our MAM-L-ND5 DNA integrated in the genome. The expression of the MAM-L-ND5 RNA was induced for 72 h prior to RNA extraction. Figure 14a shows a relative abundance of MAM and scrambled RNA within the total RNA extracted from the isolated mitochondria, treated or untreated with RNase A. Better mitochondrial import efficiency was obtained during transient transfection plasmid DNA with our probe (Figure 14b). The MAM-L-ND5 RNA was three orders of magnitudes more frequent than natural matrix ND5 mRNA (Figure 14c). Finally, Figure 2D verifies the validity of isolated mitochondrial fractions, demonstrating the enrichment of MAM and natural matrix ND5 mRNA in RNA extracted from the isolated mitochondria, in contrast to cytoplasmic marker β -actin, which was found negligible in mitochondrial fractions.

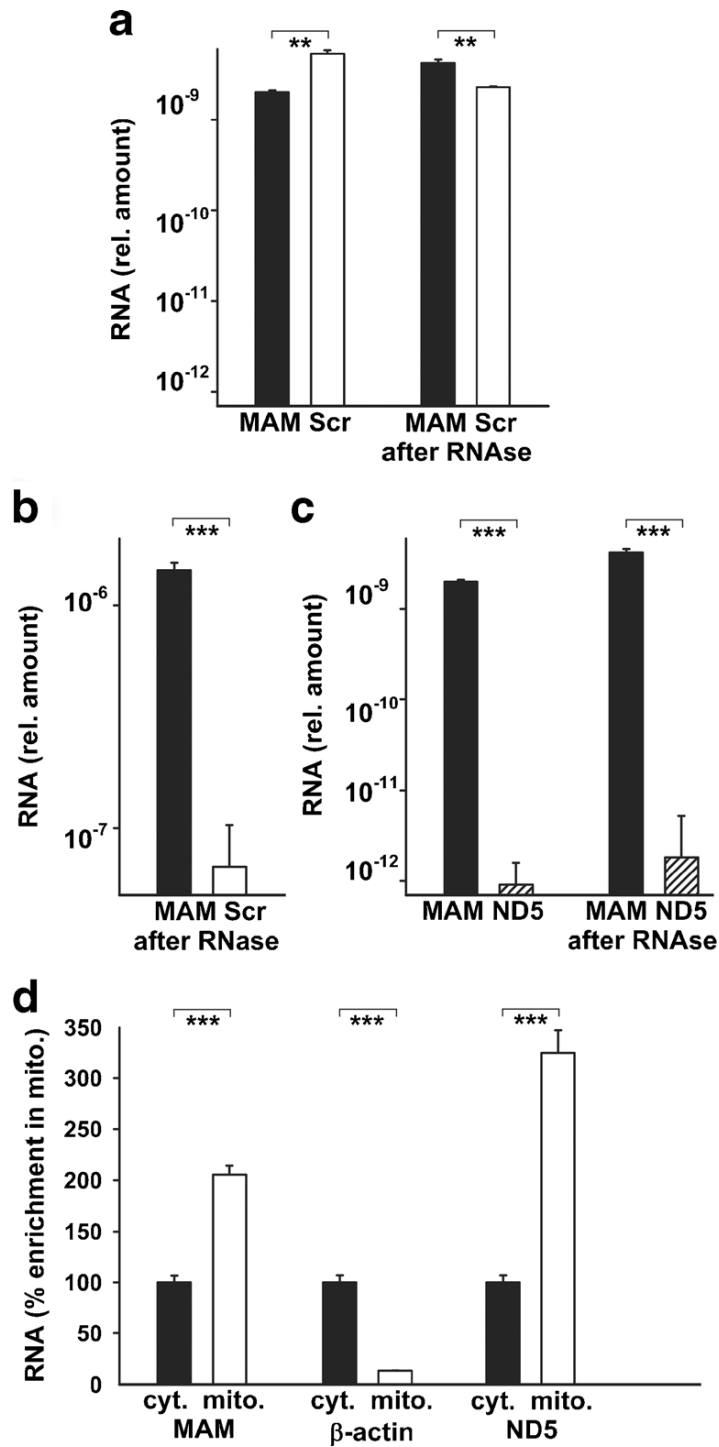


Figure 14. Import of recombinant MAM-L-ND5 RNA into mitochondria as expressed from a Tetracycline-inducible pENTRH1/TO vector. *a*, *c*, *d* permanently transfected T-REx®-293 cell line, 72 h induction, *b* transient transfection in HepG2 cells; Relative abundance within total RNA extracted from mitochondria isolated with RNase-A treatment when indicated after expression from pENTRH1/TO vector containing the MAM-L-ND5 probe, or Scrambled probe. *c* compares the natural ND5 mRNA content (distinct primer sequences from the “cargo ND5 probe” part of ND5 sequence) with the imported MAM-L-ND5 probe. Panel *d* displays the relative mitochondrial enrichment vs. cytosol of MAM-L-ND5 RNA (“MAM”), cytoplasmic marker β -actin, and natural ND5 mRNA in RNAs extracted from the isolated mitochondria vs. those extracted from the whole cells. Standard deviations are indicated. ANOVA (Student’s *t*-test for a pair) yielded ** $p < 0.05$; *** $p < 0.001$ ($n = 4$ for T-REx®-293 cells; $n = 6$ for HepG2 cells).

In order to facilitate mitochondrial import of fluorescent RNA probes, the probes were designed as those expressed by a vector, but contained Alexa Fluor 647 (Fig. 15b, e, h) or Alexa Fluor 488 dye (Fig. 15k) attached to the 3' end. The successful cases exhibited Manders' coefficient for the mitochondria/probe colocalization of 0.71 ± 0.11 (n=6), whereas those failed attempts 0.16 ± 0.14 (scrambled RNA). Likewise, the Pearson's correlation coefficients reached 0.58 ± 0.22 for successful colocalizations, whereas values for failed attempts were -0.10 ± 0.05 . The negative values indicate the mutual exclusivity of the two imaged channels.

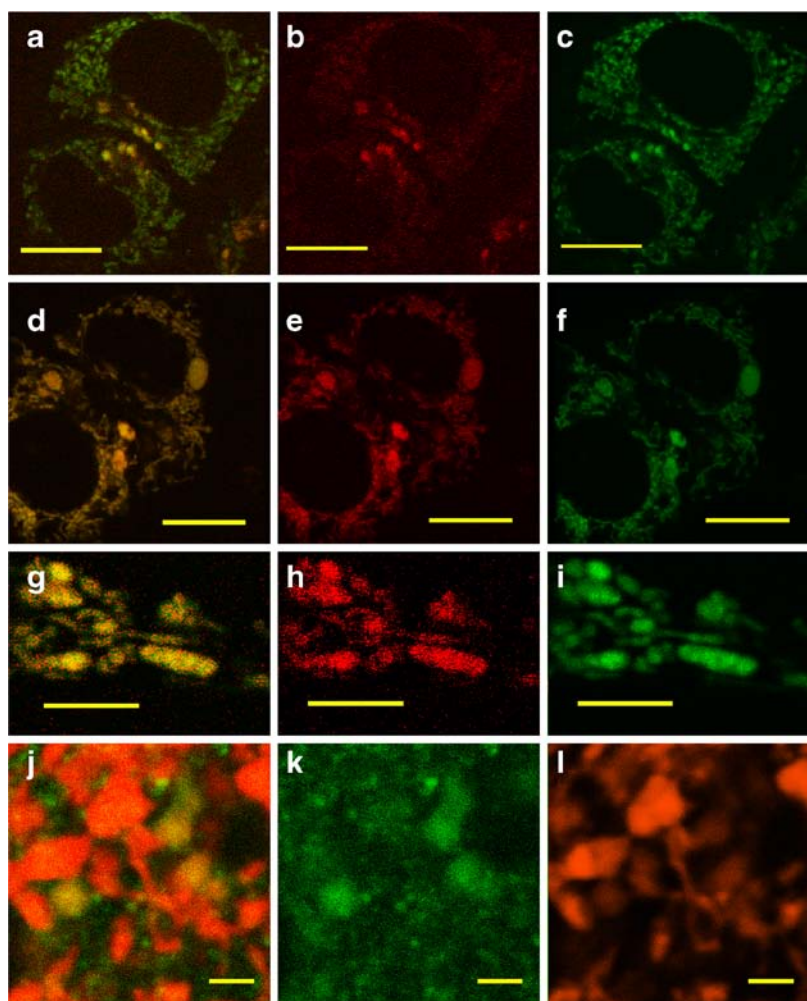


Figure 15. Successful cases of mitochondrial fluorescent in vivo hybridization (mtFIVH) by MAML-ND5 ribonucleoid probes in mtGFP- or mtKEIMA-transfected HepG2 cells. a–l) Probe with Alexa Fluor 647: confocal images or a, d, g overlays after 24 h of DQA-some facilitated delivery of a MAM-L-ND5 Alexa Fluor 647 ribonucleoid probe. The probe was imaged in the red channel (b, e, h), while the corresponding mitochondrial network in the cells expressing mtGFP in the green channel (c, f, i). Scale bars 10 μ m; 50 μ m in (g, h, i). j, k, l Probe with Alexa Fluor 488: confocal images or overlay (j) after 4 h of DQAsome facilitated delivery of a MAM-L-ND5 Alexa Fluor 488 ribonucleoid probe, which is individually displayed within a green channel (k); and corresponding yet fragmented mitochondrial network in the cells expressing mtKEIMA (l), red. Scale bars 2 μ m. Pearson's correlation coefficients were 0.53, 0.79, 0.78 and 0.24 for images of panels a, d, g, and j, respectively; while Manders' colocalization coefficients were 0.79, 0.86, 0.82 and 0.64, respectively.

Our constructs represent a prototype of mitochondrial fluorescent *in vivo* hybridization (mtFIVH). Possible annealing *in vivo* of RNA probes bearing the ND5 sequences with the corresponding ND5 L-strand mtDNA is demonstrated in Fig 16a, b, c showing that when both ND5 and MAM sequences are present on a probe, colocalization of probe fluorescence with the mitochondrial nucleoids is recognized.

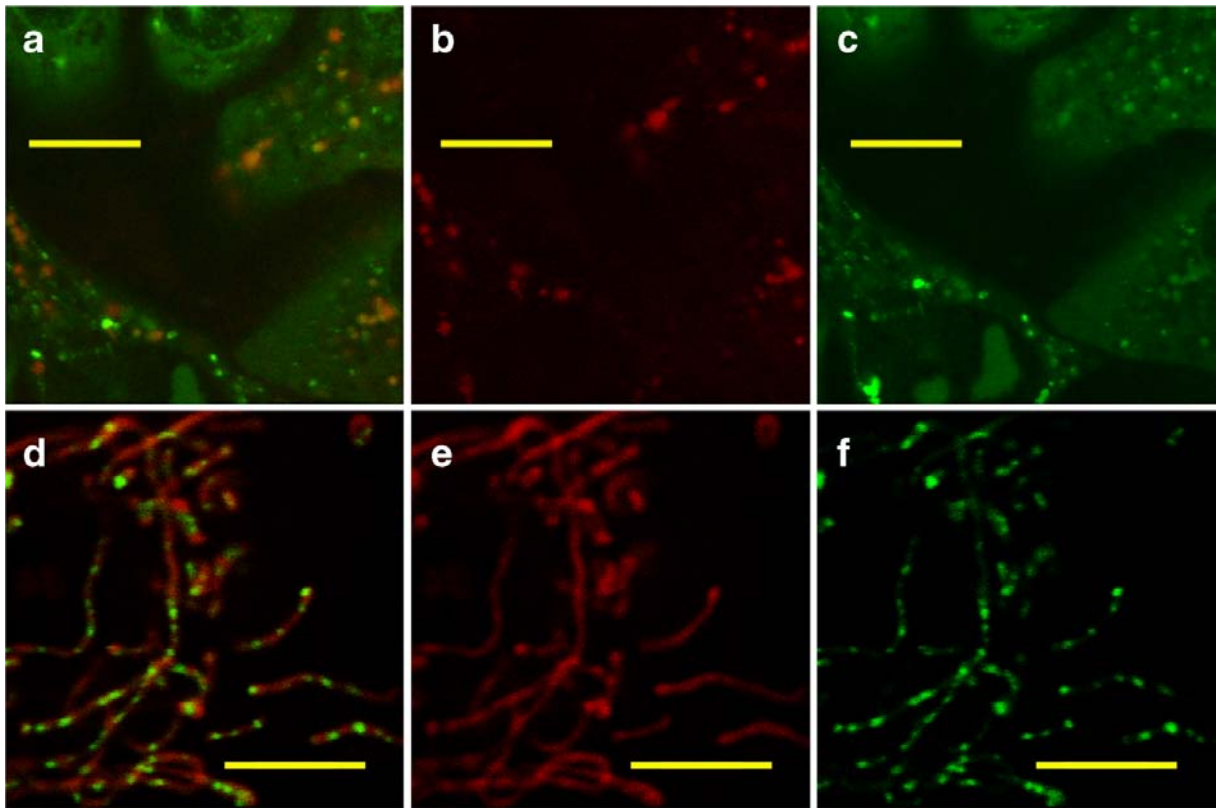


Figure 16. Mitochondrial fluorescent *in vivo* hybridization (mtFIVH) by ribonucleotide probes bearing a short antisense ND5 annealing portion enables nucleoid visualization. a, b, c Confocal images: a overlay of b and c after 24 h of DQA-some facilitated delivery of a MAM-L-ND5 Alexa Fluor 647 ribonucleotide probe, which is individually imaged in the red channel (b); c SYTO9 DNA staining of mitochondrial nucleoids (green). Scale bars 10 μ m. Pearson's correlation coefficient was 0.36 while Manders' colocalization coefficient was 0.89 for panel a. d, e, f Confocal images of mtKEIMA transfected-mitochondria (e) and SYTO 9 DNA stained-nucleoids (f) in cells untreated with DQA-somes; d overlay of e and f. Scale bars 5 μ m.

My contributions to this work were the preparation of negative probe for mitochondrial import and performance of selected RT-PCR experiments.

5.3 Publication C: Distribution of mitochondrial nucleoids upon mitochondrial network fragmentation and network reintegration in HEPG2 cells

Synopsis of results of the article “**Distribution of mitochondrial nucleoids upon mitochondrial network fragmentation and network reintegration in HEPG2 cells**”, by Tauber et al., published in The International Journal of Biochemistry and Cell Biology 2013.

The main goal of this project was to study nucleoid redistribution upon fission and following reintegration of the mitochondrial network and to compare the mtDNA distribution between cells grown on glucose and cells grown on galactose (Oxphos).

The mitochondrial fission in human hepatocellular carcinoma HepG2 cells was induced by (1) oxidative stress at respiration inhibition by rotenone or (2) elimination of the protonmotive force by uncoupling or (3) cancelling its electrical component, $\Delta\Psi_m$, by valinomycin; and (4) by silencing of mitofusin MFN2. We have established several techniques for visualization of mitochondrial network and nucleoids. For *in vivo* staining of mitochondria we have used mitochondrially targeted RoGFP, Keima fluorescent protein and tetramethylrhodamine, ethyl ester (TMRE). Mitochondrial nucleoids were *in vivo* visualized by Syto 16 and by TFAM-EGFP. Alternatively, the nucleoids were stained after fixation by anti-TFAM, anti-mtSSB or anti-DNA. All of the above mentioned situations were imaged on confocal microscope and all of the data were analyzed in ImageJ. We also established amount of mtDNA in each experiment.

First, we have analyzed mitochondrial nucleoids in glycolytic vs. so called Oxphos cells (cells grown on the non-fermentable sugar - 10 mM galactose and relied on glutaminolysis) (Rossignol et al., 2004; Smolková et al., 2011). The Oxphos cells exhibited similar numbers of nucleoids in selected continuous tubule segments (1.08 ± 0.18 nucleoids per μm of mitochondrial tubules), whereas cells grown on 5mM glucose had slightly more nucleoids (1.21 ± 0.23 nucleoids per μm of mitochondrial tubules), while cells on 25mM glucose had 1.05 ± 0.08 nucleoids per μm of mitochondrial tubules (Table 5). Altogether HepG2 cells exhibited 1.10 ± 0.5 nucleoids per μm of mitochondrial tubules in 95 estimates from confocal microscopy. The mtDNA copy number was almost equal in both glucose experiments (HepG2 cells grown on 5mM glucose 920 ± 240 and 25mM glucose 920 ± 270 copies of mtDNA per cell). While, the HepG2 cells grown on galactose displayed slightly lower amounts (870 ± 100 copies of mtDNA per cell).

Table 5. Viability, mtDNA copy number and number of nucleoids in glycolytic and Oxphos HepG2 cells. Data $\pm$ SDs are listed for intact (“Untreated”) HEPG2 cells cultivated with galactose/glutamine (Oxphos cells) or at 5 mM glucose (Glc5 cells) or 25 mM glucose (Glc25 cells). Viability in quadruplicates was assayed by trypan blue in the absence and presence of 35 nM, 100 nM and 1.5 μ M rotenone for Oxphos, Glc5, and Glc25 cells, respectively, after 48 h of incubation. The copy number of mtDNA per cell was assayed for untreated cells (10–16 estimates) and 4–7 estimates of copy number for each treatment as follows: for 0.5 μ M FCCP or 0.05 μ M valinomycin after 48 h of incubation; or for rotenone assays distinguished by concentration for metabolite modes (as for the viability). The nucleoid numbers (from three to nine cells) were counted for EGFP-TFAM co-expression with mtKEIMA (“Fl.prot.imaging”) or in mtSSB- or antiDNA-immunostained mtRoGFP-transfected cells (“immunocytology”).

	OxPHOS cells	Glc5 cells	Glc25 cells
Viability			
Untreated	98% $\pm$ 0.6%	97% $\pm$ 0.5%	97% $\pm$ 1%
Rotenone	96% $\pm$ 0.8%	88% $\pm$ 0.8%	97% $\pm$ 0.8%
mtDNA copy No.			
Untreated	870 $\pm$ 100	920 $\pm$ 240	920 $\pm$ 270
FCCP	980 $\pm$ 120	1080 $\pm$ 70	990 $\pm$ 120
Rotenone	850 $\pm$ 50	870 $\pm$ 200	790 $\pm$ 130
Valinomycin	n.d.	980 $\pm$ 100	1050 $\pm$ 200
No. of nucleoids per μ m of tubule			
Fl.prot.imaging	1.12 $\pm$ 0.18	1.19 $\pm$ 0.23	1.05 $\pm$ 0.09
Immunocytology	1.13 $\pm$ 0.16	1.16 $\pm$ 0.11	1.09 $\pm$ 0.13
No. of nucleoids per μ m ² (excl. area of the nucleus)			
Fl.prot.imaging	0.39 $\pm$ 0.18	0.39 $\pm$ 0.16	0.36 $\pm$ 0.04
Immunocytology	0.33 $\pm$ 0.07	0.33 $\pm$ 0.05	0.34 $\pm$ 0.09

Agents that affect bioenergetics and namely respiration instantly induce fission of the mitochondrial network (De Vos et al., 2005). For example, an agents diminishing the mitochondrial membrane potential, such as FCCP, at optimized dose ensures maximum respiration but ultimately disrupts mitochondrial ATP synthesis (Rossignol et al., 2004). Nucleoids were tracked after FCCP-induced fission of both glycolytic and Oxphos cells. The images of partial and complete fission of the mtRoGFP-visualized mt network were analyzed while cells, fixed at particular times, were immunostained with anti-mtSSB/Alexa-568-secondary antibody (Fig 17) or a primary antibody against DNA. All FCCP-treated Oxphos cells had a completely disintegrated mitochondrial reticulum network and almost no tubular structures, even during the first 20 min of incubation with 1 μ M FCCP. Lower FCCP doses (and situation in 5mM glucose cells) led to fission at later times, but most cells exhibited (retained) this morphology after 48 h (though multidrug resistance pumps might diminish its concentration), and nucleoids filled each of the small mitochondrial fragments in these cases (Fig. 17B, C and E).

A certain fraction of cells with a completely fragmented mitochondrial reticulum, however, exhibited larger fission objects in which nucleoids clustered in groups (Fig. 17C, E). On average, we found 3.5 ± 1.0 nucleoids per $1\mu\text{m}$ – diameter circular mitochondrial fragment. No excessive reduction in the number of nucleoids or copy number was observed after treatment with FCCP, rotenone, or valinomycin (Table 5).

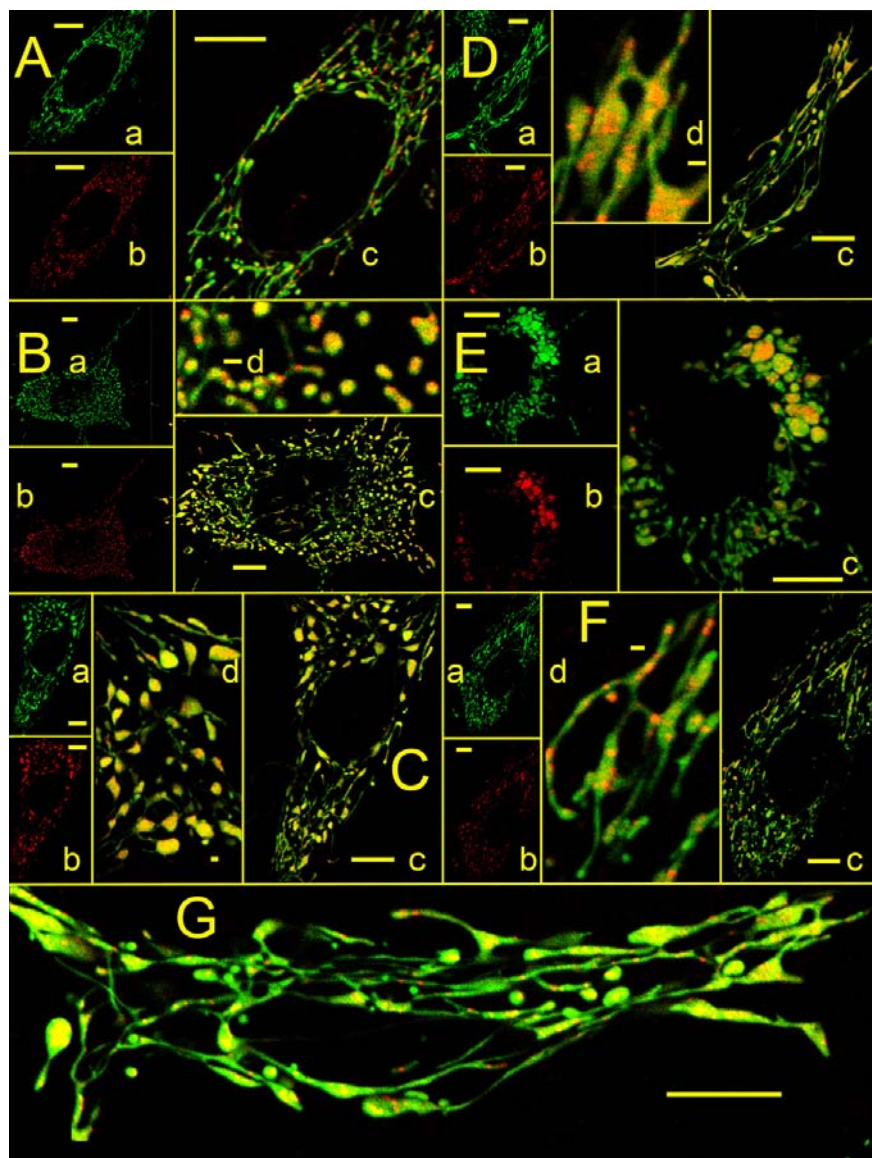


Figure 17. FCCP-induced fission and redistribution of nucleoids immunostained by mtSSB in mtRoGFP-visualized mitochondrial network. In panels A–F, three or four subdivisions (a–d) illustrate confocal images of the green (mtRoGFP) channel (a), red (mtSSB/Alexa-568) channel (b), and their overlays (c), in some cases, enlargements of overlays are displayed (d). Images show Oxphos cells (A) transfected with mtRoGFP and immunostained with mtSSB/Alexa-568 (1.17 ± 0.08 nucleoids per μm of tubule; 0.37 per μm^2) and demonstrate FCCP-induced fission and its recovery after FCCP withdrawal, as follows: (B) a 48-h incubation with $0.1\ \mu\text{M}$ FCCP ($1\text{--}6$ nucleoids per fragment; 0.22 per μm^2); (C) 48-h incubation with $0.5\ \mu\text{M}$ FCCP ($6\text{--}10$ nucleoids per fragment); (D, G) 48-h incubation with $0.5\ \mu\text{M}$ FCCP, after which FCCP was washed out and cells fixed 2 h later; (E, F) 48-h incubation with $1\ \mu\text{M}$ FCCP. Nucleoids per μm of tubule, per fragment and per μm^2 , respectively: (D = G) 1.14 ± 0.27 ; $2\text{--}6$; 0.25 ; (E) N/A; $5\text{--}19$; 0.38 ; (F) 1.28 ± 0.17 ; $1\text{--}6$; 0.36 . Scale bars: $10\ \mu\text{m}$ in a–c, $1\ \mu\text{m}$ in d.

Based on our findings we developed a new model for four stage fission/recovery of the mitochondrial reticulum network in relation to nucleoid redistribution (Fig 18).

Stage 1 – prevalent fission. The Fission after treatment with FCCP, rotenone, or valinomycin prevails due to a sudden depletion of matrix and peri-mitochondrial ATP (and hence GTP), in spite of the fact that both pro-fusion mitofusins MFN1 or MFN2 and OPA1, but also pro fission proteins such as DRP1 require GTP. When two potential fission sites on a larger mitochondrial tubule become competent to undergo fission, the resultant post-fission small cylinder/tubule subsequently takes the shape of a sphere containing a single nucleoid. If this were to happen in the entire mitochondrial network, one would exclusively observe single objects with single nucleoids, but this was only observed as a mix of network fragments containing various amounts of nucleoids (Fig. 17B).

Stage 2 – swelling of matrix volume in disintegrated objects. The valinomycin is known to assure K^+ entry into the mitochondrial matrix (Jezek et al., 1990) and as a consequence water enters the mitochondria and causes the inner mitochondrial membrane swelling. Such a swelling together with mitochondrial fission resulted in 2 μm spheres with on average 8 nucleoids. Assuming a tubule diameter 300 μm and equal distance between nucleoids 900 nm, the 10 μm tubular segment would provide a sphere with 1.75 μm (Fig 18A). Both the tubular segment and the sphere would contain 8 nucleoids in this case (Fig 18B). Similar considerations can be made for a tubule with diameter 400 nm (Fig. 18A, C) and 500 nm (Fig. 18A, D).

Stage 3 – partial reintegration of disintegrated objects. During the time ATP content may start to recover together with spontaneous (and frequently partial) reintegration of fragmented mitoreticulum objects (Fig. 17C, F, G).

Stage 4 – fusion accompanied by continuing shrinkage of the matrix space. The continuing inner mitochondrial membrane shrinkage (probably cristae formation) inside the mitochondrial tubule acts in synergy with prevailing fusion. This process causes mitochondrial tubule elongation and thinning of the inflated part of the tubule within a network. Consequently, the regular network with a unified diameter is re-established.

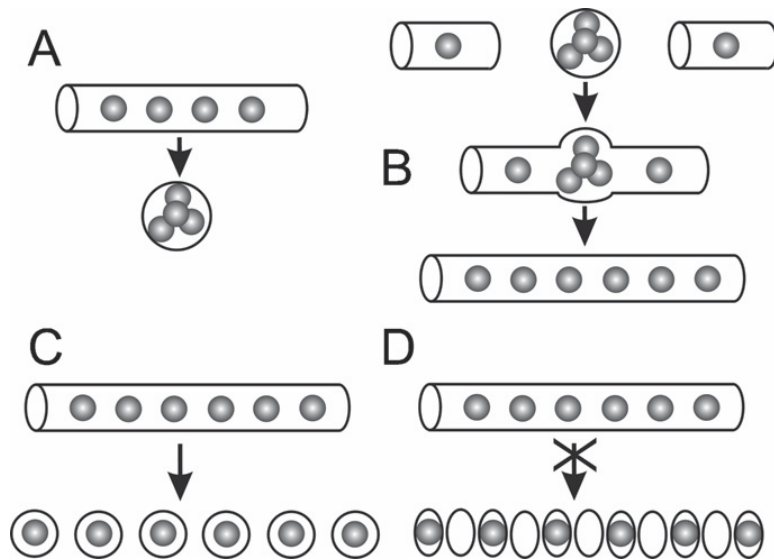


Figure 18. Schematic of nucleoid redistribution upon fission/reintegration of the mitochondrial network. Allowed (A and C) and not allowed (D) redistributions of mitochondrial nucleoids upon fission of mitochondrial tubules. (B) Reintegration of bulky fragmented objects back into the network.

My main contribution to this work was the mtDNA amount measurement in treated cells and cooperation on establishing the immunocytochemistry techniques.

5.4 Publication D: Assessment of mitochondrial DNA as an indicator of islet quality: an example in Goto Kakizaki rats

Synopsis of results of the article “**Assessment of mitochondrial DNA as an indicator of islet quality: an example in Goto Kakizaki rats**”, by Alán et al., published in The Transplantation proceedings 2011.

The objective of this study was to develop a technique for measuring the amount of mtDNA in β -cells of pancreatic islets and evaluate the potential of such technique as an indicator of islet quality in diabetic rat strain.

The Pancreatic islets were isolated from pancreata by collagenase digestion and separated on Ficoll gradient. Obtained pancreatic islets were quantified and further treated with Accutase to loosen individual cells. The mixture of pancreatic cells was stained *in vivo* by Newport Green DCF on Zn^{2+} positivity and sorted on flow cytometry with cell sorter. Obtained β -cells were lysed and quantified for amount of mtDNA.

We used 4-, 6-, and 12-month old Goto Kakizaki rats. Pancreatic islet samples were usually isolated from one Wistar rat (weighing $> 600g$ at 12 months and $> 490g$ at 4 months) and two Goto Kakizaki rats (weighing $> 420g$ at 12 months and $> 360g$ at 4 months). The Wistar rats were non-diabetic, while Goto Kakizaki rats exhibited hyperglycemia usually between 16 and 17 mmol/L glucose and impaired intravenous glucose tolerance test (~ 15 -20 mmol/L glucose remaining 60 minutes after glucose intake).

The 12-month old Goto Kakizaki rats exhibited $24 \pm 4\%$ versus the average copy number in 12-month old Wistar rats (Fig. 19). Six- and four-month-old GK rats showed reductions to $60 \pm 15\%$ and $50 \pm 20\%$, respectively, versus the age-paired Wistar rats (Fig. 19). We observed also significant mtDNA reduction in the heart muscle and liver of Goto Kakizaki rats (Fig. 20), exhibiting on average $70 \pm 25\%$ and $60 \pm 20\%$ respectively, of the levels in the heart and liver of age-paired Wistar rats.

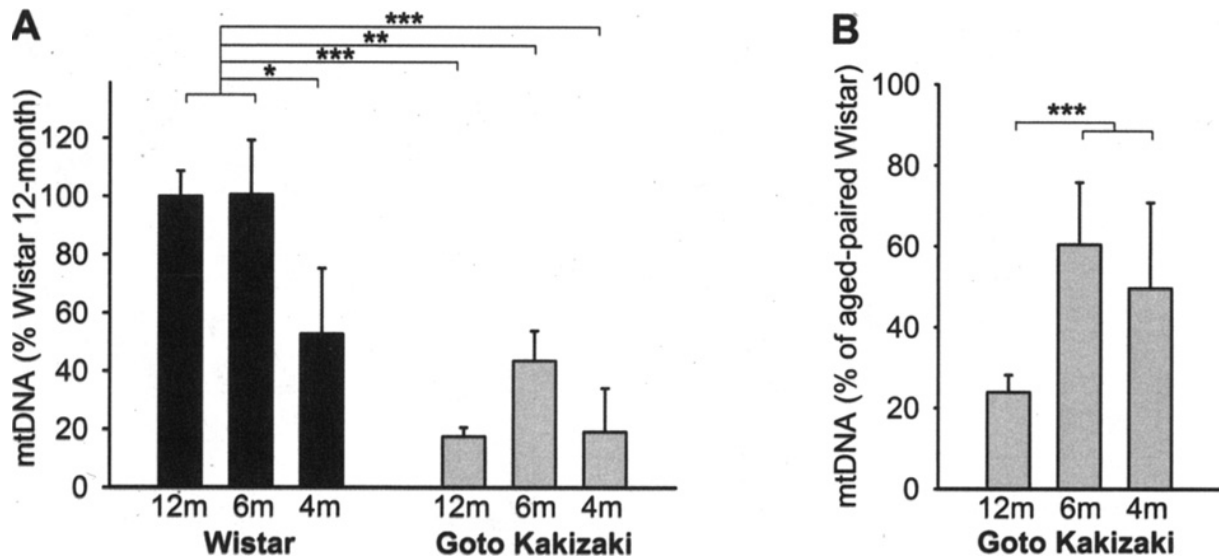


Figure 19. mtDNA copy number in PI β -cells of 12-, 6-, and 4-month old Wistar and Goto Kakizaki rats. (A) The relative scale was set such that an average copy number for 12-month old Wistar rats was ascribed as 100%. (B) The relative scale was set vs age-paired Wistar rats. * $P < .05$; ** $P < .02$; *** $P < .001$.

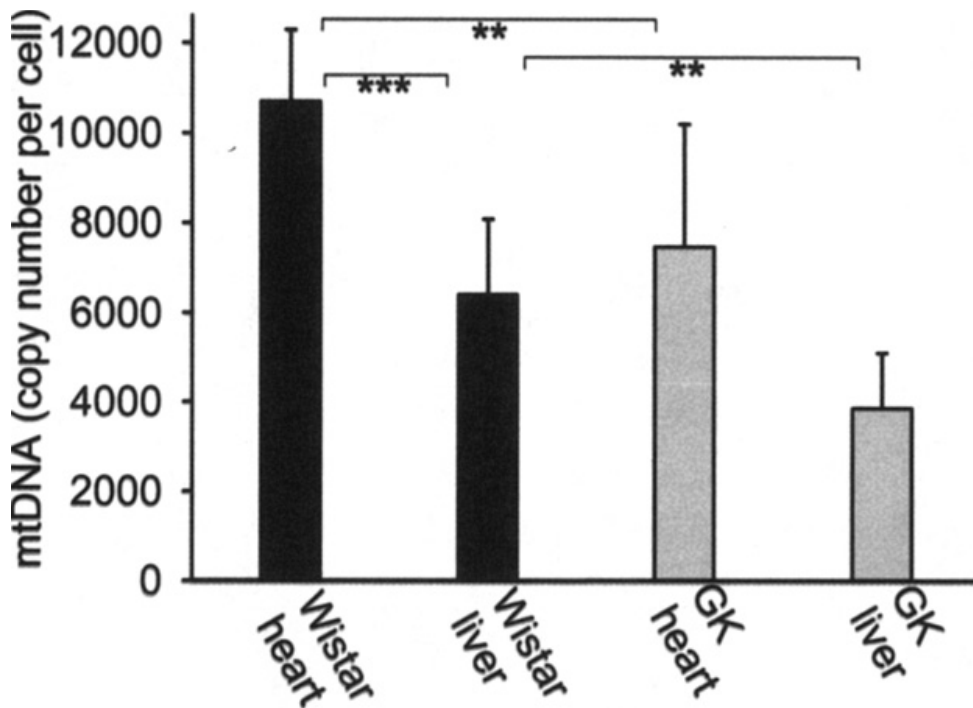


Figure 20. mtDNA copy number in hearts and liver of 6-month-old Wistar and Goto Kakizaki rats. ** $P < .02$; *** $P < .001$.

We also confirmed that the respiration test indicated a slightly impaired response to glucose that may predict insufficient GSIS (although insulin release was not measured) in the 4 PI samples collected from 6-month-old GK rat pairs exhibited hyperglycemia and impaired intravenous glucose tolerance tests (~15–20 mmol/L glucose remaining 60 minutes after glucose intake). In contrast, Wistar rat PI respired ~34% and 50% more at 5 mmol/L and 25 mmol/L glucose, respectively, than PI from age-paired 6-month-old GK rats (Fig 3). The typical respiration increase simulated by a 20 mmol/L higher glucose was 1.30 ± 0.05 in Wistar PI and 1.45 ± 0.07 in GK PI. Impaired OXPHOS among GK PI was indicated by a greater state-3/state-4 ratio of 2.0 ± 0.5 in Wistar PI versus only 1.5 ± 0.2 in GK PI.

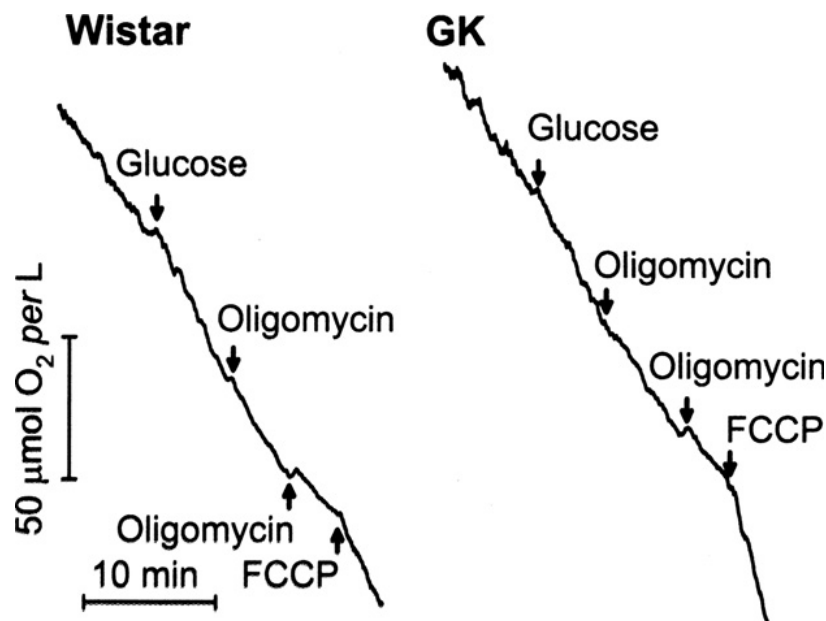


Figure 21. Respiration of pancreatic islets of 6-month-old Wistar and Goto Kakizaki rats. Typical traces of oxygen consumption are shown. Where indicated, 20 mmol/L incremental glucose was added (raising 5 mmol/L to 25 mmol/L glucose), 0.2 μ g/mL oligomycin and 3 μ mol/L FCCP, an uncoupler. GK rats exhibited hyperglycemia at 12 mmol/L glucose.

My contributions to this work were the staining and loosening of β -cells from pancreatic islets, quantification and isolation of mtDNA from β -cells and tissues.

6. Discussion

Mitochondrial disorders are a group of genetically and clinically heterogeneous diseases, due to in part, the biochemical complexity of mitochondrial respiration and the fact that two genomes participate on assembly of oxidative phosphorylation complexes. We focused our research on diagnostic techniques for detection of mitochondrial RNA species *in situ* and *in vivo* and also on quantification and distribution of mtDNA. The testing algorithm for molecular diagnostics of patients with suspected mitochondrial disease always starts with metabolic and clinical evaluation. These patients often display a malfunction of highly metabolic tissues, such as heart, muscles or nervous system and often have high concentration of lactic acid in serum. The followed analysis should search for common mtDNA mutations and then match the clinical manifestation to the already known syndromes. If previous tests fail, muscle, liver or skin biopsy is executed and checked again for common mtDNA mutations, mtDNA copy number quantification, electron transport chain defects, sequencing of all complex subunits and assembly genes, until the proper gene and diagnosis is resolved.

The first two studies of this thesis were focused on *in vivo* and *in situ* hybridization techniques, which could serve for analysis of patient's tissues with heteroplasmy of mtDNA and for visualization of proportion and distribution of mutated, and wild type mtRNA and mtDNA. Moreover, the *in vivo* hybridization might be used for further development of repression of mutated mRNA. The second diagnostic tool, mtDNA copy number, provides a powerful technique to monitor amount of mtDNA even in situations, where limited amount of diagnostic material is available, as in the case of pancreatic islets transplantations. Finally, the mtDNA distribution in response to mitochondrial fission-fusion processes is important in diagnostics of mutations in the mitodynamine, mitophagy or mitochondrial nucleoid proteins.

The distribution of different mtRNA species was studied using molecular beacon probes and confocal microscopy (Publication A). Distinct spots were present in the images visualized by ND5 probe, which were co-localized with mitochondrial nucleoids (Bogenhagen et al., 2008). The diffuse signal, most probably originating from the annealing to ND5 mRNA, disappeared after RNase A post-fixational digestion. Both D-loop probes behaved in accordance with the assumption that the corresponding LSP RNA is degraded more extensively than the HSP RNA. Indeed, both D-loop probes exhibited images of a more punctuate character, showing numerous spots resembling mitochondrial nucleoids. We also combined our mitochondrial fluorescence *in situ* hybridization protocol with post-fixation and pre-hybridization treatment with RNase A, RNase H, DNase I, and their combination. A “contrasting” action of RNase A was observed for all probes, but it was the most pronounced for ND5 probe. We expected that RNase H would digest presumed RNA-DNA hybrids around mitochondrial nucleoids. Despite our low confocal resolution we were not able to observe

significant changes. The distinction of these transcriptional states (as well as replication intermediates – RITOLS) remains a future challenge for super-resolution microscopy in combination with analogs of our developed probes. Indeed, perspectives for the use of the developed probes lie in their use in conjunction with super-resolution microscopy such as stimulated emission depletion (STED) microscopy (Willig et al., 2007), for which modified probes (conjugated with ATTO-dyes) may help to better resolve intra-nucleoid structure and might even detect RITOLS and R-loops (Yasukawa et al., 2006).

We have developed an efficient method for importing nucleic acids into the mitochondrial matrix if expressed from vectors transfected to cells and we extended this already successful approach (Comte et al., 2013; A. V Smirnov et al., 2008; Wang et al., 2010) to mitochondrial *in vivo* hybridization (mtFIVH) (Publication B). Both of our methods were based on the same RNA design, so as to contain the native import address of mitochondrial 5S rRNA, while the mtFIVH was further combined with the use of DQAsomes as micelles recruiting probes into the proximity of mitochondria within the live cells. The addressing by inherent 5S rRNA is promising, since even the mitochondrial heteroplasmy was improved in benefits of the wild-typed mtDNA, when oligoribonucleotides complementary to mutant mtDNA region were imported into the cultured transmitochondrial cybrid cells with Kearns Sayre syndrome (Comte et al., 2013). Visualization of mRNA *in vivo* can be beneficial also in studies of recently discovered mitochondrial RNA stabilization proteins such as Slirp and LRPPRC (Baughman et al., 2009; Chujo et al., 2012). Here, the visualization of RNA distribution in mitochondria may be important if these proteins stabilize wild type RNA over the mutated RNA in patients cells with a certain level of heteroplasmy. Such a stabilized wild-type mtRNA can improve its half-life and can diffuse to distant parts of mitochondria with mutated mtDNA and mtRNA.

Distribution and amount of mitochondrial nucleoids was studied by mitochondrial network fragmentation and reintegration in HepG2 cells using confocal microscopy (Publication C). First, we analyzed the nucleoid distribution in glycolytic and OXPHOS HepG2 cells. We expected lower amounts of nucleoids and mtDNA in glycolytic cells, since they display lower respiration rate (Rossignol et al., 2004). Finally there were no significant differences between glycolytic and OXPHOS cells neither in mtDNA copy number (Rossignol et al., 2004), nor nucleoid distribution. We proposed so called nucleoid code, the distance between two nucleoids, which in HepG2 cells was 1.1 ± 0.5 nucleoids per μm of mitochondrial tubule ($0.7 - 1.4 \mu\text{m}$). Induction of mitochondrial fission by FCCP, rotenone, valinomycin or by down-regulation of pro-fusion protein MFN2 resulted in four-stage model for fission/recovery of the mitochondrial network regarding the nucleoid distribution: Stage 1 – prevalent fission; Stage 2 – swelling of matrix volume in disintegrated objects; Stage 3 – partial reintegration of disintegrated objects; Stage 4 – fusion accompanied by continuing shrinkage of the matrix space (Fig 18). Interestingly, despite the finding that FCCP induces a mitophagy (Narendra et al., 2010), we did not observe any changes in the amount of mtDNA neither in glycolytic nor in

OXPHOS cells. This could be reasoned by low expression of parkin in cancer cells, since the most experiments showing mitochondrial depletion used both, parkin over-expression together with mitochondrial depolarization by FCCP or CCCP (Gilkerson et al., 2012; Narendra et al., 2010; Suen et al., 2010).

Quantification of mtDNA in diabetic rats showed marked decrease during ageing and can be used as a marker of pancreatic islet quality (Publication D). The number of pancreatic β -cells is substantially reduced in hypertrophied islets of Goto Kakizaki rats. Moreover, in the remaining β -cells, the mtDNA copy number per cell was dramatically reduced, down to one-fourth in 12-month-old Goto Kakizaki rats, and to 50% and 60%, respectively in 4- and 6-month-old Goto Kakizaki rats. Indeed, the results of our respiratory tests fit the expected correlation. There was a ~42% reduction of state-3/state-4 ratio in 6-month-old Goto Kakizaki rats compared with age-paired Wistar rats. The effect of ageing on the amount of mtDNA in pancreatic islets was further indicated by mtDNA copy number of old non-diabetic islet donors (more than 50 years old), which had significantly reduced amount of mtDNA in comparison with younger donors (less than 50 years old) (Cree et al., 2008). The other question is whether the decrease in mtDNA copy number in diabetes is a cause or a consequence of diabetes. This issue was addressed by down-regulation of mitochondrial transcription factor A (TFAM), which originally binds mtDNA in a histone like manner (Hallberg and Larsson, 2011) and initiate mitochondrial transcription (Takamatsu et al., 2002). The TFAM down-regulation in mouse pancreatic beta cell line MIN6 resulted in significantly impaired mitochondrial gene transcription and translation, as well as mitochondrial oxidative respiration and glucose stimulated insulin secretion (Nile et al., 2014) and also in tissue specific β -cells knock-out mice (Silva et al., 2000). All of these results implicate involvement of mtDNA decrease in progression of the type 2 diabetes.

In conclusion, this thesis provides development and establishment of new visualization techniques with high potential in diagnostics of mitochondrial diseases. The new *in situ* and *in vivo* techniques can be used for monitoring heteroplasmy levels in patients' cells and mtDNA copy number analysis can be used for monitoring the fluctuations of the mtDNA amount in several conditions and pathological states including cancer, senescence, diabetes or autophagy.

7. Conclusions

- A. We have developed fluorescent *in situ* hybridization for monitoring mitochondrial RNA distribution. Our ND5 molecular beacon displayed mostly mitochondrial pattern, which showed RNA clouds after mild RNase A treatment. Other D-loop probes had naturally punctuated character of signal, which was weaker after treatment with DNase I in combination with RNase H.
- B. We have employed mitochondrial 5S rRNA mitochondria-addressing domain sequence and developed an import system as expressed either from the vector transfected to the cell or artificial DQAsome facilitating import system for fluorescent *in vivo* hybridization. It allowed to us monitor ND5 RNA real time inside mitochondria.
- C. We defined the “mitochondrial nucleoid code”, which established the average distance between nucleoids as 1 μm as found in HepG2 cells. Based on our experiments with induced mitochondrial fission and fusion, we suggest a four stage model of nucleoid distribution. We predict that a 10 μm wide mitochondrial tubule containing 9 nucleoids will turn after fission into a 2 μm spheroid containing those nine nucleoids. The inspection of such a “mitochondrial nucleoid code” may provide a basis for novel mitochondrial morphology diagnostics.
- D. Diabetic Goto Kakizaki rats showed dramatic decrease in the amount of mtDNA in pancreatic β -cells accompanied with impaired respiration responses to glucose. We suggested the use of mtDNA quantification to quickly assess pancreatic islets quality before transplantation.

8. List of all my publications

Alán L, Smolková K, Kronusová E, Santorová J, Jezek P., Absolute levels of transcripts for mitochondrial uncoupling proteins UCP2, UCP3, UCP4, and UCP5 show different patterns in rat and mice tissues. *J Bioenerg Biomembr.* 2009 Feb;41(1):71-8. IP: 4,015 (2009)

Alán L, Zelenka J, Ježek J, Dlasková A, Ježek P., Fluorescent in situ hybridization of mitochondrial DNA and RNA. *Acta Biochim Pol.* 2010;57(4):403-8. IP: 1,234 (2010)

Alán L, Špaček T, Zelenka J, Tauber J, Berková Z, Zacharovová K, Saudek F, Ježek P., Assessment of mitochondrial DNA as an indicator of islet quality: an example in Goto Kakizaki rats. *Transplant Proc.* 2011 Nov;43(9):3281-4. IP: 1,005 (2011)

Slaninová J, Mlsová V, Kroupová H, Alán L, Tůmová T, Monincová L, Borovičková L, Fučík V, Ceřovský V., Toxicity study of antimicrobial peptides from wild bee venom and their analogs toward mammalian normal and cancer cells. *Peptides.* 2012 Jan;33(1):18-26. IP: 2,52 (2012)

Tauber J, Dlasková A, Šantorová J, Smolková K, Alán L, Špaček T, Plecítá-Hlavatá L, Jabůrek M, Ježek P., Distribution of mitochondrial nucleoids upon mitochondrial network fragmentation and network reintegration in HEPG2 cells. *Int J Biochem Cell Biol.* 2013 Mar;45(3):593-603. IP: 4,24 (2013)

Zelenka J, Alán L, Jabůrek M, Ježek P., Import of desired nucleic acid sequences using addressing motif of mitochondrial ribosomal 5S-rRNA for fluorescent in vivo hybridization of mitochondrial DNA and RNA. *J Bioenerg Biomembr.* 2014 Apr;46(2):147-56. IP: 2,7 (2013)

9. Reference list

- Al Rawi, S., Louvet-Vallée, S., Djeddi, A., Sachse, M., Culetto, E., Hajjar, C., Boyd, L., Legouis, R., Galy, V., 2011. Postfertilization autophagy of sperm organelles prevents paternal mitochondrial DNA transmission. *Sci. (New York, NY)* 334, 1144–1147. doi:10.1126/science.1211878
- Alán, L., Smolková, K., Kronusová, E., Šantorová, J., Ježek, P., 2009. Absolute levels of transcripts for mitochondrial uncoupling proteins UCP2, UCP3, UCP4, and UCP5 show different patterns in rat and mice tissues. *J. Bioenerg. Biomembr.* 41, 71–78.
- Alán, L., Zelenka, J., Ježek, J., Dlasková, A., Ježek, P., 2010. Fluorescent in situ hybridization of mitochondrial DNA and RNA. *Acta Biochim. Pol.* 57, 403–8.
- Alfonzo, J.D., Söll, D., 2009. Mitochondrial tRNA import - The challenge to understand has just begun. *Biol. Chem.*
- Alkhaja, A.K., Jans, D.C., Nikolov, M., Vukotic, M., Lytovchenko, O., Ludewig, F., Schliebs, W., Riedel, D., Urlaub, H., Jakobs, S., Deckers, M., 2012. MINOS1 is a conserved component of mitofilin complexes and required for mitochondrial function and cristae organization. *Mol. Biol. Cell.*
- Anderson, C.T.M., Friedberg, E.C., 1980. The presence of nuclear and mitochondrial uracil-DNA glycosylase in extracts of human KB cells. *Nucleic Acids Res.* 875–888.
- Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M.H., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J., Staden, R., Young, I.G., 1981. Sequence and organization of the human mitochondrial genome. *Nature* 290, 457–465.
- Andrews, R.M., Kubacka, I., Chinnery, P.F., Lightowlers, R.N., Turnbull, D.M., Howell, N., 1999. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. *Nat. Genet.* 23, 147. doi:10.1038/13779
- Area-Gomez, E., de Groof, A.J.C., Boldogh, I., Bird, T.D., Gibson, G.E., Koehler, C.M., Yu, W.H., Duff, K.E., Yaffe, M.P., Pon, L.A., Schon, E.A., 2009. Presenilins are enriched in endoplasmic reticulum membranes associated with mitochondria. *Am. J. Pathol.* 175, 1810–1816.
- Ashcroft, F.M., Proks, P., Smith, P.A., Ammälä, C., Bokvist, K., Rorsman, P., 1994. Stimulus-secretion coupling in pancreatic beta cells. *J. Cell. Biochem.* 55 Suppl, 54–65.
- Ashley, N., Poulton, J., 2009. Anticancer DNA intercalators cause p53-dependent mitochondrial DNA nucleoid re-modelling. *Oncogene* 28, 3880–3891. doi:10.1038/onc.2009.242
- Ashrafi, G., Schwarz, T.L., 2012. The pathways of mitophagy for quality control and clearance of mitochondria. *Cell Death Differ.*
- Bai, R.-K., Wong, L.-J.C., 2005. Simultaneous detection and quantification of mitochondrial DNA deletion(s), depletion, and over-replication in patients with mitochondrial disease. *J. Mol. Diagn.* 7, 613–622.
- Bandiera, S., Rüberg, S., Girard, M., Cagnard, N., Hanein, S., Chrétien, D., Munnich, A., Lyonnet, S., Henrion-Caude, A., 2011. Nuclear outsourcing of RNA interference components to human mitochondria. *PLoS One* 6.
- Bandy, B., Davison, A.J., 1990. Mitochondrial mutations may increase oxidative stress: Implications for carcinogenesis and aging? *Free Radic. Biol. Med.*
- Ban-Ishihara, R., Ishihara, T., Sasaki, N., Mihara, K., Ishihara, N., 2013. Dynamics of nucleoid structure regulated by mitochondrial fission contributes to cristae reformation and release of cytochrome c. *Proc. Natl. Acad. Sci. U. S. A.* 110, 11863–8. doi:10.1073/pnas.1301951110
- Baricault, L., Ségui, B., Guégand, L., Olichon, A., Valette, A., Larminat, F., Lenaers, G., 2007. OPA1 cleavage depends on decreased mitochondrial ATP level and bivalent metals. *Exp. Cell Res.* 313, 3800–3808.
- Barrientos, A., Casademont, J., Cardellach, F., Estivill, X., Urbano-Marquez, A., Nunes, V., 1997. Reduced steady-state levels of mitochondrial RNA and increased mitochondrial DNA amount in human brain with aging. *Mol. Brain Res.* 52, 284–289.

- Baughman, J.M., Nilsson, R., Gohil, V.M., Arlow, D.H., Gauhar, Z., Mootha, V.K., 2009. A computational screen for regulators of oxidative phosphorylation implicates SLIRP in mitochondrial RNA homeostasis. *PLoS Genet.* 5, e1000590. doi:10.1371/journal.pgen.1000590
- Baughman, J.M., Perocchi, F., Girgis, H.S., Plovanich, M., Belcher-Timme, C.A., Sancak, Y., Bao, X.R., Strittmatter, L., Goldberger, O., Bogorad, R.L., Koteliensky, V., Mootha, V.K., 2011. Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. *Nature* 476, 341–345.
- Bereiter-Hahn, J., Vöth, M., 1994. Dynamics of mitochondria in living cells: Shape changes, dislocations, fusion, and fission of mitochondria. *Microsc. Res. Tech.* 27, 198–219. doi:10.1002/jemt.1070270303
- Beyer, W., Imlay, J., Fridovich, I., 1991. Superoxide dismutases. *Prog. Nucleic Acid Res. Mol. Biol.* 40, 221–253.
- Bjelakovic, G., Nikolova, D., Gluud, L.L., Simonetti, R.G., Gluud, C., 2007. Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. *JAMA* 297, 842–857.
- Blokhin, A., Vyshkina, T., Komoly, S., Kalman, B., 2008. Variations in mitochondrial DNA copy numbers in MS brains. *J. Mol. Neurosci.* 35, 283–287.
- Bogenhagen, D., Clayton, D.A., 1974. The number of mitochondrial deoxyribonucleic acid genomes in mouse L and human HeLa cells. Quantitative isolation of mitochondrial deoxyribonucleic acid. *J. Biol. Chem.* 249, 7991–7995.
- Bogenhagen, D.F., 2012. Mitochondrial DNA nucleoid structure. *Biochim. Biophys. Acta* 1819, 914–20. doi:10.1016/j.bbagr.2011.11.005
- Bogenhagen, D.F., 1999. Repair of mtDNA in vertebrates. *Am. J. Hum. Genet.* 64, 1276–81. doi:10.1086/302392
- Bogenhagen, D.F., Martin, D.W., Koller, A., 2014. Initial steps in RNA processing and ribosome assembly occur at mitochondrial DNA nucleoids. *Cell Metab.* 19, 618–29. doi:10.1016/j.cmet.2014.03.013
- Bogenhagen, D.F., Rousseau, D., Burke, S., 2008. The Layered Structure of Human Mitochondrial DNA Nucleoids. *J. Biol. Chem.* 283, 3665–3675. doi:10.1074/jbc.M708444200
- Bonawitz, N.D., Chatenay-Lapointe, M., Pan, Y., Shadel, G.S., 2007. Reduced TOR Signaling Extends Chronological Life Span via Increased Respiration and Upregulation of Mitochondrial Gene Expression. *Cell Metab.* 5, 265–277.
- Brown, T.A., Tkachuk, A.N., Shtengel, G., Kopek, B.G., Bogenhagen, D.F., Hess, H.F., Clayton, D.A., 2011. Superresolution Fluorescence Imaging of Mitochondrial Nucleoids Reveals Their Spatial Range, Limits, and Membrane Interaction. *Mol. Cell. Biol.*
- Brown, W.M., Shine, J., Goodman, H.M., 1978. Human mitochondrial DNA: analysis of 7S DNA from the origin of replication. *Proc. Natl. Acad. Sci. U. S. A.* 75, 735–739.
- Busch, C.P., Hegele, R.A., 2001. Genetic determinants of type 2 diabetes mellitus. *Clin. Genet.* 60, 243–254.
- Butow, R.A., Zhu, H., Perlman, P., Conrad-Webb, H., 1989. The role of a conserved dodecamer sequence in yeast mitochondrial gene expression. *Genome* 31, 757–760.
- Cámara, Y., Asin-Cayuela, J., Park, C.B., Metodiev, M.D., Shi, Y., Ruzzenente, B., Kukat, C., Habermann, B., Wibom, R., Hulténby, K., Franz, T., Erdjument-Bromage, H., Tempst, P., Hallberg, B.M., Gustafsson, C.M., Larsson, N.G., 2011. MTERF4 regulates translation by targeting the methyltransferase NSUN4 to the mammalian mitochondrial ribosome. *Cell Metab.* 13, 527–539.
- Cavelier, L., Johannisson, A., Gyllenstein, U., 2000. Analysis of mtDNA copy number and composition of single mitochondrial particles using flow cytometry and PCR. *Exp. Cell Res.* 259, 79–85.
- Cipolat, S., Martins de Brito, O., Dal Zilio, B., Scorrano, L., 2004. OPA1 requires mitofusin 1 to promote mitochondrial fusion. *Proc. Natl. Acad. Sci. U. S. A.* 101, 15927–15932.
- Clay Montier, L.L., Deng, J.J., Bai, Y., 2009. Number matters: control of mammalian mitochondrial DNA copy number. *J. Genet. Genomics* 36, 125–131. doi:10.1016/S1673-8527(08)60099-5

- Coffey, G., Lakshmiopathy, U., Campbell, C., 1999. Mammalian mitochondrial extracts possess DNA end-binding activity. *Nucleic Acids Res.* 27, 3348–3354.
- Comte, C., Tonin, Y., Heckel-Mager, A.-M., Boucheham, A., Smirnov, A., Auré, K., Lombès, A., Martin, R.P., Entelis, N., Tarassov, I., 2013. Mitochondrial targeting of recombinant RNAs modulates the level of a heteroplasmic mutation in human mitochondrial DNA associated with Kearns Sayre Syndrome. *Nucleic Acids Res.* 41, 418–33. doi:10.1093/nar/gks965
- Cotney, J., Wang, Z., Shadel, G.S., 2007. Relative abundance of the human mitochondrial transcription system and distinct roles for h-mtTFB1 and h-mtTFB2 in mitochondrial biogenesis and gene expression. *Nucleic Acids Res.* 35, 4042–4054.
- Cree, L.M., Patel, S.K., Pyle, A., Lynn, S., Turnbull, D.M., Chinnery, P.F., Walker, M., 2008. Age-related decline in mitochondrial DNA copy number in isolated human pancreatic islets. *Diabetologia* 51, 1440–1443. doi:10.1007/s00125-008-1054-4
- Crofts, A.R., 2004. Proton-coupled electron transfer at the Qo-site of the bc1 complex controls the rate of ubihydroquinone oxidation. *Biochim. Biophys. Acta* 1655, 77–92. doi:10.1016/j.bbabi.2003.10.012
- Curth, U., Urbanke, C., Greipel, J., Gerberding, H., Tiranti, V., Zeviani, M., 1994. Single-stranded-DNA-binding proteins from human mitochondria and *Escherichia coli* have analogous physicochemical properties. *Eur. J. Biochem.* 221, 435–443.
- De Brito, O.M., Scorrano, L., 2008. Mitofusin 2 tethers endoplasmic reticulum to mitochondria. *Nature* 456, 605–610.
- De Souza-Pinto, N.C., Mason, P.A., Hashiguchi, K., Weissman, L., Tian, J., Guay, D., Lebel, M., Stevnsner, T., V., Rasmussen, L.J., Bohr, V.A., 2009. Novel DNA mismatch-repair activity involving YB-1 in human mitochondria. *DNA Repair (Amst.)* 8, 704–719.
- De Vos, K.J., Allan, V.J., Grierson, A.J., Sheetz, M.P., 2005. Mitochondrial function and actin regulate dynamin-related protein 1-dependent mitochondrial fission. *Curr. Biol.* 15, 678–83. doi:10.1016/j.cub.2005.02.064
- De vos, K.J., Mórotz, G.M., Stoica, R., Tudor, E.L., Lau, K.F., Ackerley, S., Warley, A., Shaw, C.E., Miller, C.C.J., 2012. VAPB interacts with the mitochondrial protein PTPIP51 to regulate calcium homeostasis. *Hum. Mol. Genet.* 21, 1299–1311.
- Denton, R.M., 2009. Regulation of mitochondrial dehydrogenases by calcium ions. *Biochim. Biophys. Acta - Bioenerg.*
- Di Re, M., Sembongi, H., He, J., Reyes, a, Yasukawa, T., Martinsson, P., Bailey, L.J., Goffart, S., Boyd-Kirkup, J.D., Wong, T.S., Fersht, a R., Spelbrink, J.N., Holt, I.J., 2009. The accessory subunit of mitochondrial DNA polymerase gamma determines the DNA content of mitochondrial nucleoids in human cultured cells. *Nucleic Acids Res.* 37, 5701–13. doi:10.1093/nar/gkp614
- Dianov, G.L., Souza-Pinto, N., Nyaga, S.G., Thybo, T., Stevnsner, T., Bohr, V.A., 2001. Base excision repair in nuclear and mitochondrial DNA. *Prog. Nucleic Acid Res. Mol. Biol.* 68, 285–297.
- Dodson, M.L., Lloyd, R.S., 2002. Mechanistic comparisons among base excision repair glycosylases. *Free Radic. Biol. Med.*
- Doonan, R., McElwee, J.J., Matthijssens, F., Walker, G.A., Houthoofd, K., Back, P., Matscheski, A., Vanfleteren, J.R., Gems, D., 2008. Against the oxidative damage theory of aging: Superoxide dismutases protect against oxidative stress but have little or no effect on life span in *Caenorhabditis elegans*. *Genes Dev.* 22, 3236–3241.
- Ehse, S., Raschke, I., Mancuso, G., Bernacchia, A., Geimer, S., Tondera, D., Martinou, J.C., Westermann, B., Rugarli, E.I., Langer, T., 2009. Regulation of OPA1 processing and mitochondrial fusion by m-AAA protease isoenzymes and OMA1. *J. Cell Biol.* 187, 1023–1036.
- Ekstrand, M.I., Falkenberg, M., Rantanen, A., Park, C.B., Gaspari, M., Hultenby, K., Rustin, P., Gustafsson, C.M., Larsson, N.G., 2004. Mitochondrial transcription factor A regulates mtDNA copy number in mammals. *Hum. Mol. Genet.* 13, 935–944.
- Elachouri, G., Vidoni, S., Zanna, C., Pattyn, A., Boukhaddaoui, H., Gaget, K., Yu-Wai-Man, P., Gasparre, G., Sarzi, E., Delettre, C., Olichon, A., Loiseau, D., Reynier, P., Chinnery, P.F., Rotig, A., Carelli, V., Hamel,

- C.P., Rugolo, M., Lenaers, G., 2011. OPA1 links human mitochondrial genome maintenance to mtDNA replication and distribution. *Genome Res.* 21, 12–20.
- Esterházy, D., King, M.S., Yakovlev, G., Hirst, J., 2008. Production of reactive oxygen species by complex I (NADH:ubiquinone oxidoreductase) from *Escherichia coli* and comparison to the enzyme from mitochondria. *Biochemistry* 47, 3964–71. doi:10.1021/bi702243b
- Farr, C.L., Wang, Y., Kaguni, L.S., 1999. Functional interactions of mitochondrial DNA polymerase and single-stranded DNA-binding protein. Template-primer DNA binding and initiation and elongation of DNA strand synthesis. *J. Biol. Chem.* 274, 14779–14785.
- Fernández-Silva, P., Enriquez, J.A., Montoya, J., 2003. Replication and transcription of mammalian mitochondrial DNA. *Exp. Physiol.* 88, 41–56.
- Finsterer, J., 2008. Leigh and Leigh-Like Syndrome in Children and Adults. *Pediatr. Neurol.*
- Fisher, N., Meunier, B., 2001. Effects of mutations in mitochondrial cytochrome b in yeast and man: Deficiency, compensation and disease. *Eur. J. Biochem.*
- Frezza, C., Cipolat, S., Martins de Brito, O., Micaroni, M., Beznoussenko, G. V, Rudka, T., Bartoli, D., Polishuck, R.S., Danial, N.N., De Strooper, B., Scorrano, L., 2006. OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. *Cell* 126, 177–89. doi:10.1016/j.cell.2006.06.025
- Friedman, J.R., Lackner, L.L., West, M., DiBenedetto, J.R., Nunnari, J., Voeltz, G.K., 2011. ER Tubules Mark Sites of Mitochondrial Division. *Science* (80-.).
- Fukai, T., Ushio-Fukai, M., 2011. Superoxide dismutases: role in redox signaling, vascular function, and diseases. *Antioxid. Redox Signal.* 15, 1583–1606.
- Gilkerson, R.W., De vries, R.L.A., Lebot, P., Wikstrom, J.D., Torgykes, E., Shirihai, O.S., Przedborski, S., Schon, E.A., 2012. Mitochondrial autophagy in cells with mtDNA mutations results from synergistic loss of transmembrane potential and mTORC1 inhibition. *Hum. Mol. Genet.* 21, 978–990.
- Gilkerson, R.W., Schon, E.A., Hernandez, E., Davidson, M.M., 2008. Mitochondrial nucleoids maintain genetic autonomy but allow for functional complementation. *J. Cell Biol.* 181, 1117–1128.
- Goffart, S., Cooper, H.M., Tynismaa, H., Wanrooij, S., Suomalainen, A., Spelbrink, J.N., 2009. Twinkle mutations associated with autosomal dominant progressive external ophthalmoplegia lead to impaired helicase function and in vivo mtDNA replication stalling. *Hum. Mol. Genet.* 18, 328–340. doi:10.1093/hmg/ddn359
- Goto, Y., Nonaka, I., Horai, S., 1990. A mutation in the tRNA^{Leu(UUR)} gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. *Nature* 348, 651–653.
- Gray, M.W., 2012. Mitochondrial evolution. *Cold Spring Harb. Perspect. Biol.* 4, a011403. doi:10.1101/cshperspect.a011403
- Hallberg, B.M., Larsson, N.-G., 2011. TFAM forces mtDNA to make a U-turn. *Nat Struct Mol Biol* 18, 1179–1181.
- Harman, D., 1956. Aging: a theory based on free radical and radiation chemistry. *J. Gerontol.* 11, 298–300.
- Harner, M., Körner, C., Walther, D., Mokranjac, D., Kaesmacher, J., Welsch, U., Griffith, J., Mann, M., Reggiori, F., Neupert, W., 2011. The mitochondrial contact site complex, a determinant of mitochondrial architecture. *EMBO J.*
- Hayashi, J., Ohta, S., Kikuchi, A., Takemitsu, M., Goto, Y., Nonaka, I., 1991. Introduction of disease-related mitochondrial DNA deletions into HeLa cells lacking mitochondrial DNA results in mitochondrial dysfunction. *Proc. Natl. Acad. Sci. U. S. A.* 88, 10614–10618.
- Herrmann, J.M., 2011. MINOS Is Plus: A Mitofilin Complex for Mitochondrial Membrane Contacts. *Dev. Cell.*
- Holt, I.J., 2009. Mitochondrial DNA replication and repair: all a flap. *Trends Biochem. Sci.*
- Holt, I.J., Reyes, A., 2012. Human mitochondrial DNA replication. *Cold Spring Harb. Perspect. Biol.* 4.
- Holzenberger, M., Dupont, J., Ducos, B., Leneuve, P., Gélœn, A., Even, P.C., Cervera, P., Le Bouc, Y., 2003. IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. *Nature* 421, 182–187.

- Holzmann, J., Frank, P., Löffler, E., Bennett, K.L., Gerner, C., Rossmannith, W., 2008. RNase P without RNA: Identification and Functional Reconstitution of the Human Mitochondrial tRNA Processing Enzyme. *Cell* 135, 462–474.
- Hoppins, S., Collins, S.R., Cassidy-Stone, A., Hummel, E., DeVay, R.M., Lackner, L.L., Westermann, B., Schuldiner, M., Weissman, J.S., Nunnari, J., 2011. A mitochondrial-focused genetic interaction map reveals a scaffold-like complex required for inner membrane organization in mitochondria. *J. Cell Biol.* 195, 323–340.
- Hoppins, S., Nunnari, J., 2009. The molecular mechanism of mitochondrial fusion. *Biochim. Biophys. Acta* 1793, 20–6. doi:10.1016/j.bbamcr.2008.07.005
- Hu, J., de Souza-Pinto, N.C., Haraguchi, K., Hogue, B.A., Jaruga, P., Greenberg, M.M., Dizdaroglu, M., Bohr, V.A., 2005. Repair of formamidopyrimidines in DNA involves different glycosylases: role of the OGG1, NTH1, and NEIL1 enzymes. *J. Biol. Chem.* 280, 40544–51. doi:10.1074/jbc.M508772200
- Huang, H., Gao, Q., Peng, X., Choi, S.Y., Sarma, K., Ren, H., Morris, A.J., Frohman, M.A., 2011. PiRNA-Associated Germline Nuage Formation and Spermatogenesis Require MitoPLD Profusogenic Mitochondrial-Surface Lipid Signaling. *Dev. Cell* 20, 376–387.
- Hütter, E., Skovbro, M., Lener, B., Prats, C., Rabøl, R., Dela, F., Jansen-Dürr, P., 2007. Oxidative stress and mitochondrial impairment can be separated from lipofuscin accumulation in aged human skeletal muscle. *Aging Cell* 6, 245–256.
- Chacinska, A., Koehler, C.M., Milenkovic, D., Lithgow, T., Pfanner, N., 2009. Importing Mitochondrial Proteins: Machineries and Mechanisms. *Cell*.
- Chan, N.C., Salazar, A.M., Pham, A.H., Sweredoski, M.J., Kolawa, N.J., Graham, R.L.J., Hess, S., Chan, D.C., 2011. Broad activation of the ubiquitin-proteasome system by Parkin is critical for mitophagy. *Hum. Mol. Genet.* 20, 1726–1737.
- Chandel, N.S., 2010. Mitochondrial regulation of oxygen sensing. *Adv. Exp. Med. Biol.* 661, 339–54. doi:10.1007/978-1-60761-500-2_22
- Chappie, J.S., Acharya, S., Leonard, M., Schmid, S.L., Dyda, F., 2010. G domain dimerization controls dynamin's assembly-stimulated GTPase activity. *Nature* 465, 435–440.
- Chen, D., Gao, F., Li, B., Wang, H., Xu, Y., Zhu, C., Wang, G., 2010. Parkin mono-ubiquitinates Bcl-2 and regulates autophagy. *J. Biol. Chem.* 285, 38214–38223.
- Choi, S.-Y., Huang, P., Jenkins, G.M., Chan, D.C., Schiller, J., Frohman, M.A., 2006. A common lipid links Mfn-mediated mitochondrial fusion and SNARE-regulated exocytosis. *Nat. Cell Biol.* 8, 1255–1262.
- Choi, Y.S., Kim, S., Pak, Y.K., 2001. Mitochondrial transcription factor A (mtTFA) and diabetes, in: *Diabetes Research and Clinical Practice*.
- Chomyn, A., Martinuzzi, A., Yoneda, M., Daga, A., Hurko, O., Johns, D., Lai, S.T., Nonaka, I., Angelini, C., Attardi, G., 1992. MELAS mutation in mtDNA binding site for transcription termination factor causes defects in protein synthesis and in respiration but no change in levels of upstream and downstream mature transcripts. *Proc. Natl. Acad. Sci.* 89, 4221–4225. doi:10.1073/pnas.89.10.4221
- Christian, B.E., Spremulli, L.L., 2012. Mechanism of protein biosynthesis in mammalian mitochondria. *Biochim. Biophys. Acta - Gene Regul. Mech.*
- Chujo, T., Ohira, T., Sakaguchi, Y., Goshima, N., Nomura, N., Nagao, A., Suzuki, T., 2012. LRPPRC/SLIRP suppresses PNPase-mediated mRNA decay and promotes polyadenylation in human mitochondria. *Nucleic Acids Res.* 40, 8033–47. doi:10.1093/nar/gks506
- Chung, H.Y., Cesari, M., Anton, S., Marzetti, E., Giovannini, S., Seo, A.Y., Carter, C., Yu, B.P., Leeuwenburgh, C., 2009. Molecular inflammation: Underpinnings of aging and age-related diseases. *Ageing Res. Rev.*
- Iborra, F.J., Kimura, H., Cook, P.R., 2004. The functional organization of mitochondrial genomes in human cells. *BMC Biol.* 2, 9. doi:10.1186/1741-7007-2-9
- Iwasawa, R., Mahul-Mellier, A.-L., Datler, C., Pazarentzos, E., Grimm, S., 2011. Fis1 and Bap31 bridge the mitochondria-ER interface to establish a platform for apoptosis induction. *EMBO J.* 30, 556–568.

- Jans, D.C., Wurm, C. a, Riedel, D., Wenzel, D., Stagge, F., Deckers, M., Rehling, P., Jakobs, S., 2013. STED super-resolution microscopy reveals an array of MINOS clusters along human mitochondria. *Proc. Natl. Acad. Sci. U. S. A.* 110, 8936–41.
- Jenuth, J.P., Peterson, A.C., Fu, K., Shoubridge, E.A., 1996. Random genetic drift in the female germline explains the rapid segregation of mammalian mitochondrial DNA. *Nat. Genet.* 14, 146–151.
- Jezek, P., Mahdi, F., Garlid, K., 1990. Reconstitution of the beef heart and rat liver mitochondrial K⁺/H⁺ (Na⁺/H⁺) antiporter. Quantitation of K⁺ transport with the novel fluorescent probe, PBFI. *J. Biol. Chem.* 265, 10522–10526.
- Ježek, P., Hlavatá, L., 2005. Mitochondria in homeostasis of reactive oxygen species in cell, tissues, and organism. *Int. J. Biochem. Cell Biol.*
- Jia, L., Dienhart, M., Schrampp, M., McCauley, M., Hell, K., Stuart, R.A., 2003. Yeast Oxa1 interacts with mitochondrial ribosomes: The importance of the C-terminal region of Oxa1. *EMBO J.* 22, 6438–6447.
- Joo, J.H., Dorsey, F.C., Joshi, A., Hennessy-Walters, K.M., Rose, K.L., McCastlain, K., Zhang, J., Iyengar, R., Jung, C.H., Suen, D.F., Steeves, M.A., Yang, C.Y., Prater, S.M., Kim, D.H., Thompson, C.B., Youle, R.J., Ney, P.A., Cleveland, J.L., Kundu, M., 2011. Hsp90-Cdc37 Chaperone Complex Regulates Ulk1- and Atg13-Mediated Mitophagy. *Mol. Cell* 43, 572–585.
- Kaeberlein, M., Burtner, C.R., Kennedy, B.K., 2007. Recent developments in yeast aging. *PLoS Genet.*
- Karahalil, B., de Souza-Pinto, N.C., Parsons, J.L., Elder, R.H., Bohr, V.A., 2003. Compromised incision of oxidized pyrimidines in liver mitochondria of mice deficient in NTH1 and OGG1 glycosylases. *J. Biol. Chem.* 278, 33701–7. doi:10.1074/jbc.M301617200
- Kasashima, K., Sumitani, M., Endo, H., 2012. Maintenance of mitochondrial genome distribution by mitochondrial AAA+ protein ClpX. *Exp. Cell Res.* 318, 2335–43. doi:10.1016/j.yexcr.2012.07.012
- Kasashima, K., Sumitani, M., Endo, H., 2011. Human mitochondrial transcription factor A is required for the segregation of mitochondrial DNA in cultured cells. *Exp. Cell Res.* 317, 210–20. doi:10.1016/j.yexcr.2010.10.008
- Kazak, L., Reyes, A., Holt, I.J., 2012. Minimizing the damage: repair pathways keep mitochondrial DNA intact. *Nat Rev Mol Cell Biol* 13, 659–671.
- Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., Shimizu, N., 1998. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism., *Nature.*
- Kogure, T., Kawano, H., Abe, Y., Miyawaki, A., 2008. Fluorescence imaging using a fluorescent protein with a large Stokes shift. *Methods* 45, 223–226. doi:10.1016/j.ymeth.2008.06.009
- Kolesnikova, O.A., Entelis, N.S., Jacquin-Becker, C., Goltzene, F., Chrzanowska-Lightowlers, Z.M., Lightowlers, R.N., Martin, R.P., Tarasov, I., 2004. Nuclear DNA-encoded tRNAs targeted into mitochondria can rescue a mitochondrial DNA mutation associated with the MERRF syndrome in cultured human cells. *Hum. Mol. Genet.* 13, 2519–2534.
- Kopek, B.G., Shtengel, G., Xu, C.S., Clayton, D.A., Hess, H.F., 2012. Correlative 3D superresolution fluorescence and electron microscopy reveal the relationship of mitochondrial nucleoids to membranes. *Proc. Natl. Acad. Sci. U. S. A.* 109, 6136–41. doi:10.1073/pnas.1121558109
- Korhonen, J.A., Pham, X.H., Pellegrini, M., Falkenberg, M., 2004. Reconstitution of a minimal mtDNA replisome in vitro. *EMBO J.* 23, 2423–2429.
- Kornmann, B., 2013. The molecular hug between the ER and the mitochondria. *Curr. Opin. Cell Biol.*
- Korobova, F., Ramabhadran, V., Higgs, H., 2013. An actin-dependent step in mitochondrial fission mediated by the ER-associated formin INF2. *Science (80-.).* 339, 464–7.
- Korshunov, S.S., Skulachev, V.P., Starkov, A.A., 1997. High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS Lett.* 416, 15–18.
- Koshiba, T., Detmer, S.A., Kaiser, J.T., Chen, H., McCaffery, J.M., Chan, D.C., 2004. Structural basis of mitochondrial tethering by mitofusin complexes. *Science* 305, 858–862.

- Krishnan, K.J., Reeve, A.K., Samuels, D.C., Chinnery, P.F., Blackwood, J.K., Taylor, R.W., Wanrooij, S., Spelbrink, J.N., Lightowlers, R.N., Turnbull, D.M., 2008. What causes mitochondrial DNA deletions in human cells? *Nat. Genet.* 40, 275–279.
- Kukat, C., Wurm, C.A., Spähr, H., Falkenberg, M., Larsson, N.-G., Jakobs, S., 2011. Super-resolution microscopy reveals that mammalian mitochondrial nucleoids have a uniform size and frequently contain a single copy of mtDNA. *Proc. Natl. Acad. Sci. U. S. A.* 108, 13534–13539.
- Lazarou, M., Jin, S.M., Kane, L.A., Youle, R.J., 2012. Role of PINK1 Binding to the TOM Complex and Alternate Intracellular Membranes in Recruitment and Activation of the E3 Ligase Parkin. *Dev. Cell* 22, 320–333.
- Leach, K.L., Swaney, S.M., Colca, J.R., McDonald, W.G., Blinn, J.R., Thomasco, L.M., Gadwood, R.C., Shinabarger, D., Xiong, L., Mankin, A.S., 2007. The Site of Action of Oxazolidinone Antibiotics in Living Bacteria and in Human Mitochondria. *Mol. Cell* 26, 393–402.
- Lee, K.-W., Okot-Kotber, C., LaComb, J.F., Bogenhagen, D.F., 2013. Mitochondrial ribosomal RNA (rRNA) methyltransferase family members are positioned to modify nascent rRNA in foci near the mitochondrial DNA nucleoid. *J. Biol. Chem.* 288, 31386–99. doi:10.1074/jbc.M113.515692
- Legros, F., Malka, F., Frachon, P., Lombès, A., Rojo, M., 2004. Organization and dynamics of human mitochondrial DNA. *J. Cell Sci.* 117, 2653–2662.
- Lewis, M., Lewis, W., 1915. Mitochondria (and other cytoplasmic structures) in tissue cultures. *Am. J. Anat.* 17, 339–401.
- Lim, K.L., Dawson, V.L., Dawson, T.M., 2006. Parkin-mediated lysine 63-linked polyubiquitination: A link to protein inclusions formation in Parkinson's and other conformational diseases? *Neurobiol. Aging.*
- López-Lluch, G., Hunt, N., Jones, B., Zhu, M., Jamieson, H., Hilmer, S., Cascajo, M. V., Allard, J., Ingram, D.K., Navas, P., de Cabo, R., 2006. Calorie restriction induces mitochondrial biogenesis and bioenergetic efficiency. *Proc. Natl. Acad. Sci. U. S. A.* 103, 1768–1773.
- Lung, B., Zemann, A., Madej, M.J., Schuelke, M., Techritz, S., Ruf, S., Bock, R., Hüttenhofer, A., 2006. Identification of small non-coding RNAs from mitochondria and chloroplasts. *Nucleic Acids Res.* 34, 3842–3852.
- Luo, N., Kaguni, L.S., 2005. Mutations in the spacer region of *Drosophila* mitochondrial DNA polymerase affect DNA binding, processivity, and the balance between Pol and Exo function. *J. Biol. Chem.* 280, 2491–7. doi:10.1074/jbc.M411447200
- Macmillan, C.J., Shoubbridge, E.A., 1996. Mitochondrial DNA depletion: Prevalence in a pediatric population referred for neurologic evaluation. *Pediatr. Neurol.* 14, 203–210.
- Maechler, P., Wollheim, C.B., 2001. Mitochondrial function in normal and diabetic beta-cells. *Nature* 414, 807–812. doi:10.1038/414807a
- Magalhães, P.J., Andreu, A.L., Schon, E.A., 1998. Evidence for the presence of 5S rRNA in mammalian mitochondria. *Mol. Biol. Cell* 9, 2375–2382.
- Man, P.Y.W., Turnbull, D.M., Chinnery, P.F., 2002. Leber hereditary optic neuropathy. *J. Med. Genet.* 39, 162–169.
- Mandal, S.M., Hegde, M.L., Chatterjee, A., Hegde, P.M., Szczesny, B., Banerjee, D., Boldogh, I., Gao, R., Falkenberg, M., Gustafsson, C.M., Sarkar, P.S., Hazra, T.K., 2012. Role of human DNA glycosylase Nei-like 2 (NEIL2) and single strand break repair protein polynucleotide kinase 3'-phosphatase in maintenance of mitochondrial genome. *J. Biol. Chem.* 287, 2819–29. doi:10.1074/jbc.M111.272179
- Mandel, H., Szargel, R., Labay, V., Elpeleg, O., Saada, A., Shalata, A., Anbinder, Y., Berkowitz, D., Hartman, C., Barak, M., Eriksson, S., Cohen, N., 2001. The deoxyguanosine kinase gene is mutated in individuals with depleted hepatocerebral mitochondrial DNA. *Nat. Genet.* 29, 337–341.
- Mannella, C.A., 2006. The relevance of mitochondrial membrane topology to mitochondrial function. *Biochim. Biophys. Acta* 1762, 140–147. doi:10.1016/j.bbadis.2005.07.001
- Marklund, S.L., 1984. Extracellular superoxide dismutase and other superoxide dismutase isoenzymes in tissues from nine mammalian species. *Biochem. J.* 222, 649–655.

- Mason, P.A., Lightowlers, R.N., 2003. Why do mammalian mitochondria possess a mismatch repair activity? *FEBS Lett.* 554, 6–9.
- Meissner, C., Lorenz, H., Weihofen, A., Selkoe, D.J., Lemberg, M.K., 2011. The mitochondrial intramembrane protease PARL cleaves human Pink1 to regulate Pink1 trafficking. *J. Neurochem.* 117, 856–867.
- Mignotte, B., Barat, M., Mounolou, J.-C., 1985. Characterization of a mitochondrial protein binding to single-stranded DNA. *Nucleic Acids Res* 13, 1703–1716. doi:10.1093/nar/13.5.1703
- Minauro-Sanmiguel, F., Wilkens, S., García, J.J., 2005. Structure of dimeric mitochondrial ATP synthase: novel F0 bridging features and the structural basis of mitochondrial cristae biogenesis. *Proc. Natl. Acad. Sci. U. S. A.* 102, 12356–12358.
- Miquel, J., Economos, A.C., Fleming, J., Johnson, J.E., 1980. Mitochondrial role in cell aging. *Exp. Gerontol.* 15, 575–591.
- Miwa, S., Lawless, C., Von Zglinicki, T., 2008. Mitochondrial turnover in liver is fast in vivo and is accelerated by dietary restriction: Application of a simple dynamic model. *Aging Cell* 7, 920–923.
- Morten, K.J., Ashley, N., Wijburg, F., Hadzic, N., Parr, J., Jayawant, S., Adams, S., Bindoff, L., Bakker, H.D., Mieli-Vergani, G., Zeviani, M., Poulton, J., 2007. Liver mtDNA content increases during development: A comparison of methods and the importance of age- and tissue-specific controls for the diagnosis of mtDNA depletion. *Mitochondrion* 7, 386–395.
- Nagaike, T., Suzuki, T., Tomari, Y., Takemoto-Hori, C., Negayama, F., Watanabe, K., Ueda, T., 2001. Identification and characterization of mammalian mitochondrial tRNA nucleotidyltransferases. *J. Biol. Chem.* 276, 40041–40049. doi:10.1074/jbc.M106202200
- Narendra, D.P., Jin, S.M., Tanaka, A., Suen, D.F., Gautier, C. a., Shen, J., Cookson, M.R., Youle, R.J., 2010. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. *PLoS Biol.* 8. doi:10.1371/journal.pbio.1000298
- Nass, M.M., 1966. The circularity of mitochondrial DNA. *Proc. Natl. Acad. Sci. U. S. A.* 56, 1215–22.
- Nègre-Salvayre, A., Hirtz, C., Carrera, G., Cazenave, R., Troly, M., Salvayre, R., Pénicaud, L., Casteilla, L., 1997. A role for uncoupling protein-2 as a regulator of mitochondrial hydrogen peroxide generation. *FASEB J.* 11, 809–815.
- Ngo, H.B., Kaiser, J.T., Chan, D.C., 2011. The mitochondrial transcription and packaging factor Tfam imposes a U-turn on mitochondrial DNA. *Nat. Struct. Mol. Biol.* 18, 1290–6. doi:10.1038/nsmb.2159
- Niazi, A.K., Milesina, D., Cosset, A., Val, R., Weber-Lotfi, F., Dietrich, A., 2013. Targeting nucleic acids into mitochondria: Progress and prospects. *Mitochondrion* 13, 548–558.
- Nile, D.L., Brown, A.E., Kumaheri, M. a., Blair, H.R., Heggie, A., Miwa, S., Cree, L.M., Payne, B., Chinnery, P.F., Brown, L., Gunn, D. a., Walker, M., 2014. Age-Related Mitochondrial DNA Depletion and the Impact on Pancreatic Beta Cell Function. *PLoS One* 9, e115433. doi:10.1371/journal.pone.0115433
- O'Brien, T.W., 1971. The General Occurrence of 55 S Ribosomes in Mammalian Liver Mitochondria. *J. Biol. Chem.* 246, 3409–3417.
- Ohtsubo, T., 2000. Identification of human MutY homolog (hMYH) as a repair enzyme for 2-hydroxyadenine in DNA and detection of multiple forms of hMYH located in nuclei and mitochondria. *Nucleic Acids Res.* 28, 1355–1364. doi:10.1093/nar/28.6.1355
- Ojala, D., Montoya, J., Attardi, G., 1981. tRNA punctuation model of RNA processing in human mitochondria. *Nature* 290, 470–474.
- Olichon, A., Baricault, L., Gas, N., Guillou, E., Valette, A., Belenguer, P., Lenaers, G., 2003. Loss of OPA1 perturbs the mitochondrial inner membrane structure and integrity, leading to cytochrome c release and apoptosis. *J. Biol. Chem.* 278, 7743–7746.
- Oliveira, M.T., Kaguni, L.S., 2010. Functional roles of the N- and C-terminal regions of the human mitochondrial single-stranded DNA-binding protein. *PLoS One* 5, e15379. doi:10.1371/journal.pone.0015379

- Östenson, C.G., Efendic, S., 2007. Islet gene expression and function in type 2 diabetes; studies in the Goto-Kakizaki rat and humans, in: *Diabetes, Obesity and Metabolism*. pp. 180–186.
- Otera, H., Wang, C., Cleland, M.M., Setoguchi, K., Yokota, S., Youle, R.J., Mihara, K., 2010. Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells. *J. Cell Biol.* 191, 1141–1158.
- Palmer, C.S., Osellame, L.D., Laine, D., Koutsopoulos, O.S., Frazier, A.E., Ryan, M.T., 2011. MiD49 and MiD51, new components of the mitochondrial fission machinery. *EMBO Rep.* 12, 565–573.
- Park, C.B., Asin-Cayuela, J., Cámara, Y., Shi, Y., Pellegrini, M., Gaspari, M., Wibom, R., Hultenby, K., Erdjument-Bromage, H., Tempst, P., Falkenberg, M., Gustafsson, C.M., Larsson, N.G., 2007. MTERF3 Is a Negative Regulator of Mammalian mtDNA Transcription. *Cell* 130, 273–285.
- Petersen, K.F., Dufour, S., Savage, D.B., Bilz, S., Solomon, G., Yonemitsu, S., Cline, G.W., Befroy, D., Zeman, L., Kahn, B.B., Papademetris, X., Rothman, D.L., Shulman, G.I., 2007. The role of skeletal muscle insulin resistance in the pathogenesis of the metabolic syndrome. *Proc. Natl. Acad. Sci. U. S. A.* 104, 12587–94. doi:10.1073/pnas.0705408104
- Plecitá-Hlavatá, L., Lessard, M., Santorová, J., Bewersdorf, J., Jezek, P., 2008. Mitochondrial oxidative phosphorylation and energetic status are reflected by morphology of mitochondrial network in INS-1E and HEP-G2 cells viewed by 4Pi microscopy. *Biochim. Biophys. Acta* 1777, 834–46. doi:10.1016/j.bbabi.2008.04.002
- Pohjoismäki, J.L.O., Goffart, S., 2011. Of circles, forks and humanity: Topological organisation and replication of mammalian mitochondrial DNA. *BioEssays*.
- Praefcke, G.J.K., McMahon, H.T., 2004. The dynamin superfamily: universal membrane tubulation and fission molecules? *Nat. Rev. Mol. Cell Biol.* 5, 133–147.
- Puranam, R.S., Attardi, G., 2001. The RNase P associated with HeLa cell mitochondria contains an essential RNA component identical in sequence to that of the nuclear RNase P. *Mol. Cell. Biol.* 21, 548–561.
- Rabl, R., Soubannier, V., Scholz, R., Vogel, F., Mendl, N., Vasiljev-Neumeyer, A., Körner, C., Jagasia, R., Keil, T., Baumeister, W., Cyrklaff, M., Neupert, W., Reichert, A.S., 2009. Formation of cristae and crista junctions in mitochondria depends on antagonism between Fcj1 and Su e/g. *J. Cell Biol.* 185, 1047–63. doi:10.1083/jcb.200811099
- Rajala, N., Gerhold, J.M., Martinsson, P., Klymov, A., Spelbrink, J.N., 2014. Replication factors transiently associate with mtDNA at the mitochondrial inner membrane to facilitate replication. *Nucleic Acids Res.* 42, 952–967.
- Rera, M., Bahadorani, S., Cho, J., Koehler, C.L., Ulgherait, M., Hur, J.H., Ansari, W.S., Lo, T., Jones, D.L., Walker, D.W., 2011. Modulation of longevity and tissue homeostasis by the drosophila PGC-1 homolog. *Cell Metab.* 14, 623–634.
- Reyes, A., He, J., Mao, C.C., Bailey, L.J., Di Re, M., Sembongi, H., Kazak, L., Dzionek, K., Holmes, J.B., Cluett, T.J., Harbour, M.E., Fearnley, I.M., Crouch, R.J., Conti, M.A., Adelstein, R.S., Walker, J.E., Holt, I.J., 2011. Actin and myosin contribute to mammalian mitochondrial DNA maintenance. *Nucleic Acids Res.* 39, 5098–5108.
- Robberson, D.L., Kasamatsu, H., Vinograd, J., 1972. Replication of mitochondrial DNA. Circular replicative intermediates in mouse L cells. *Proc. Natl. Acad. Sci. U. S. A.* 69, 737–741.
- Robertson, J.D., 1960. The molecular structure and contact relationships of cell membranes. *Prog Biophys Mol Biol* 343–418.
- Robin, E.D., Wong, R., 1988. Mitochondrial DNA molecules and virtual number of mitochondria per cell in mammalian cells. *J. Cell. Physiol.* 136, 507–513.
- Rorbach, J., Minczuk, M., 2012. The post-transcriptional life of mammalian mitochondrial RNA. *Biochem. J.*
- Rorsman, P., 1997. The pancreatic beta-cell as a fuel sensor: An electrophysiologist's viewpoint. *Diabetologia*.
- Rossignol, R., Gilkerson, R., Aggeler, R., Yamagata, K., Remington, S.J., Capaldi, R.A., 2004. Energy Substrate Modulates Mitochondrial Structure and Oxidative Capacity in Cancer Cells. *Cancer Res.* 64, 985–993.

- Rossmannith, W., 2011. Localization of human RNase Z isoforms: Dual nuclear/mitochondrial targeting of the ELAC2 gene product by alternative translation initiation. *PLoS One* 6.
- Rubio-Cosials, A., Sidow, J.F., Jiménez-Menéndez, N., Fernández-Millán, P., Montoya, J., Jacobs, H.T., Coll, M., Bernadó, P., Solà, M., 2011. Human mitochondrial transcription factor A induces a U-turn structure in the light strand promoter. *Nat. Struct. Mol. Biol.* doi:10.1038/nsmb.2160
- Rusiñol, A.E., Cui, Z., Chen, M.H., Vance, J.E., 1994. A unique mitochondria-associated membrane fraction from rat liver has a high capacity for lipid synthesis and contains pre-Golgi secretory proteins including nascent lipoproteins. *J. Biol. Chem.* 269, 27494–27502.
- Saada, A., Shaag, A., Mandel, H., Nevo, Y., Eriksson, S., Elpeleg, O., 2001. Mutant mitochondrial thymidine kinase in mitochondrial DNA depletion myopathy. *Nat. Genet.* 29, 342–344.
- Sage, J.M., Gildemeister, O.S., Knight, K.L., 2010. Discovery of a novel function for human Rad51: maintenance of the mitochondrial genome. *J Biol Chem* 285, 18984–18990. doi:10.1074/jbc.M109.099846
- Sandoval, H., Thiagarajan, P., Dasgupta, S.K., Schumacher, A., Prchal, J.T., Chen, M., Wang, J., 2008. Essential role for Nix in autophagic maturation of erythroid cells. *Nature* 454, 232–235.
- Santangelo, P.J., Nitin, N., Bao, G., 2005. Direct visualization of mRNA colocalization with mitochondria in living cells using molecular beacons. *J. Biomed. Opt.* 10, 44025. doi:10.1117/1.2011402
- Santra, S., Gilkerson, R.W., Davidson, M., Schon, E.A., 2004. Ketogenic treatment reduces deleted mitochondrial DNAs in cultured human cells. *Ann. Neurol.* 56, 662–669.
- Satoh, M., Kuroiwa, T., 1991. Organization of multiple nucleoids and DNA molecules in mitochondria of a human cell. *Exp. Cell Res.* 196, 137–140.
- Seidel-Rogol, B.L., McCulloch, V., Shadel, G.S., 2003. Human mitochondrial transcription factor B1 methylates ribosomal RNA at a conserved stem-loop. *Nat. Genet.* 33, 23–24.
- Sharma, M.R., Koc, E.C., Datta, P.P., Booth, T.M., Spemulli, L.L., Agrawal, R.K., 2003. Structure of the mammalian mitochondrial ribosome reveals an expanded functional role for its component proteins. *Cell* 115, 97–108.
- Shmookler Reis, R.J., Goldstein, S., 1983. Mitochondrial DNA in mortal and immortal human cells. Genome number, integrity, and methylation. *J. Biol. Chem.* 258, 9078–9085.
- Shoffner, J.M., Lott, M.T., Lezza, A.M., Seibel, P., Ballinger, S.W., Wallace, D.C., 1990. Myoclonic epilepsy and ragged-red fiber disease (MERRF) is associated with a mitochondrial DNA tRNA(Lys) mutation. *Cell* 61, 931–937.
- Schneider, A., Ebert, D., 2004. Covariation of mitochondrial genome size with gene lengths: evidence for gene length reduction during mitochondrial evolution. *J. Mol. Evol.* 59, 90–96.
- Schon, E.A., Area-Gomez, E., 2013. Mitochondria-associated ER membranes in Alzheimer disease. *Mol. Cell. Neurosci.*
- Schon, E.A., DiMauro, S., Hirano, M., 2012. Human mitochondrial DNA: roles of inherited and somatic mutations. *Nat Rev Genet* 13, 878–890.
- Silva, J.P., Kohler, M., Graff, C., Oldfors, A., Magnuson, M.A., Berggren, P.-O., Larsson, N.-G., 2000. Impaired insulin secretion and [beta]-cell loss in tissue-specific knockout mice with mitochondrial diabetes. *Nat Genet* 26, 336–340.
- Smirnov, A., Comte, C., Mager-Heckel, A.M., Addis, V., Krashennnikov, I.A., Martin, R.P., Entelis, N., Tarassov, I., 2010. Mitochondrial enzyme rhodanese is essential for 5 S ribosomal RNA import into human mitochondria. *J. Biol. Chem.* 285, 30792–30803.
- Smirnov, A., Tarassov, I., Mager-Heckel, A.-M., Letzelter, M., Martin, R.P., Krashennnikov, I.A., Entelis, N., 2008. Two distinct structural elements of 5S rRNA are needed for its import into human mitochondria. *RNA* 14, 749–759.
- Smirnov, A. V., Entelis, N.S., Krashennnikov, I.A., Martin, R., Tarassov, I.A., 2008. Specific features of 5S rRNA structure - its interactions with macromolecules and possible functions. *Biochemistry. (Mosc).* 73, 1418–1437.

- Smirnova, E., Griparic, L., Shurland, D.L., van der Bliek, A.M., 2001. Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. *Mol. Biol. Cell* 12, 2245–2256.
- Smolková, K., Plecitá-Hlavatá, L., Bellance, N., Benard, G., Rossignol, R., Ježek, P., 2011. Waves of gene regulation suppress and then restore oxidative phosphorylation in cancer cells. *Int. J. Biochem. Cell Biol.* 43, 950–68. doi:10.1016/j.biocel.2010.05.003
- Song, Z., Chen, H., Fiket, M., Alexander, C., Chan, D.C., 2007. OPA1 processing controls mitochondrial fusion and is regulated by mRNA splicing, membrane potential, and Yme1L. *J. Cell Biol.* 178, 749–55. doi:10.1083/jcb.200704110
- Spelbrink, J.N., Li, F.-Y., Tiranti, V., Nikali, K., Yuan, Q.-P., Tariq, M., Wanrooij, S., Garrido, N., Comi, G., Morandi, L., Santoro, L., Toscano, A., Fabrizi, G.-M., Somer, H., Croxen, R., Beeson, D., Poulton, J., Suomalainen, A., Jacobs, H.T., Zeviani, M., Larsson, C., 2001. Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria. *Nat Genet* 28, 223–231.
- St. John, J., 2014. The control of mtDNA replication during differentiation and development. *Biochim. Biophys. Acta - Gen. Subj.*
- Suen, D.-F., Narendra, D.P., Tanaka, A., Manfredi, G., Youle, R.J., 2010. Parkin overexpression selects against a deleterious mtDNA mutation in heteroplasmic cybrid cells. *Proc. Natl. Acad. Sci. U. S. A.* 107, 11835–11840.
- Szabadkai, G., Bianchi, K., Várnai, P., De Stefani, D., Wieckowski, M.R., Cavagna, D., Nagy, A.I., Balla, T., Rizzuto, R., 2006. Chaperone-mediated coupling of endoplasmic reticulum and mitochondrial Ca²⁺ channels. *J. Cell Biol.* 175, 901–911.
- Szczesny, B., Tann, A.W., Longley, M.J., Copeland, W.C., Mitra, S., 2008. Long patch base excision repair in mammalian mitochondrial genomes. *J. Biol. Chem.* 283, 26349–56. doi:10.1074/jbc.M803491200
- Szczesny, R.J., Wojcik, M.A., Borowski, L.S., Szewczyk, M.J., Skrok, M.M., Golik, P., Stepien, P.P., 2013. Yeast and human mitochondrial helicases. *Biochim. Biophys. Acta* 1829, 842–53. doi:10.1016/j.bbagr.2013.02.009
- Szyrach, G., Ott, M., Bonnefoy, N., Neupert, W., Herrmann, J.M., 2003. Ribosome binding to the Oxa1 complex facilitates co-translational protein insertion in mitochondria. *EMBO J.* 22, 6448–6457.
- Taguchi, N., Ishihara, N., Jofuku, A., Oka, T., Mihara, K., 2007. Mitotic phosphorylation of dynamin-related GTPase Drp1 participates in mitochondrial fission. *J. Biol. Chem.* 282, 11521–11529.
- Takamatsu, C., Umeda, S., Ohsato, T., Ohno, T., Abe, Y., Fukuoh, A., Shinagawa, H., Hamasaki, N., Kang, D., 2002. Regulation of mitochondrial D-loops by transcription factor A and single-stranded DNA-binding protein. *EMBO Rep.* 3, 451–456.
- Takao, M., Aburatani, H., Kobayashi, K., Yasui, A., 1998. Mitochondrial targeting of human DNA glycosylases for repair of oxidative DNA damage. *Nucleic Acids Res.* 26, 2917–2922. doi:10.1093/nar/26.12.2917
- Tang, Y., Schon, E.A., Wilichowski, E., Vazquez-Memije, M.E., Davidson, E., King, M.P., 2000. Rearrangements of human mitochondrial DNA (mtDNA): new insights into the regulation of mtDNA copy number and gene expression. *Mol. Biol. Cell* 11, 1471–1485.
- Tanida, I., 2011. Autophagy basics. *Microbiol. Immunol.*
- Tatuch, Y., Christodoulou, J., Feigenbaum, A., Clarke, J.T., Wherret, J., Smith, C., Rudd, N., Petrova-Benedict, R., Robinson, B.H., 1992. Heteroplasmic mtDNA mutation (T----G) at 8993 can cause Leigh disease when the percentage of abnormal mtDNA is high. *Am. J. Hum. Genet.* 50, 852–858.
- Tauber, J., Dlasková, A., Šantorová, J., Smolková, K., Alán, L., Špaček, T., Plecitá-Hlavatá, L., Jabůrek, M., Ježek, P., 2013. Distribution of mitochondrial nucleoids upon mitochondrial network fragmentation and network reintegration in HEPG2 cells. *Int. J. Biochem. Cell Biol.* 45, 593–603. doi:10.1016/j.biocel.2012.11.019
- Temperley, R., Richter, R., Dennerlein, S., Lightowlers, R.N., Chrzanowska-Lightowlers, Z.M., 2010. Hungry codons promote frameshifting in human mitochondrial ribosomes. *Science* 327, 301.

- Thomas, D.D., Liu, X., Kantrow, S.P., Lancaster, J.R., 2001. The biological lifetime of nitric oxide: implications for the perivascular dynamics of NO and O₂. *Proc. Natl. Acad. Sci. U. S. A.* 98, 355–360.
- Thyagarajan, B., Padua, R.A., Campbell, C., 1996. Mammalian mitochondria possess homologous DNA recombination activity. *J. Biol. Chem.* 271, 27536–27543.
- Tondera, D., Grandemange, S., Jourdain, A., Karbowski, M., Mattenberger, Y., Herzig, S., Da Cruz, S., Clerc, P., Raschke, I., Merkwirth, C., Ehses, S., Krause, F., Chan, D.C., Alexander, C., Bauer, C., Youle, R., Langer, T., Martinou, J.-C., 2009. SLP-2 is required for stress-induced mitochondrial hyperfusion. *EMBO J.* 28, 1589–1600.
- Tooze, S.A., Yoshimori, T., 2010. The origin of the autophagosomal membrane. *Nat. Cell Biol.* 12, 831–835.
- Tsuchiya, S., Sueoka, K., Matsuda, N., Tanigaki, R., Asada, H., Hashiba, T., Kato, S., Yoshimura, Y., 2005. The “spanning protocol”: A new DNA extraction method for efficient single-cell genetic diagnosis. *J. Assist. Reprod. Genet.* 22, 407–414.
- Tuppen, H.A.L., Blakely, E.L., Turnbull, D.M., Taylor, R.W., 2010. Mitochondrial DNA mutations and human disease. *Biochim. Biophys. Acta* 1797, 113–128.
- Twig, G., Elorza, A., Molina, A.J.A., Mohamed, H., Wikstrom, J.D., Walzer, G., Stiles, L., Haigh, S.E., Katz, S., Las, G., Alroy, J., Wu, M., Py, B.F., Yuan, J., Deeney, J.T., Corkey, B.E., Shirihai, O.S., 2008. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. *EMBO J.* 27, 433–446.
- Van Bruggen, E.F.J., Borst, P., Ruttenberg, G.J.C.M., Gruber, M., Kroon, A.M., 1966. Circular mitochondrial DNA. *Biochim. Biophys. Acta - Nucleic Acids Protein Synth.* 119, 437–439. doi:10.1016/0005-2787(66)90210-3
- Van der Bliek, A.M., Payne, G.S., 2010. Dynamin subunit interactions revealed. *Dev. Cell.*
- Van der Bliek, A.M., Shen, Q., Kawajiri, S., 2013. Mechanisms of mitochondrial fission and fusion. *Cold Spring Harb. Perspect. Biol.* 5.
- Van Goethem, G., Martin, J.-J., Van Broeckhoven, C., 2003. Progressive external ophthalmoplegia characterized by multiple deletions of mitochondrial DNA. *NeuroMolecular Med.* 3, 129–146. doi:10.1385/NMM:3:3:129
- Vance, J.E., 1990. Phospholipid synthesis in a membrane fraction associated with mitochondria. *J. Biol. Chem.* 265, 7248–7256.
- Villegas, J., Burzio, V., Villota, C., Landerer, E., Martinez, R., Santander, M., Martinez, R., Pinto, R., Vera, M.I., Boccardo, E., Villa, L.L., Burzio, L.O., 2007. Expression of a novel non-coding mitochondrial RNA in human proliferating cells. *Nucleic Acids Res.* 35, 7336–47. doi:10.1093/nar/gkm863
- Voelker, D., 2004. Lipid synthesis and transport in mitochondrial biogenesis. *Mitochondrial Funct. Biog.* doi:10.1007/b95719
- Wakabayashi, J., Zhang, Z., Wakabayashi, N., Tamura, Y., Fukaya, M., Kensler, T.W., Iijima, M., Sesaki, H., 2009. The dynamin-related GTPase Drp1 is required for embryonic and brain development in mice. *J. Cell Biol.* 186, 805–816.
- Wallace, D.C., 2013. Bioenergetics in human evolution and disease: implications for the origins of biological complexity and the missing genetic variation of common diseases. *Phil. Trans. R. Soc* 368, 20120267. doi:10.1098/rstb.2012.0267
- Wallace, D.C., 2010. Mitochondrial DNA mutations in disease and aging. *Environ. Mol. Mutagen.*
- Wallace, D.C., 2008. Mitochondria as Chi. *Genetics.*
- Wallace, D.C., 2007. Why do we still have a maternally inherited mitochondrial DNA? Insights from evolutionary medicine. *Annu. Rev. Biochem.* 76, 781–821. doi:10.1146/annurev.biochem.76.081205.150955
- Wallace, D.C., 2005. Mitochondria and cancer: Warburg addressed, in: *Cold Spring Harbor Symposia on Quantitative Biology.* pp. 363–374.

- Wang, G., Chen, H.W., Oktay, Y., Zhang, J., Allen, E.L., Smith, G.M., Fan, K.C., Hong, J.S., French, S.W., McCaffery, J.M., Lightowlers, R.N., Morse, H.C., Koehler, C.M., Teitell, M.A., 2010. PNPASE regulates RNA import into mitochondria. *Cell* 142, 456–467.
- Wang, Y., Bogenhagen, D.F., 2006. Human Mitochondrial DNA Nucleoids Are Linked to Protein Folding Machinery and Metabolic Enzymes at the Mitochondrial Inner Membrane. *J. Biol. Chem.* 281, 25791–25802. doi:10.1074/jbc.M604501200
- Weber, T.A., Koob, S., Heide, H., Wittig, I., Head, B., van der Blik, A., Brandt, U., Mittelbronn, M., Reichert, A.S., 2013. APOOL Is a Cardiolipin-Binding Constituent of the Mitofilin/MINOS Protein Complex Determining Cristae Morphology in Mammalian Mitochondria. *PLoS One* 8.
- Weindruch, R., Walford, R.L., Fligiel, S., Guthrie, D., 1986. The retardation of aging in mice by dietary restriction: longevity, cancer, immunity and lifetime energy intake. *J. Nutr.* 116, 641–654.
- Weisiger, R.A., Fridovich, I., 1973. Superoxide dismutase. Organelle specificity. *J. Biol. Chem.* 248, 3582–3592.
- Wiesner, R.J., Rösen, P., Grieshaber, M.K., 1988. Pathways of succinate formation and their contribution to improvement of cardiac function in the hypoxic rat heart. *Biochem. Med. Metab. Biol.* 40, 19–34.
- Willig, K.I., Harke, B., Medda, R., Hell, S.W., 2007. STED microscopy with continuous wave beams. *Nat. Methods* 4, 915–8. doi:10.1038/nmeth1108
- Wollen Steen, K., Doseth, B., P. Westbye, M., Akbari, M., Kang, D., Falkenberg, M., Slupphaug, G., 2012. MtSSB may sequester UNG1 at mitochondrial ssDNA and delay uracil processing until the dsDNA conformation is restored. *DNA Repair (Amst)*. 11, 82–91.
- Xing, J., Chen, M., Wood, C.G., Lin, J., Spitz, M.R., Ma, J., Amos, C.I., Shields, P.G., Benowitz, N.L., Gu, J., De Andrade, M., Swan, G.E., Wu, X., 2008. Mitochondrial DNA content: Its genetic heritability and association with renal cell carcinoma. *J. Natl. Cancer Inst.* 100, 1104–1112.
- Xu, Y., DeMott, M.S., Hwang, J.T., Greenberg, M.M., Demple, B., 2003. Action of human apurinic endonuclease (Ape1) on C1'-oxidized deoxyribose damage in DNA. *DNA Repair (Amst)*. 2, 175–185. doi:10.1016/S1568-7864(02)00194-5
- Yang, M.Y., Bowmaker, M., Reyes, A., Vergani, L., Angeli, P., Gringeri, E., Jacobs, H.T., Holt, I.J., 2002. Biased Incorporation of Ribonucleotides on the Mitochondrial L-Strand Accounts for Apparent Strand-Asymmetric DNA Replication. *Cell* 111, 495–505. doi:10.1016/S0092-8674(02)01075-9
- Yasukawa, T., Reyes, A., Cluett, T.J., Yang, M.-Y., Bowmaker, M., Jacobs, H.T., Holt, I.J., 2006. Replication of vertebrate mitochondrial DNA entails transient ribonucleotide incorporation throughout the lagging strand. *EMBO J.* 25, 5358–5371.
- Ylikallio, E., Tyynismaa, H., Tsutsui, H., Ide, T., Suomalainen, A., 2010. High mitochondrial DNA copy number has detrimental effects in mice. *Hum. Mol. Genet.* 19, 2695–705. doi:10.1093/hmg/ddq163
- Yoon, Y., Krueger, E.W., Oswald, B.J., McNiven, M.A., 2003. The mitochondrial protein hFis1 regulates mitochondrial fission in mammalian cells through an interaction with the dynamin-like protein DLP1. *Mol. Cell. Biol.* 23, 5409–5420.
- Youle, R.J., Narendra, D.P., 2011. Mechanisms of mitophagy. *Nat. Rev. Mol. Cell Biol.* 12, 9–14.
- Yu, M., Zhou, Y., Shi, Y., Ning, L., Yang, Y., Wei, X., Zhang, N., Hao, X., Niu, R., 2007. Reduced mitochondrial DNA copy number is correlated with tumor progression and prognosis in Chinese breast cancer patients. *IUBMB Life* 59, 450–457.
- Zerbes, R.M., Der Klei, I.J. Van, Veenhuis, M., Pfanner, N., Laan, M. Van Der, Bohnert, M., 2012. Mitofilin complexes: Conserved organizers of mitochondrial membrane architecture. *Biol. Chem.*
- Zhadanov, S.I., Grechanina, E.Y., Grechanina, Y.B., Gusar, V.A., Fedoseeva, N.P., Lebon, S., Münnich, A., Schurr, T.G., 2007. Fatal manifestation of a de novo ND5 mutation: Insights into the pathogenetic mechanisms of mtDNA ND5 gene defects. *Mitochondrion* 7, 260–266.
- Zhang, Y., Ikeno, Y., Qi, W., Chaudhuri, A., Li, Y., Bokov, A., Thorpe, S.R., Baynes, J.W., Epstein, C., Richardson, A., Van Remmen, H., 2009. Mice deficient in both Mn superoxide dismutase and glutathione

- peroxidase-1 have increased oxidative damage and a greater incidence of pathology but no reduction in longevity. *Journals Gerontol. - Ser. A Biol. Sci. Med. Sci.* 64, 1212–1220.
- Zhu, Z., Yao, J., Johns, T., Fu, K., De Bie, I., Macmillan, C., Cuthbert, A.P., Newbold, R.F., Wang, J., Chevrette, M., Brown, G.K., Brown, R.M., Shoubbridge, E.A., 1998. SURF1, encoding a factor involved in the biogenesis of cytochrome c oxidase, is mutated in Leigh syndrome. *Nat. Genet.* 20, 337–343.
- Zoccarato, F., Toscano, P., Alexandre, A., 2005. Dopamine-derived dopaminochrome promotes H₂O₂ release at mitochondrial Complex I: Stimulation by rotenone, control by Ca²⁺, and relevance to Parkinson disease. *J. Biol. Chem.* 280, 15587–15594.
- Zorov, D.B., Juhaszova, M., Sollott, S.J., 2014. Mitochondrial Reactive Oxygen Species (ROS) and ROS-Induced ROS Release. *Physiol. Rev.* 94, 909–950.

10. Publications enclosed in full

Fluorescent *in situ* hybridization of mitochondrial DNA and RNA

Lukáš Alán, Jaroslav Zelenka, Jan Ježek, Andrea Dlasková and Petr Ježek[✉]

Department No. 75, Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic

To reveal nucleic acid localization in mitochondria, we designed molecular beacon fluorescent probes against: i) the light strand complementary to ND5 mitochondrial DNA (mtDNA) gene (annealing also to corresponding mRNA); ii) displacement (D) loop 7S DNA (annealing also to parallel heavy strand mtDNA and corresponding light strand transcript); iii) the proximal D-loop heavy strand displaced by the light strand promoter minor RNA. Confocal microscopy demonstrated ND5 probe spreading (less for other probes) in mitochondrial reticulum tubules but upon RNase A treatment all probes contoured mtDNA nucleoid localization. DNase I spread the signal over mitochondrial tubules. Future applications are discussed.

Keywords: mitochondrial DNA and RNA, nucleoids of mitochondrial DNA, molecular beacon fluorescent hybridization probes, confocal microscopy, transcription and replication intermediates

Received: 30 August, 2010; **revised:** 04 November, 2010; **accepted:** 25 November, 2010; **available on-line:** 29 November, 2010

INTRODUCTION

Mitochondrial DNA (mtDNA) most probably determines aging to some extent and hence the lifespan of living organisms (Holt, 2010; Tuppen *et al.*, 2010). In turn, mtDNA genetics is characterized by robustness due to multiple mtDNA copies (typically 1000 to 10000) existing in a single cell (Holt *et al.*, 2007; Holt, 2010; Tuppen *et al.*, 2010) and due to a purifying bottleneck effect during embryonic and fetal development (Cree *et al.* 2008; Stewart *et al.*, 2008). Moreover, mtDNA mutations have been implicated in mitopathies and other diseases, namely those originating from oxidative stress — a constant inevitable superoxide production is a by-product of respiratory chain function and „tax for life“ (Lu *et al.*, 2000; Zhadanov *et al.*, 2007; Scaglia & Wong, 2008; Holt, 2010; Tuppen *et al.*, 2010). It is surprising how little information has been gathered concerning mitochondrial genetics, about details of transcription and replication mechanisms of mtDNA (Fig. 1A, B), and the structure of mtDNA organized in so-called mt nucleoids (Holt *et al.* 2007; Bogenhagen *et al.*, 2008).

An originally proposed *asynchronous strand displacement replication* model for mtDNA was later questioned for the lacking existence of its single strand asynchronous intermediates (Yasukawa *et al.*, 2006; Hyvärinen *et al.*, 2007; Holt, 2009). This model assumed that DNA synthesis is primed by transcription through the H strand origin (OriH) within the displacement (D) loop (Shadel & Clayton, 1997; Fig. 1B). Unidirectional synthesis is supposed to proceed along the template while displacing the opposite strand. After two-thirds around the 16-kb circular

human mt genome, the nascent H strand should be replicated and the L strand origin OriL should be exposed, allowing initiation of the nascent L strand synthesis. Elongation of the second strand then should continue along the previously displaced single-stranded (ss) template in the opposite direction. The leading strand and lagging strand synthesis are supposed to be independent in mitochondria. Both strands should be extended in an asymmetric manner until two daughter molecules are formed (Clayton, 2003; Falkenberg *et al.*, 2007). Mitochondrial RNA polymerase serves as a primase required for DNA synthesis at OriL (Fusté *et al.*, 2010).

Mitochondria contain the apparatus for Okazaki fragment maturation, although it is not clear whether this mechanism is in effect (Holt, 2009). Here, a transcript from the L strand promoter (LSP) serves as a primer for H strand replication while forming the RNA/DNA hybrid prior to OriH, and hence forming the D-loop (Fig. 1A, B). The existence of two promoters, LSP_{major} and LSP_{minor} (Chang *et al.*, 1986), has been suggested to produce both a polycistronic transcript (LSP RNA) and replication primer. According to a processing model assuming self-cleavage of polycistronic RNA based on self-splicing sequences including those in tRNA genes (Chang & Clayton, 1985), one transcriptional event gives rise to the priming RNA plus the precursor RNA for structural genes (ND6 in the case of LSP RNA, remaining on HSP RNA). Transcription from the LSP_{major} promoter, when it is self-cleaved from the transcript, could produce priming RNA preceding 7S DNA (Fig. 1A). Thus the priming RNA coincides with the supposed 5' end of starting H strand replication and, consequently, it should compete with the unprocessed (uncleaved polycistronic) transcript and regulate replication of OriH. In the case of LSP_{minor}, no such regulation has been observed and probably the corresponding RNA serves as a primer in all occasions (Chang & Clayton, 1985).

One of the alternatives, the *strand-coupled bidirectional replication* model (Bowmaker *et al.*, 2003; Brown *et al.*, 2005; 2008; Brown & Clayton, 2006; Yasukawa *et al.*, 2006; Pohjoismäki *et al.*, 2010) assumes that bidirectional replication is initiated from a zone near OriH followed by the progression of the two forks around the mtDNA circle (Bowmaker *et al.*, 2003). This model proposes that

[✉]e-mail: jezek@biomed.cas.cz

This work was presented in the poster form at the 16th European Bioenergetics Conference (Warsaw, 2010); abstract in *Biochim Biophys Acta*, **1797** (Suppl): 105 (2010).

Abbreviations: D-loop, displacement loop; ds, double-stranded; EGFP, enhanced green fluorescence protein; FISH, fluorescence *in situ* hybridization; HSP, H strand promoter; LSP, L strand promoter; mt, mitochondrial; mtDNA, mitochondrial DNA; OriH, H strand origin; OriL, L strand origin; PBS, phosphate buffered-saline; RITOLS, RNA incorporations throughout of lagging strand; ss, single-stranded; TFAM, transcription factor-A, mitochondrial.

L strand replication initiation can occur at various locations other than OriL, as alternative L strand origins (altOriL; Brown & Clayton, 2006; Holt *et al.*, 2007; Holt, 2009; Fig. 1B). These altOriL modifications require, on occasion, a reduction in the asymmetry and a mechanism to initiate L strand replication at locations other than OriL. This model is supported by the revealed two classes of double-stranded (ds) replication intermediates, the first class consisting of the predicted products of conventional strand-coupled replication (remaining after RNA loss during isolation and processing; Holt *et al.*, 2000; Kajander *et al.*, 2001; Bowmaker *et al.*, 2003; Reyes *et al.*, 2005); and the second, extended RNA segments encompassing the entire lagging strand, i.e., RNA incorporations throughout of lagging strand (RITOLS; Yang *et al.*, 2002; Bowmaker *et al.*, 2003). The mechanism by which the RNA lagging strand is created is unknown. The nascent RNA strand merely protects the lagging strand template until the second-strand DNA synthesis is initiated. Other remnant RNA/DNA hybrid structures, called R-loops, have been identified to exist at a variety of locations throughout the mt genome due to retention of the RNA paired to its DNA template (Xu & Clayton, 1996; Brown *et al.*, 2008). The original role of R-loops should lie in priming the leading strand DNA synthesis. However, an RNase H-like activity exists which digests RNA only when it is hybridized to DNA. Hence, the putative RNase H probably generates short RNA primers when it comes in contact with the nascent RNA strands of mtDNA replicating *via* RITOLS. Consequently, potential primers are formed at dispersed sites. In conclusion, it is currently accepted that the conventional leading and lagging strand DNA synthesis may occur in parallel with RITOLS replication in mammalian mitochondria (Pohjoismäki *et al.*, 2010).

The above described knowledge has been gathered mostly with isolated mtDNA and other entities such as isolated mt nucleoids. Visualization of, e.g., RNA/DNA hybrids *in situ* might contribute to confirming the gathered knowledge or obtaining more details. Also mt RNA existing in various forms, e.g., as polycistronic RNA (nascent at transcription) and as cleavage/self-cleavage products, i.e., tRNAs and mRNAs, have rarely been visualized and localized within the mitochondrial matrix. Therefore, we have initiated an effort in this path. Attempting to visualize mtDNA and certain forms of mitochondrial RNA, we designed three fluorescent probes for *in situ* hybridization (in fixed cells) based on the principle of molecular beacon (Santangelo *et al.*, 2005). This is constructed so that complementary oligonucleotides flank the desired hybridizing sequence with the quencher located at the 3' end and a desired fluorophore (Cy3 dye in our case) located at the 5' end of resulting constructs. The self-hybridization of the complementary oligonucleotides within the formed loop brings the quencher towards the fluorophore, so that the beacon probe does not emit fluorescence. Upon hybridization, the loop structure is aborted, hence the quencher does not quench the fluorescence of the Cy3 dye anymore. Note also that conventional confocal microscopy has a resolution of about 250 nm, i.e., the space interval in which no two or more points could be distinguished from a single point. Consequently, no structure within a single mt nucleoid can be resolved. In spite of this, one still may use it in order to develop methodology before adapting these probes for super-resolution microscopic techniques. Here, we designed at first the "ND5 probe" to be annealed to the light (L, i.e., antisense) strand com-

plementary to the ND5 mtDNA gene (H strand mtDNA sequence), but also to RNAs containing the ND5 sequence. We clearly demonstrated that such a molecular beacon ND5 probe visualized objects co-localizing with tubules of mitochondrial reticulum (matrix space), thus reflecting HSP RNA and ND5 mRNA spreading localization in the matrix space. Two other probes, termed "7S DNA probe" and "proximal D-loop probe", designed against the H strand D-loop regions controlled by the light (L) strand promoter major (LSP_{major}) and LSP_{minor}, respectively, hybridize preferentially to mtDNA, since structures co-localizing with mitochondrial nucleoids were visualized by them. Nevertheless, LSP RNA, either within transcribing/replicating nucleoids or spread in the matrix space could also be visualized. In conclusion, we have laid out methodology required for future extension to super-resolution microscopy that might be able to resolve even distinct mt nucleoid types and structures such as RITOLS and R-loops on nucleoids of mtDNA.

METHODS

Cell model used. Human hepatocellular carcinoma HepG2 cells obtained from European culture collection (ECACC 85011430) were cultivated in DMEM without glucose supplemented with 3 mM glutamine, 10% (v/v) glucose-free dialyzed fetal calf serum, 10 mM Hepes, 10 IU/ml penicillin, 100 µg/ml streptomycin and 10 mM galactose, in order to force cells to oxidative phosphorylation (Rossignol *et al.*, 2004). This treatment also activates mitochondrial biogenesis including mtDNA replication, hence it leads to more mt nucleoids.

Molecular beacon design and preparation. Revised Cambridge Reference Sequence of the human mtDNA (Andrews *et al.*, 1999) was used and probes were designed against its regions as illustrated in Fig. 1A, B. The molecular beacon ND5 probe (No. 3 in Fig. 1) for annealing to the L strand (antisense) base pairs (bp) 12924 to 12948 complementary to a portion of the ND5 gene (H strand bp 12337 to 14148) was designed as follows (with complementary nucleotides in normal font and the ND5 sequence part in bold):

5'-Cy3-CGA CGG AAG GGC TAT TTG TTG TGG GTC-TCG TCG-BHQ2-3'.

This probe should also anneal to the mitochondrial polycistronic HSP RNA (Fig. 1A) which might be also a part of RITOLS (Fig. 1B) and the corresponding ND5 mRNA. The ND5 mRNA should not be localized only at mt nucleoids, but should be spread all over the matrix space and in mt ribosomes. In addition, replicating nucleoids are expected to give a more intensive signal, due to the doubling of the L strand.

Two other probes were designed for annealing to the D-loop region of H strand (bp 16024 to 574). Probe No. 2 (Fig. 1A), called "7S DNA probe", should anneal to the H strand bp 16109 to 16127, which is the part of the nascent 7S DNA displacing this H strand (Fig. 1A, B). Naturally, the probe should anneal also to the 7S DNA. Moreover, the corresponding region of non-coding LSP RNA (controlled by the LSP_{major}) should bind this probe as well, either before self-cleavage at the first tRNA gene location or afterwards (Fig. 1A). Therefore, the 7S DNA probe was designed as follows (with complementary pentanucleotides in normal font and the 7S DNA sequence part in bold):

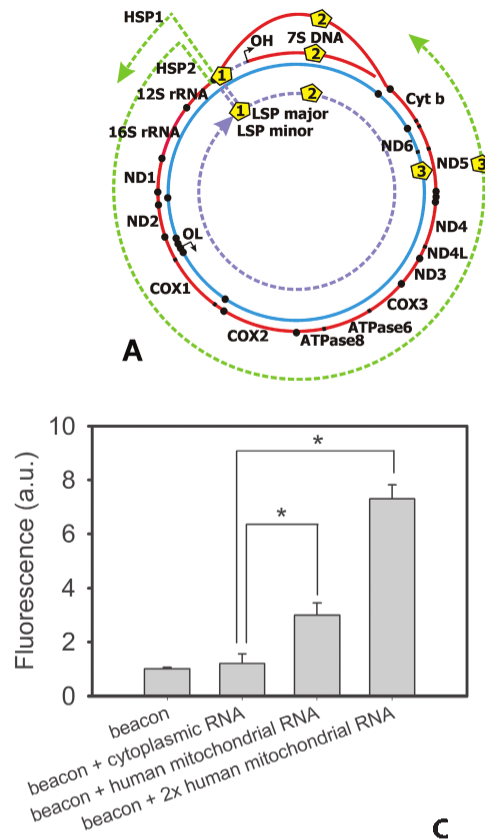


Figure 1. Scheme of possible hybridization of the developed probes to mitochondrial DNA and RNA upon transcription (A) and replication (B) and demonstration of hybridization specificity (C).

(A) Transcription schema illustrates the heavy (H, red) and light (L, blue) strands of ds circular mtDNA, where borders between mt genes (their common abbreviations used in bold) are indicated by small black squares and tRNA genes as black dots; two possible polycistronic RNAs derived from them (HSP RNA, green, and LSP RNA, violet dashed lines) are illustrated with indicated transcription direction. The displacement (D) loop region contains permanent third strand with OriH („OH“). The possible hybridization positions of molecular beacon probes are denoted by numbers in yellow pentagons: 1) proximal D-loop probe, also termed LSP_{minor} RNA probe; 2) 7S DNA probe; and 3) ND5 probe. (B) Replication schema uses the same graphics as A). Here also RITOL5 are illustrated by blue dashed line, while nascent H and L strand by red and blue dot-dashed line, respectively. For the strand-coupled bidirectional replication model, alternative origin sites („Alt OL“) besides the classic OriL („OL“) are depicted as the nascent L strand starts to be synthesized at them and replaces protecting RNA, i.e., RITOL5. Theoretically, in replicating nucleoids the ND5 probe (No.3) could anneal to the corresponding RITOL5, to the nascent L strand plus to the original L strand. (C) Demonstration of hybridization specificity for the ND5 molecular beacon probe by comparing fluorescence of the ND5 probe mixed with RNA extract from mitochondria and cells depleted of mitochondria.

5'-Cy3-ACC GTA GCC ACC ATG AAT ATT GTA CGG-T-BHQ2-3'.

We expect that when, after cleavage, this non-coding LSP RNA diffuses out of mt nucleoids, its degradation might be faster than the degradation of mRNA. Faster degradation is also expected for the corresponding LSP RNA_{minor} located in the very proximal part of D loop. For this ultimate proximal part of D loop, we designed the third probe (probe No. 1, Fig. 1A) which should anneal to the H strand bp 524 to 547. This is the portion of the D-loop initially displaced by the priming RNA portion of LSP RNA, to which this probe has to anneal as well (Fig. 1A). This “proximal D-loop probe” or “LSP_{minor} RNA probe” was designed as follows (with complementary pentanucleotides in normal font and the proximal D-loop sequence part in bold):

5'-Cy3-ACC TGA CAC ACA CCG CTG CTA ACC CCA-TCA GGT-BHQ2-3'.

Figure 1C demonstrates by fluorescence intensities that all three probes were fluorescent exclusively when hybridized to the sequences against which they were designed. In addition, *in situ* hybridization with a molecular beacon

probe designed against 28S rRNA (Alán *et al.*, unpublished) and a random molecular beacon (Santangelo *et al.*, 2005) gave no fluorescent signal in mitochondria.

Standardized mtFISH protocol. The standardized protocol for fluorescent *in situ* hybridization of mtDNA or mtRNA (mtFISH) was adjusted as follows: Cells were fixed in 0.3% glutaraldehyde in PBS for 20 min at 37°C, followed by two washes with 1 mg/ml NaBH₄ in PBS for 15 min in order to reduce unbound glutaraldehyde. Afterwards, *post*-fixation nuclease treatment was performed in some experiments. Partial treatment with RNase A (400 µg per coverslip; Invitrogen), removing ssRNA was performed for two hours in PBS at 37°C. To remove RNA from RNA/DNA hybrids we used digestion with RNase H (15 U per coverslip; Biolabs) for 30 min at 37°C. Treatment with DNase I (200 U per coverslip; Top-Bio, Czech Republic), which digests ds-DNA, proceeded for 30 min at 37°C. The DNase I and RNase H treatments were performed in a reaction buffer prepared according to the manufacturer's protocol. Coverslips were pre-hybridized in 50% formamide with salmon sperm DNA and 0.2% Tween 20 in 2-fold concentrated saline sodium citrate (2×SSC) buffer for 5 min at 72°C. Molecular beacon (100 ng) in 50% formamide with 0.2% Tween in 2×SSC buffer was added on the coverslip and hybridized overnight at 37°C. Coverslips

were then washed in 0.2% Tween with 50% formamide in 2×SSC for 5 min at 42°C, followed by three washes in 0.2% Tween in 2×SSC.

Confocal microscopy. An inverted confocal fluorescent Leica TCS SP2 AOBS microscope was employed with a PL APO 100×/1.40–0.70 oil immersion objective (a pinhole of 1 Airy unit). For Cy3 dye the excitation by a 1.2 mW He/Ne laser was set at 543 nm. Matrix-addressed RoGFP, a modified EGFP of "type 1", a kind gift from Dr. Rodrigue Rossignol, Univ. Bordeaux 2 (France), was excited at 488 nm with a 20-mW argon laser, to visualize tubules of mitochondrial reticulum (Rossignol *et al.*, 2004). HepG2 cells pre-transfected with baculoviral particles (a kind gift of Dr. Werner Koopman, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands) coding for EGFP conjugated with mitochondrial transcription factor-A (TFAM) were used for visualization of mt nucleoids. Baculoviral particles were prepared on the basis of a modified *Baculovirus* vector containing a mammalian-active promoter (Koopman *et al.*, 2007). Transfection was performed by incubating cells with baculoviral particles for 20 h to 24 h. The culture medium contained 1.5 mM sodium butyrate for proper expression of EGFP-TFAM. After transfection the cells were washed with PBS and fixed.

RESULTS

Mitochondrial localization of hybridized molecular beacons

Successful *in situ* hybridization is suggested by co-localization of the fluorescence of the ND5 molecular beacon with that of mitochondria addressed-RoGFP (Fig. 2A, B, C) and with EGFP-conjugated mitochondrial transcription factor-A (TFAM, Fig. 2D, E, F). The RoGFP-stained tubules of mitochondrial reticulum are only partly covered by the ND5 probe signal. In turn, the EGFP-TFAM-stained nucleoids of mitochondrial DNA (mt nucleoids) appear as numerous small sphere projections. In certain samples, the ND5 probe signal was apparently more intense (concentrated) in those mt nucleoid spots (Fig. 2D). The EGFP-TFAM fluorescence also co-localized with the 7S DNA probe emission (Fig. 2G, H, I) and with the proximal D-loop probe emission (termed also LSP_{minor} RNA probe, not shown). Although hardly distinguishable from the background, the signal of these two D-loop probes was less spread within the region of apparent mitochondrial reticulum tubules, i.e., the matrix space.

Distinction between the three molecular beacon probes

A comparison of Fig. 2D *vs.* 2G illustrates the difference between the ND5 and 7S DNA probes. Besides mt nucleoids, the ND5 probe hybridizes against the vast amount of ND5 mRNA (and polycistronic HSP RNA detached from the site of transcription, i.e., from mt nucleoids) spread inside the tubules of mitochondrial reticulum. Unlike for the ND5 probe, a more punctate character of images was observed for the 7S DNA probe and the proximal D-loop probe (termed also the LSP_{minor} RNA probe; Fig. 3E, 3I, *vs.* 3A). The numerous small spots most probably represent mt nucleoids as suggested by their overlay with EGFP-TFAM (Fig. 2G, H, I). The stronger mt nucleoid signal relative to the overall mitochondrial (matrix space) tubule staining

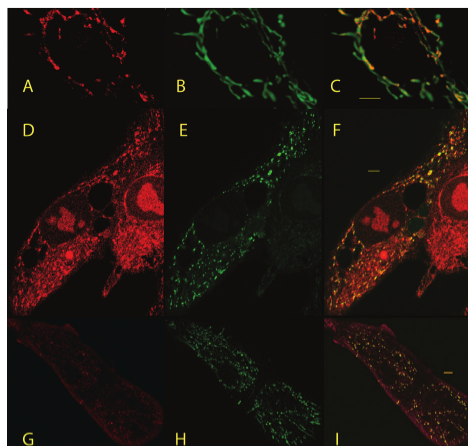


Figure 2. Colocalization of molecular beacon probes with tubules of mitochondrial reticulum and with mtDNA nucleoids. Confocal images of (A) ND5 molecular beacon probe; (B) matrix-addressed RoGFP; (C) overlay of A plus B illustrate co-localization of the ND5 probe with tubules of mitochondrial reticulum. Confocal images of (D) ND5 probe and (G) 7S DNA probe show more punctate character of the 7S DNA probe image and its similarity to the EGFP-TFAM-visualized mt nucleoids (E and H). Overlays show clearly that the ND5 probe hybridizes also to ND5 mRNA spread (plus to HSP RNA released from mt nucleoids) all over the matrix of mitochondrial reticulum tubules (F), whereas the 7S DNA probe is more concentrated in mt nucleoids (I). Scale bars at images represent 5 μ m. The red pseudo color is used for molecular beacon probes.

observed for both D-loop probes would be compatible with a stronger degradation of LSP RNA in comparison with that of ND5 mRNA (or else HSP RNA).

Transcribed polycistronic RNAs as possibly resolved structures

Despite the only about 250 nm resolution of conventional confocal microscopy, distinct images were gathered with the three molecular beacon probes in combination with *post*-fixation (but *pre*-hybridization) treatment with RNases A and H and DNase I. In order to ascertain these differences, we performed the following three digestion treatments:

i) with RNase A, digesting ssRNA, which converted the ND5 probe images covering nearly whole mitochondrial tubules (Fig. 3A) into a punctate pattern observed in the mt nucleoid images (Fig. 3B). The *pre*-hybridization treatment with RNase A affected images obtained with the 7S DNA probe (Fig. 3E *vs.* F) and with the proximal D-loop probe (termed also LSP_{minor} RNA; Fig. 3I *vs.* J) to a lesser extent. This confirms that these two probes mainly visualize mt nucleoids.

ii) The treatment with RNase A plus RNase H (the latter digesting RNA from RNA/DNA hybrids) led in general to a partial reversal of the punctate effect, giving a more diffuse signal of all three probes in comparison with that obtained after treatment with RNase A alone (Fig. 3B *vs.* C; 3F *vs.* G; J *vs.* K). We speculate that partially digested RNA structures spread in the mitochondrial tubules (in spite of the sample being fixed). Partial resistance to RNase A could be a feature of structures such as polycistronic LSP RNA (in the case of D-loop probes). Since

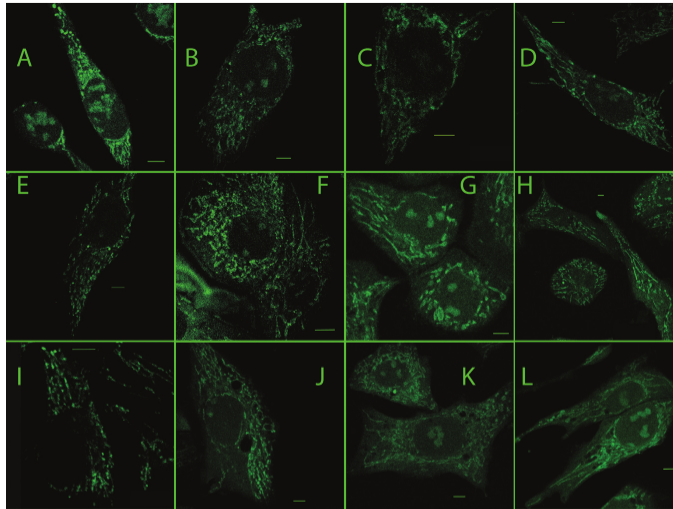


Figure 3. Confocal images of beacon probes before and after treatment with RNase A, RNase A plus RNase H, or DNase I. Confocal images are shown for ND5 probe (A) to (D); 7S DNA probe (E) to (H); and proximal D-loop probe (annealing also to LSP_{minor} RNA) (I) to (L) in the absence of any further treatment (A), (E), (I); after *post-fixation pre-hybridization* treatment with RNase A, digesting ssRNA (B), (F), (J); after treatment with RNase A plus RNase H, the latter digesting RNA from RNA-DNA hybrids (C), (G), (K); and after treatment with DNase I plus RNase H (D), (H), (L). Scale bars at each image represent 5 μ m. Note that unlike in Fig. 2, now the green channel is used for molecular beacon probes.

ND5 mRNA could be digested by RNase A alone, only HSP RNA might be released by the additional action of RNase H in the case of the ND5 probe, and thus the effect was less pronounced in this case (Fig. 3C).

Finally, iii) treatment with DNase I plus RNase H without (Fig. 3D, H, L) or with a preceding RNase A treatment (not shown) converted images of mt nucleoids to the images of the whole mitochondrial network reticulum for all three probes. It reflects the distribution of the partially digested RNA and DNA all over the mitochondrial tubules in these fixed samples.

DISCUSSION

We have developed an *in situ* hybridization procedure for mitochondrial nucleic acids (mtFISH) with molecular beacon probes and verified it using conventional confocal microscopy. The *anti* L strand ND5 DNA&RNA probe most likely anneal to the L-strand of ds mtDNA, thus highlighting the mt nucleoids, plus to the single-gene (ND5) mRNA spread all over the matrix space, i.e., within the tubules of mitochondrial reticulum. A minor contribution to a similar pattern may arise from hybridization with polycistronic HSP-RNA (that might be still attached to nucleoids and may be present in RITOLS, but mostly is spread into the entire matrix space). Distinct spots were present in the images visualized by the ND5 probe, which were co-localized with mt nucleoids (Holt *et al.*, 2007; Bogenhagen *et al.*, 2008). Moreover, the diffuse signal, most probably originating from the annealing to HSP-RNA and ND5 messenger RNA, disappeared after the RNase A *pre-hybridization* diges-

tion of ssRNAs. Some signal was also observed in nuclei, most likely due to the presence of multiple insertions of mitochondrial genes into the nuclear chromosomes in non-transcribed regions.

Both D-loop probes used behaved in accordance with the simple assumption that the corresponding polycistronic and cleaved LSP RNA (the cleavage occurs due to self-processing at tRNA gene sites upon transcription) is degraded more extensively than the polycistronic and cleaved HSP RNA (tRNAs plus messenger RNAs for all mitochondrial protein coding genes but ND6). Indeed, both D-loop probes exhibited images of a more punctate character, showing numerous spots resembling mt nucleoids. The EGFP-TFAM-visualized mt nucleoids colocalized with the majority of signal of both D-loop probes. We expected that the 7S DNA probe, which could anneal to three nucleic acid entities (Fig. 1A, B), would provide less distinct mt nucleoid images than the proximal D-loop probe (annealing also to the corresponding LSP_{minor} RNA, Fig. 1A, B). This assumption can only be

verified after adaptation of the molecular beacon technique to super-resolution microscopy.

We also combined our mtFISH protocol with the *post-fixation* and *pre-hybridization* treatment with RNase A, RNase H, DNase I, and their combinations. A “contrasting” action of RNase A was observed for all probes, but it was most pronounced for the ND5 probe. The RNase simply eliminated messenger RNAs and other RNAs spread in the matrix space of mitochondrial tubules within the fixed sample. We speculate that some mt nucleoids exhibiting a higher signal might be replicating (but the presence of replication machinery had to be proven in them — such a search is out of scope of the current work). The “contrasting” action of RNase H, however, indicates the presence of polycistronic LSP RNA (in the case of two D-loop probes) and HSP RNA (in the case of the ND5 DNA&RNA probe) attached to mt nucleoids. We expect that RNase H would digest presumed “transcription clouds” (not to be resolved in principle by the conventional confocal microscopy) around the mt nucleoids, representing those attached RNAs. The distinction of these transcriptional states (as well as RITOLS and replicating nucleoids) remains a future challenge for super-resolution microscopy in combination with analogs of our developed probes. Indeed, perspectives for the use of the developed probes lie in their use in conjunction with super-resolution microscopy such as stimulated emission depletion (STED) microscopy (Hell, 2007), for which modified probes (conjugated with STED-active dyes) may help to better resolve intra-nucleoid structure and might even detect RITOLS and R-loops, which are, unlike regular transcripts, pres-

ent in single copies. This would broaden our knowledge of these structures and their relations to the resting state, transcribing state, and replicating state of mt nucleoids. In conclusion, the two developed D-loop molecular beacon probes represent a novel tool for mt nucleoid visualization.

Acknowledgements

Dr. Gabrielle Saretzki (Newcastle University, UK), for introducing us to molecular beacon system, and Dr. Werner Koopman (Nijmegen Centre for Molecular Life Sciences, The Netherlands), for providing baculoviral particles coding for EGFP-TFAM, are gratefully acknowledged, as well as cell culturing by Lenka Jusková.

Financial support: This work was supported by grants No. P302/10/0346 (to P.J.) and P304/10/P204 (to A.D.) from the Grant Agency of the Czech Republic; and grants No. KJB500110902 (to J.J.) and AV0Z50110509 (to Inst. Physiology) from the Academy of Sciences of the Czech Republic.

REFERENCES

- Andrews RM, Kubacka I, Chinnery PF, Lightowlers RN, Turnbull DM, Howell N (1999) Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. *Nat Genet* **23**: 147.
- Bogenhagen DP, Rousseau D, Burke S (2008) The layered structure of human mitochondrial DNA nucleoids. *J Biol Chem* **283**: 3665–3675.
- Bowmaker M, Yang MY, Yasukawa T, Reyes A, Jacobs HT, Huberman JA, Holt IJ (2003) Mammalian mitochondrial DNA replicates bidirectionally from an initiation zone. *J Biol Chem* **278**: 50961–50969.
- Brown TA, Clayton DA (2006) Genesis and wanderings: origins and migrations in asymmetrically replicating mitochondrial DNA. *Cell Cycle* **5**: 917–921.
- Brown TA, Cecconi C, Tkachuk AN, Bustamante C, Clayton DA (2005) Replication of mitochondrial DNA occurs by strand displacement with alternative light-strand origins, not via a strand-coupled mechanism. *Genes Dev* **19**: 2466–2476.
- Brown TA, Tkachuk AN, Clayton DA (2008) Native R-loops persist throughout the mouse mitochondrial DNA genome. *J Biol Chem* **283**: 36743–36751.
- Chang DD, Clayton DA (1985) Priming of human mitochondrial DNA replication occurs at the light-strand promoter. *Proc Natl Acad Sci USA* **82**: 351–355.
- Chang DD, Hixson JE, Clayton DA (1986) Minor transcription initiation events indicate that both human mitochondrial promoters function bidirectionally. *Mol Cell Biol* **6**: 294–301.
- Clayton DA (2003) Mitochondrial DNA replication: what we know. *JUBMB Life* **55**: 213–217.
- Cree LM, Samuels DC, de Sousa Lopes SC, Rajasimha HK, Wonnapijit P, Mann JR, Dahl HH, Chinnery PF (2008) A reduction of mitochondrial DNA molecules during embryogenesis explains the rapid segregation of genotypes. *Nat Genet* **40**: 249–254.
- Falkenberg M, Larsson NG, Gustafsson CM (2007) DNA replication and transcription in mammalian mitochondria. *Annu Rev Biochem* **76**: 679–699.
- Fusté JM, Wantooij S, Jemt E, Granycome CE, Cluett TJ, Shi Y, Atanassova N, Holt IJ, Gustafsson CM, Falkenberg M (2010) Mitochondrial RNA polymerase is needed for activation of the origin of light-strand DNA replication. *Mol Cell* **37**: 67–78.
- Hell SW (2007) Far-field optical nanoscopy. *Science* **316**: 1153–1158.
- Holt IJ (2009) Mitochondrial DNA replication and repair: all a flap. *Trends Biochem Sci* **34**: 358–365.
- Holt IJ (2010) Zen and the art of mitochondrial DNA maintenance. *Trends Genet* **26**: 103–109.
- Holt IJ, Lorimer HE, Jacobs HT (2000) Coupled leading- and lagging-strand synthesis of mammalian mitochondrial DNA. *Cell* **100**: 515–524.
- Holt IJ, He J, Mao C-C, Boyd-Kitkup JD, Martinsson P, Sembongi H, Reyes A, Spelbrink JN (2007) Mammalian mitochondrial nucleoids: organizing an independently minded genome. *Mitochondrion* **7**: 311–321.
- Hyvärinen AK, Pohjoismäki JL, Reyes A, Wantooij S, Yasukawa T, Kathunen PJ, Spelbrink JN, Holt IJ, Jacobs HT (2007) The mitochondrial transcription termination factor mTERF modulates replication pausing in human mitochondrial DNA. *Nucleic Acids Res* **35**: 6458–6474.
- Kajander OA, Kathunen PJ, Holt IJ, Jacobs HT (2001) Prominent mitochondrial DNA recombination intermediates in human heart muscle. *EMBO Rep* **2**: 1007–1012.
- Koopman WJH, Hink MA, Verkaar S, Visch HJ, Smeitink JAM, Willem PHGM (2007) Partial complex I inhibition decreases mitochondrial motility and increases matrix protein diffusion as revealed by fluorescence correlation spectroscopy. *Biochim Biophys Acta* **1767**: 940–947.
- Lu F, Selak M, O'Connor J, Croul S, Lorenzana C, Butunoi C, Kalman B. J. (2000) Oxidative damage to mitochondrial DNA and activity of mitochondrial enzymes in chronic active lesions of multiple sclerosis. *Neural Sci* **177**: 95–103.
- Pohjoismäki JL, Holmes JB, Wood SR, Yang MY, Yasukawa T, Reyes A, Bailey LJ, Cluett TJ, Goffart S, Willcox S, Rigby RE, Jackson AP, Spelbrink JN, Griffith JD, Crouch RJ, Jacobs HT, Holt IJ (2010) Mammalian mitochondrial DNA replication intermediates are essentially duplex but contain extensive tracts of RNA/DNA hybrid. *J Mol Biol* **397**: 1144–1155.
- Reyes A, Yang MY, Bowmaker M, Holt IJ (2005) Bidirectional replication initiates at sites throughout the mitochondrial genome of birds. *J Biol Chem* **280**: 3242–3250.
- Rosignol R, Gilekson R, Aggeler R, Yamagata K, Remington SJ, Capaldi RA (2004) Energy substrate modulates mitochondrial structure and oxidative capacity in cancer cells. *Cancer Res* **64**: 985–993.
- Santangelo PJ, Niin N, Bao G (2005) Direct visualization of mRNA colocalization with mitochondria in living cells using molecular beacons. *J Biomed Opt* **10**: 044025:1–16.
- Scaglia F, Wong L-JC (2008) Human mitochondrial transfer RNAs: Role of pathogenic mutation in disease. *Muscle Nerve* **37**: 150–171.
- Shadel GS, Clayton DA (1997) Mitochondrial DNA maintenance in vertebrates. *Annu Rev Biochem* **66**: 409–435.
- Stewart JB, Treter C, Elson JL, Wredenberg A, Cansu Z, Trifunovic A, Larsson N-G (2008) Strong purifying selection in transmission of mammalian mitochondrial DNA. *PLoS Biology* **6**: e10.
- Tuppen HA, Blakely EL, Turnbull DM, Taylor RW (2010) Mitochondrial DNA mutations and human disease. *Biochim Biophys Acta* **1797**: 113–128.
- Xu B, Clayton DA (1996) RNA-DNA hybrid formation at the human mitochondrial heavy-strand origin ceases at replication start sites: an implication for RNA-DNA hybrids serving as primers. *EMBO J* **15**: 3135–3143.
- Yang MY, Bowmaker M, Reyes A, Vergani L, Angeli P, Gringeri E, Jacobs HT, Holt IJ (2002) Biased incorporation of ribonucleotides on the mitochondrial L-strand accounts for apparent strand-asymmetric DNA replication. *Cell* **111**: 495–505.
- Yasukawa T, Reyes A, Cluett TJ, Yang MY, Bowmaker M, Jacobs HT, Holt IJ (2006) Replication of vertebrate mitochondrial DNA entails transient ribonucleotide incorporation throughout the lagging strand. *EMBO J* **25**: 5358–5371.
- Zhadanov SI, Grechanina EY, Grechanina YB, Gusar VA, Pedoseeva NP, Lebon S, Munnich A, Schurr TG (2007) Fatal manifestation of a de novo NDS mutation: Insights into the pathogenic mechanisms of mtDNA NDS gene defects. *Mitochondrion* **7**: 260–266.

Import of desired nucleic acid sequences using addressing motif of mitochondrial ribosomal 5S-rRNA for fluorescent in vivo hybridization of mitochondrial DNA and RNA

Jaroslav Zelenka · Lukáš Alán · Martin Jabůrek · Petr Ježek

Received: 5 December 2013 / Accepted: 29 January 2014 / Published online: 23 February 2014
© Springer Science+Business Media New York 2014

Abstract Based on the matrix-addressing sequence of mitochondrial ribosomal 5S-rRNA (termed MAM), which is naturally imported into mitochondria, we have constructed an import system for in vivo targeting of mitochondrial DNA (mtDNA) or mt-mRNA, in order to provide fluorescence hybridization of the desired sequences. Thus DNA oligonucleotides were constructed, containing the 5'-flanked T7 RNA polymerase promoter. After in vitro transcription and fluorescent labeling with Alexa Fluor® 488 or 647 dye, we obtained the fluorescent "L-ND5 probe" containing MAM and exemplar cargo, i.e., annealing sequence to a short portion of ND5 mRNA and to the light-strand mtDNA complementary to the heavy strand *nd5* mt gene (5'-end 21 base pair sequence). For mitochondrial in vivo fluorescent hybridization, HepG2 cells were treated with dequalinium micelles, containing the fluorescent probes, bringing the probes proximally to the mitochondrial outer membrane and to the natural import system. A verification of import into the mitochondrial matrix of cultured HepG2 cells was provided by confocal microscopy colocalizations. Transfections using lipofectamine or probes without 5S-rRNA addressing MAM sequence or with MAM only were ineffective. Alternatively, the same DNA oligonucleotides with 5'-CACC overhang (substituting T7 promoter) were transcribed from the tetracycline-inducible pENTRH1/TO vector in human embryonic kidney T-REx®-293 cells, while mitochondrial matrix localization after import of the resulting unlabeled RNA was detected by PCR. The MAM-containing probe was then enriched by three-order of magnitude over the

natural ND5 mRNA in the mitochondrial matrix. In conclusion, we present a proof-of-principle for mitochondrial in vivo hybridization and mitochondrial nucleic acid import.

Keywords 5S-rRNA · Mitochondrial DNA · Nucleic acid import into mitochondria · Fluorescent in vivo hybridization of mtDNA · Mitochondrial nucleoids

Introduction

Genetics of mitochondrial DNA (mtDNA) seems to be the least understood part of genetics (Li et al. 2012; Kasiviswanathan et al. 2012; Holt 2009; Falkenberg et al. 2007). Methods of manipulations with mtDNA are virtually absent, though a progress is being made (Niazi et al. 2013; Durazo and Kompella 2012; Comte et al. 2013; Jash and Adhya 2012; Sieber et al. 2011a; Weber-Lotfi et al. 2009; Mahata et al. 2006; D'Souza et al. 2003). Considering in vivo manipulations with mtDNA, addressing to the target organ or tissue, transport across the plasma membrane and across the two mitochondrial membranes must be ensured (Niazi et al. 2013; Durazo and Kompella 2012), besides possibly required unwinding of mtDNA which is highly compacted in nucleoids (Bogenhagen 2012; Bogenhagen et al. 2008). Even for targeting of mtDNA by oligonucleotides in cultured cells, carriers containing positive charges are required, such as DQAsomes (micelles of dequalinium chloride, (D'Souza et al. 2003)), or positively charged liposomes, such as the MitoPorter system based on lecithin liposomes doped with octylarginine stearate (Yamada et al. 2008). Nevertheless, these carriers do not deliver oligonucleotide cargo directly to the mitochondrial matrix and hence are quite inefficient. Likewise strategies using the uptake of peptide nucleic acid by transporter of outer membrane plus transporter of inner membrane (TOM & TIM), employing peptide conjugates of protein matrix-addressing sequences with oligonucleotides have a limited use (Muratovska et al. 2001).

J. Zelenka · L. Alán · M. Jabůrek · P. Ježek
Institute of Physiology, Academy of Sciences of the Czech Republic,
Prague, Czech Republic

P. Ježek (✉)
Department No.75, Membrane Transport Biophysics, Institute of
Physiology, Academy of Sciences of the Czech Republic, Václavská
1083, 14220 Prague 4, Czech Republic
e-mail: jezek@biomed.cas.cz

It has been, however, demonstrated that plant, yeast, and mammalian 5S rRNA (Magalhaes et al. 1998; Rorbach and Minczuk 2012) is readily imported into the mitochondrial matrix just by the TOM & TIM import pathway (Comte et al. 2013; Smimov et al. 2008a, b, 2011; Tarassov et al. 2007), as facilitated by rhodanese (Smimov et al. 2010) and PNPase proteins (Vedrenne et al. 2012; Wang et al. 2010, 2012a, b). Native 5S rRNA import is essential for proper function of mitochondrial ribosomes (Magalhaes et al. 1998; Rorbach and Minczuk 2012). In isolated plant mitochondria, another shuttle protein, dihydrofolate reductase (DHFR), has been used to import up to 815-nt-long mRNAs. DHFR was able to import of 5 % load of human 815-nt-long *nad3* mRNA into rat liver mitochondria (Sieber et al. 2011b). Alternatively, short synthetic RNAs (referred to as FD-RNA) comprising two domains of the tRNA exhibit also high efficiency of mitochondrial import (Kolesnikova et al. 2010) and likewise the carriers based on 5S rRNA were successfully used to balance heteroplasmy in cells with portion of mtDNA bearing a large deletion associated with the Kearns Sayre syndrome (Comte et al. 2013). Nevertheless, in order to work with cultured cells, systems of fluorescent in vivo hybridization should be developed to allow direct visualization of the selected nucleic acids entities in mitochondria.

Consequently, we have turned our attention towards the successfully adapted natural mt 5S rRNA import pathway into mitochondria (Comte et al. 2013; Smimov et al. 2008a, b, 2011; Tarassov et al. 2007). We have employed mt 5S rRNA mitochondria-addressing motif (MAM) sequence portion and developed a carrier system to target mtDNA and its structures, or to target the relevant derived mt-mRNAs. In addition, on the basis of such facilitated import system, we have developed a concept of mt fluorescent in vivo hybridization (mtFIVH), i.e. targeting one of the heavy (H) and light (L) complementary mtDNA strands plus any types of relevant mtRNAs containing the targeted sequence. Except of *nd6* gene, mt mRNAs are derived from (being complementary to) the H strand. Note that polycistronic mt-RNA is transcribed first which is subsequently cleaved to the mt-mRNAs. Due to the specificity of mtDNA genetics and high compaction of mtDNA within the nucleoids, we expect that the system might be dependent on the accessibility of desired sequence in the mitochondrial matrix. In this work, we present a proof-of-principle for mitochondrial in vivo hybridization in conjunction with the mitochondrial nucleic acid import based on 5S rRNA mitochondrial-addressing sequence.

Materials and methods

Design of nucleic acid import into mitochondrial matrix

To achieve import of desired nucleic acid sequence into the mitochondrial matrix, we have employed a defined pathway

that facilitates import of 5S rRNA (Comte et al. 2013; Smimov et al. 2008a, b, 2010, 2011; Tarassov et al. 2007; Vedrenne et al. 2012; Wang et al. 2010, 2012a, b). Although 5S rRNA has not yet been definitively confirmed to be component of mt ribosomes (Magalhaes et al. 1998; Rorbach and Minczuk 2012), it is translocated naturally into mitochondria and even certain mutations of its mitochondrial matrix-addressing motif (MAM) sequence improve such an import (Smimov et al. 2008b). We have designed an RNA probe system as a carrier for the desired RNA sequence (principally DNA sequence as well), employing the optimized MAM of 5S rRNA (Smimov et al. 2008b), i.e., mutated to its most efficient version (Fig. 1a). Thus our fluorescent oligoribonucleotide probes contained the mutated most efficient version of MAM and an exemplar cargo RNA sequence (Fig. 1a). In our case, the exemplar cargo was the RNA sequence annealing to a short portion of ND5 mRNA which also has the ability to anneal to the L-strand mtDNA complementary to the H-strand *nd5* mt gene (21 base pair sequence of the 5'-end) (Zhadanov et al. 2007) (Fig. 1d), as the prototype targeting sequence (Alán et al. 2010). A fragment of the human mt genome (NC_012920) (describing the L-strand, cf. Ref. (Andrews et al. 1999)) corresponding to the base pairs 12 341 to 12 361 was designed to be attached after the MAM in the sequence of the resulting "MAM-L-ND5" RNA probe (Fig. 1a). Note that the *nad5* gene is located within the H strand between the base pairs 12 337 to 14 148.

Fluorescent in vivo hybridization (FIVH) probe construction/preparation

The custom-synthesized DNA template, containing the 5'-flanked T7 RNA polymerase promoter TAATACGACTCA CTATAG was designed so to obtain the MAM-L-ND5 ribonucleotide probe (Fig. 1a) after in vitro transcription with the aid of RiboMax Kit (Promega, Madison WI, USA). After purification (Fig. 1b), 3'-end of the resulting RNA was activated by sodium periodate and subsequently labeled with Alexa Fluor® 647 hydrazide or AlexaFluor® 488 hydrazide (Life technologies, Carlsbad, CA, USA). The probe was purified by LiCl/ethanol precipitation. This approach ensures the defined, pure and correctly folded fluorescent probe. For direct transfections of the constructed fluorescent RNA probes into the cells (Fig. 1c), the following probes were used (Fig. 1a): "full probe", termed MAM-L-ND5, theoretically capable of binding to the above specified short portion of ND5 mRNA plus to the light-strand mtDNA complementary to the same corresponding portion of the heavy strand *nd5* mt gene (Fig. 1d). As a second probe, termed MAM-000, was the probe without the ND5 cargo; whereas the third probe was lacking the MAM address, as termed 000-L-ND5. Finally the fourth probe was designed to contain the scrambled sequence, as termed "Scrambled". Fluorescent probes were transfected into mitochondria of HepG2 cells expressing mitochondrial-matrix-

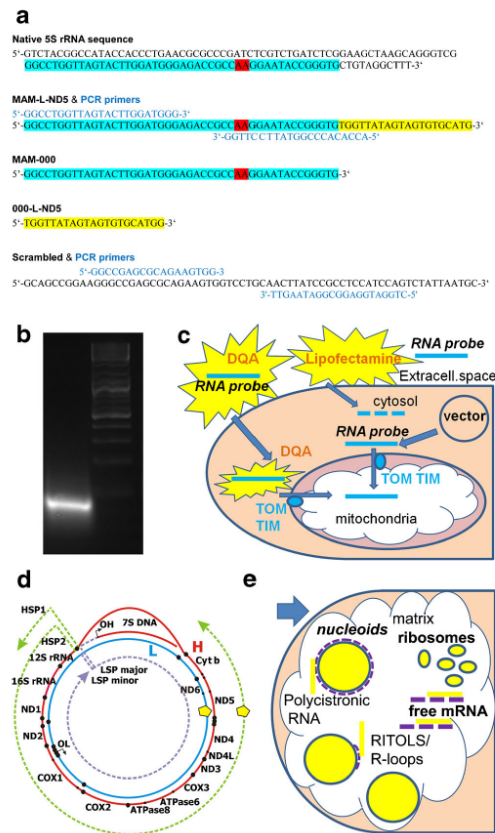


Fig. 1 Design and concepts: **a** Sequences of RNA probes for achieving import of nucleic acids. *Blue fonts*: PCR primers employed for detection of imported probes in the mitochondrial matrix. *Blue background* shows the native import address of 5S rRNA (MAM) in which two residues (*red background*) were mutated to get the most efficient import (Smirnov et al. 2008b). *Yellow background* indicates the cargo, an exemplar, 21 bases long, RNA sequence, annealing to ND5 mt-mRNA (Zhadanov et al. 2007) and to the corresponding region of L-strand mtDNA complementary to the *nd5* gene of the H-strand (Alán et al. 2010). **b** Purity of RNA probe; **c** Concept of mitochondrial fluorescent in vivo hybridization (mtFIVH). **d** Gene and probe location on mtDNA and its transcripts – schema illustrates the heavy (H, *red*) and light (L, *blue*) strands of ds circular mtDNA, where borders between mt genes (their common abbreviations used in *bold*) are indicated by *small black squares* and tRNA genes as *black dots*; two possible polycistronic RNAs derived from them (HSP RNA, *green*, and LSP RNA, *violet dashed lines*) are illustrated with indicated transcriptional direction. The displacement D-loop region contains permanent third strand with OriH (“OH”). The expected hybridization positions of MAM-L-ND5 probe are denoted by yellow pentagons; **e** Expected loci visualized by MAM-L-ND5 probe (*yellow*)—nucleoids containing mtDNA, and either attached polycistronic RNA, or RITOLS or R-loops should be fluorescently visualized, plus ND5 mRNA bound to ribosomes plus free ND5 mRNA

addressed (mt) GFP or KEIMA (Kogure et al. 2008; Tauber et al. 2013) using either Lipofectamine® (Life technologies) or DQAsomes (Kasiviswanathan et al. 2012) (Fig. 1c). Colocalizations of mtGFP with Alexa Fluor® 647 or mt KEIMA with Alexa Fluor® 488 was analyzed by confocal microscopy (Leica TSC SP2, Microsystems, Wetzlar, Germany). When desired, mitochondrial nucleoids were stained with a DNA stain SYTO-9 (Life technologies).

Vectors for expression of MAM RNA recombinant constructs

Alternatively, all four above mentioned ribonucleotide probes, but non-fluorescent, were expressed upon the tetracycline induction from the pENTRH1/TO vector of the BLOCK-iT® kit (Invitrogen, Carlsbad, CA, USA). The corresponding DNA oligonucleotides, now with 5'-CACC overhang instead of the T7 promoter, were inserted into the vector and the resulting construct was transfected into the human embryonic kidney cells, *pre*-disposed for tetracycline-induced expression (T-REx®-293 cell line, Invitrogen, Carlsbad, CA, USA), thus creating permanently transfected cell line with zeocin selection. Alternatively, transient transfections were performed for HepG2 cells. Both type of transfections enable cellular RNA polymerase III to transcribe the RNA oligonucleotide from the vector. Accumulated cytosolic RNA oligonucleotides were supposed to be subsequently imported into the mitochondrial matrix by the natural mechanism, as insured by the proper MAM address (Fig. 1c).

Import verification by qRT-PCR

The import of the designed RNA probes into the mitochondrial matrix was tested by qRT-PCR (Roche) of (RNase-A washed) mitochondrial fraction isolated from the cells or from whole cell lysate. The import efficiency was taken as the relative amount of amplicon for the MAM-L-ND5 construct. Standardization has been achieved by employing human β -actin (base pairs 699–930), i.e., forward primer 5'-GCGGGA AATCGTG CGTGACAT-3' and reverse primer 5'-GATGGA GTTGAAGGTAGTTTC-3', as the cytosolic marker; and the native ND5 mRNA (base pairs 13202 to 13373) as the mitochondrial matrix marker, i.e., forward primer 5'-CAGTCTGC GCCCTTACACAAA-3' and reverse primer 5'-TGGACC CGGAGCACATAAATA-3'.

Agarose electrophoresis and Southern blots with RNA probes

Isolated mitochondria were prepared by homogenization of HepG2 cells with Dounce homogenizer in 180 mM KCl, 5 mM K-MOPS, 2 mM K-EGTA pH 7.2. Homogenate was centrifuged at 1,000×g for 10 min to pellet the cell debris while mitochondria were harvested at 15,000×g for 15 min. From the obtained mitochondrial fraction, mtDNA was prepared by

alkaline lysis followed by isopropanol precipitation. Resulting mtDNA was separated by agarose electrophoresis (0.4 % gel) with GeneRuler DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA). DNA was subsequently blotted to Amersham HyBond N⁺ membrane (GE Healthcare, Fairfield, CT, USA) using semidry blotting equipment TE77XP (Hoefer, Holliston, MA, USA). DNA was stabilized on the membrane by heating to 80 °C for 2 h. Membrane was then *pre*-hybridized at 42 °C for 2 h in 5×SSC buffer with 50 % formamide and 0.2 % SDS containing 10 µg.mL⁻¹ of *pre*-boiled salmon sperm DNA as a blocking agent. Hybridization proceeded in the same buffer with 1 µg.mL⁻¹ of the MAM-L-ND5 probe conjugated with AlexaFluor 488 at 42 °C overnight. Membrane was then washed and analyzed. While a direct densitometric detection failed to show any signal, the membrane was cut into defined pieces, boiled in distilled water for 10 min and the fluorescence of particular supernatants was measured by spectrofluorimetry (RF-5301-PC, Shimadzu, Kyoto, Japan).

Cell models

Besides the T-REx®-293 human embryonic kidney cell line (Life Technologies), also human hepatocellular carcinoma (HepG2) cells were used (Tauber et al. 2013), as obtained from European culture collection (ECACC 85011430) while cultivated in DMEM supplemented with 3 mM glutamine, 10 % (v/v) fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin and 5 mM glucose.

Confocal microscopy

A confocal inverted fluorescent microscope Leica TCS SP2 AOBS using a PL APO 100×/ 1.40–0.70 oil immersion objective (a pinhole 1 Airy unit) and a 20-mW argon laser for excitation was used with a thermostable sample chamber set to 37 °C supplied with 5 % CO₂, thus mimicking normal cultivation conditions. For GFP, KEIMA, SYTO-9, and AlexaFluor 488 -conjugated probes, excitation was at 488 nm, and 30-min time series of three-dimensional data stacks were recorded, with one three-dimensional stack recorded every 5 min. For Alexa Fluor 647-conjugated probes excitation was at 633 nm. Confocal microscope images were taken together with their overlays with either appropriate pairs of import/FIVH probe and mitochondrial matrix stain.

Results

Import of recombinant MAM-L-ND5 RNA into mitochondria as expressed from a vector

We have first attempted to employ sole native 5S RNA import pathway in human embryonic kidney (HEK) 293 cells *pre*-

disposed for tetracycline-induced expression (T-REx®-293 cells). After transfection and induction of MAM-L-ND5 RNA expression ongoing for 72 h, RNA was extracted from cells or isolated mitochondria and was inspected by qRT-PCR for the presence of the construct. Figure 2a shows a relative abundance within total RNA extracted from the isolated mitochondria, unwashed or washed by RNase-A, for the MAM-containing RNA or the above described scrambled-sequence containing RNA after 72 h of tetracycline-induced expression

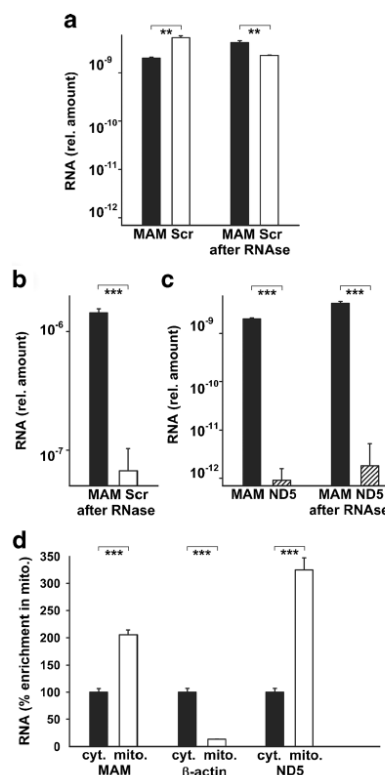


Fig. 2 Import of recombinant MAM-L-ND5 RNA into mitochondria as expressed from a Tetracycline-inducible pENTRH1/TO vector. **a**, **c**, **d** permanently transfected T-REx®-293 cell line, 72 h induction, **b** transient transfection in HepG2 cells; Relative abundance within total RNA extracted from mitochondria isolated with RNase-A treatment when indicated after expression from pENTRH1/TO vector containing the MAM-L-ND5 probe (cf. Fig. 1a; “MAM”), or Scrambled probe (cf. Fig. 1a; “Scr”). **c** compares the natural ND5 mRNA content (distinct primer sequences from the “cargo ND5 probe” part of ND5 sequence) with the imported MAM-L-ND5 probe. Panel **d** displays the relative mitochondrial enrichment vs. cytosol of MAM-L-ND5 RNA (“MAM”), cytoplasmic marker beta-actin, and natural ND5 mRNA in RNAs extracted from the isolated mitochondria vs. those extracted from the whole cells. Standard deviations are indicated. ANOVA (Student's t-test for a pair) yielded ** $p < 0.05$; *** $p < 0.001$ ($n = 4$ for T-REx®-293 cells; $n = 6$ for HepG2 cells)

from pENTRH1/TO and subsequent import into the mitochondrial matrix. Alternatively, we have transiently transfected HepG2 cells for 72 h with the same vector containing either MAM-L-ND5 coding or scrambled sequence and quantified their content in isolated mitochondria washed by RNase-A (Fig. 2b). Figure 2c demonstrates how sufficient, three-order of magnitude, excess of the MAM-L-ND5 imported probe over the natural ND5 mRNA was enriched after the import into the mitochondrial matrix. Finally, Fig. 2d verifies the validity of isolated mitochondrial fractions, demonstrating relative enrichment of MAM, and natural matrix ND5 mRNA in RNA extracted from the isolated mitochondria, in contrast to the great reduction of cytoplasmic marker β -actin, which was found negligible in mitochondrial fractions. These data suggest the existence of a quantitative mitochondrial import mechanism for the constructed RNA probes. The amount of imported RNA into T-REx®-293 cells would be satisfactory for the great enrichment of the imported probe over the native ND5 mRNA (Fig. 2c) and thus should be sufficient for an efficient delivery of nucleic acids into mitochondria, if one needs to achieve hybridization of predominant fraction of targeted nucleic acid types.

In vitro probe binding to ND5 target sequences

Verification of hybridization of predominant fraction of targeted nucleic acid types was tested in vitro by Southern blots as the ability of the constructed MAM-L-ND5 RNA probes to anneal to the ND5-containing sequence. As an example in Fig. 3 shows, the MAM-L-ND5 RNA probe

anneals to the mtDNA template, but not to off-target DNA from the ladder. Also, the MAM-000 probe, lacking ND5 annealing sequence, does not bind.

Import of mtFIVH probes into the mitochondria in cell cultures

In order to facilitate mitochondrial import of fluorescent RNA probes (see Fig. 4 for successful examples and Fig. 5 for failed attempts), the probes were designed equally as those expressed by a vector, but contained Alexa Fluor 647 (Fig. 4b, e, h) or AlexaFluor 488 dye (Fig. 4k) attached to the 3' end. The successful cases exhibited Manders' coefficient for the mitochondria/probe colocalization of 0.71 ± 0.11 ($n=6$), whereas those failed attempts 0.16 ± 0.14 . Likewise, the Pearson's correlation coefficients reached 0.58 ± 0.22 for successful colocalizations, whereas their values were -0.10 ± 0.05 for failed attempts. The negative values indicate the mutual exclusivity of the two imaged channels.

In developing the methodology, we had to first adjust the fluorescent probe delivery into the cells. Since lipid-mediated RNA transfection using commercially available Lipofectamine® was not effective (Fig. 5d, e, f), we had to adapt methodology of DQAsomes (dequalinium chloride micelles, (D'Souza et al. 2003)) to ensure probe import into the cells and simultaneously also to the proximity of mitochondria (Fig. 4). This is presumably achieved due to the mitochondriotropic amphiphilic nature of the resulting cationic micelles engulfing the RNA probes. Likewise DQAsomes ensure protection for RNA. As revealed by confocal

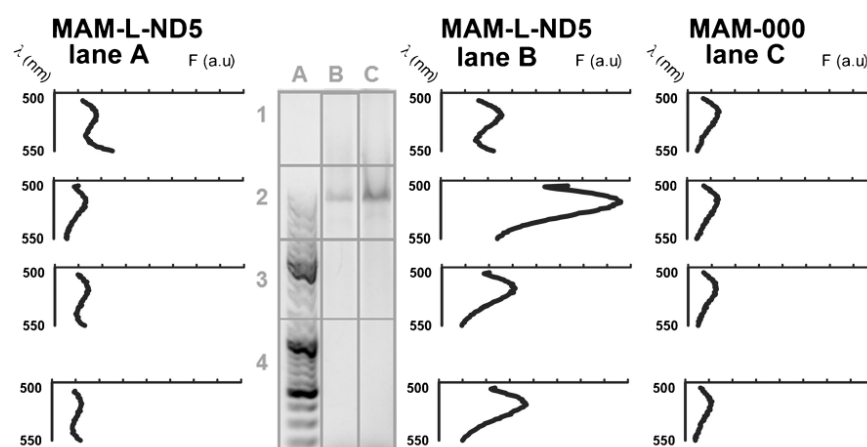
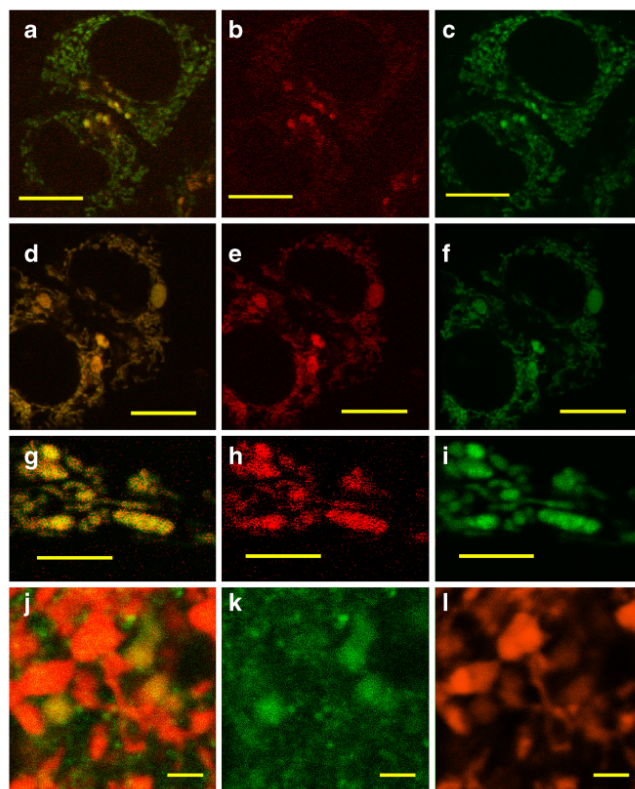


Fig. 3 In vitro MAM-L-ND5 probe binding to ND5 target sequence and lack of binding for MAM-000 probe. Membrane with separated DNA ladder (lane A) and mtDNA (lanes B, C) and was incubated with the fluorescent MAM-L-ND5 probe (lanes A, B) or MAM-000 probe (lane C), i.e., the probes labeled at 3' end by Alexa Fluor® 488, and

fluorescence of designated segments of the gel was quantified by measuring spectra. Though a rather high background with dye traces is indicated for lanes A and C, the realistic Alexa Fluor® emission is recognized specifically on the segment 2 of the lane B, the one containing mtDNA

Fig. 4 Successful cases of mitochondrial fluorescent *in vivo* hybridization (mtFIVH) by MAM-L-ND5 ribonucleoid probes in mtGFP- or mtKEIMA-transfected HepG2 cells. **a–i**) Probe with Alexa Fluor 647: confocal images or **a, d, g** overlays after 24 h of DQA-some facilitated delivery of a MAM-L-ND5 Alexa Fluor 647 ribonucleoid probe. The probe was imaged in the red channel (**b, e, h**), while the corresponding mitochondrial network in the cells expressing mtGFP in the green channel (**c, f, i**). Scale bars 10 μ m; 50 μ m in (**g, h, i**). **j, k, l**) Probe with Alexa Fluor 488: confocal images or overlay (**j**) after 4 h of DQA-some facilitated delivery of a MAM-L-ND5 Alexa Fluor 488 ribonucleoid probe, which is individually displayed within a green channel (**k**); and corresponding yet fragmented mitochondrial network in the cells expressing mtKEIMA (**l**, red). Scale bars 2 μ m. Pearson's correlation coefficients were 0.53, 0.79, 0.78 and 0.24 for images of panels **a, d, g**, and **j**, respectively; while Manders' colocalization coefficients were 0.79, 0.86, 0.82 and 0.64, respectively



microscopy, three conditions to be simultaneously fulfilled were required to facilitate the import into the mitochondrion for the fluorescent RNA probes, added to the HepG2 cell culture—*i*) DQA-some facilitated delivery; *ii*) the MAM address for the native mt SS RNA import pathway; and *iii*) annealing sequence to the target present within the probe, such as our ND5 exemplar sequence. Thus, DQA-facilitated import of probes with scrambled MAM failed (Fig. 5a, b, c; g, h, i) as well as import failed when the RNA probe contained only MAM sequence and not the ND5 annealing portion (Fig. 5j, k, l). The scrambled MAM probe was rapidly sequestered and excluded from the mitochondrial network and usually concentrated in extramitochondrial structures, lysosomes or vacuoles (Fig. 5g, h, i). As mentioned above, even the full probe was localized into the cytosol with Lipofectamine®-facilitated attempts for import (Fig. 5d, e, f). Such a Lipofectamine®-facilitated import of MAM-L-ND5 RNA did not work perhaps due to the inability to locate cell-imported RNA to the proximity of mitochondria. Note, we have also attempted a system of MitoPorter (Yamada et al.

2008; Nakamura et al. 2012) with our probes (not shown), but we were unable to detect the probe fluorescence in mitochondria.

Our constructs represent a prototype of mitochondrial fluorescent *in vivo* hybridization (mtFIVH). Possible annealing *in vivo* of RNA probes bearing the ND5 sequences with the corresponding ND5 L-strand mtDNA is demonstrated in Fig. 6a, b, c showing that when both ND5 and MAM sequences are present on a probe, colocalization of probe fluorescence with the mitochondrial nucleoids (definitively units containing mtDNA, Fig. 1e) is recognized (Fig. 6a, b, c; cf. (Alán et al. 2010)).

Discussion

We have developed an efficient method for importing nucleic acids into the mitochondrial matrix if expressed from vectors transfected to cells and we extended this already successful approach (Comte et al. 2013; Smimov et al. 2008a, b, 2010,

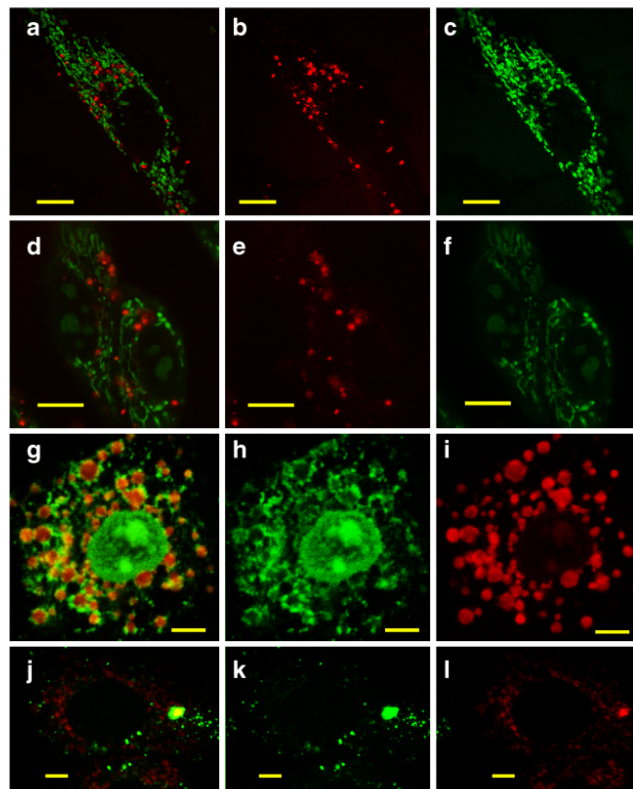


Fig. 5 Failed cases of mitochondrial fluorescent *in vivo* hybridization (mtFIVH) by ribonucleoid probes in mtGFP- or mtKEIMA-transfected HepG2 cells. **a, b, c** Confocal images illustrating a failed attempt for mitochondrial 24 h import of a 000-L-ND5 Alexa Fluor 647 ribonucleoid probe, i.e., probe lacking MAM, which is individually displayed (**b**) within a red channel; **c** corresponding mitochondrial network in the cells expressing mtGFP (green channel); **a** overlay of **c** and **b**. Scale bars 10 μ m. **d, e, f** Confocal images illustrating attempts of lipofectamine-facilitated 24 h delivery of a MAM-L-ND5 Alexa Fluor 647 ribonucleoid probe, which is individually displayed (**e**) within a red channel; **f** corresponding yet partly fragmented mitochondrial network in the cells expressing mtGFP (green channel); **d** overlay of **e** and **f**. Scale bars 10 μ m. **g, h, i** Confocal images illustrating a failed attempt for mitochondrial 4 h

import of a 000-L-ND5 Alexa Fluor 488 ribonucleoid probe, i.e., probe lacking MAM, which is individually displayed (**h**) within a green channel; **i** corresponding yet fragmented mitochondrial network in the cells expressing mtKEIMA (red channel); **g** overlay of **i** and **h**. Scale bars 5 μ m. **j, k, l** Confocal images illustrating a failed attempt for mitochondrial 4 h import of a MAM-000 Alexa Fluor 488 ribonucleoid probe, i.e., probe lacking NDS annealing sequence, which is individually displayed (**k**) within a green channel; **l** corresponding yet fragmented mitochondrial network in the cells expressing mtKEIMA (red channel); **j** overlay of **k** and **l**. Scale bars 5 μ m. Pearson's correlation coefficients were -0.173 , -0.065 , -0.052 and 0.025 for images of panels **a, d, g**, and **j**, respectively; while Manders' colocalization coefficients were 0.017 , 0.048 , 0.239 and 0.15 , respectively

2011; Tarassov et al. 2007; Vedrenne et al. 2012; Wang et al. 2010, 2012a, b) to mitochondrial fluorescent *in vivo* hybridization (mtFIVH). Both methods were based on the same RNA probe design, so to contain the native import address of mt 5S RNA, while the mtFIVH was further combined with the use of DQAsomes as micelles recruiting probes into the proximity of mitochondria within the cell. The first system, based on efficiently expressed form, i.e., on a vehicle, which can be rather easily transfected into the cells, can be predicted to have a higher practical and medical significance. Probably, it could

be provided by the future systems based on viral transfections, when similar probe design could be combined with viral expression. The addressing by the inherent 5S RNA or FD-RNA is promising, since even heteroplasmy was improved in benefits of wild-type, when oligoribonucleotides complementary to mutant mtDNA region were imported into the cultured transmitochondrial cybrid cells with a portion of mtDNA bearing a large deletion associated with the Kearns Sayre syndrome (Comte et al. 2013). Likewise, human mtDNA mutations were corrected using such an approach (Wang et al. 2012b).

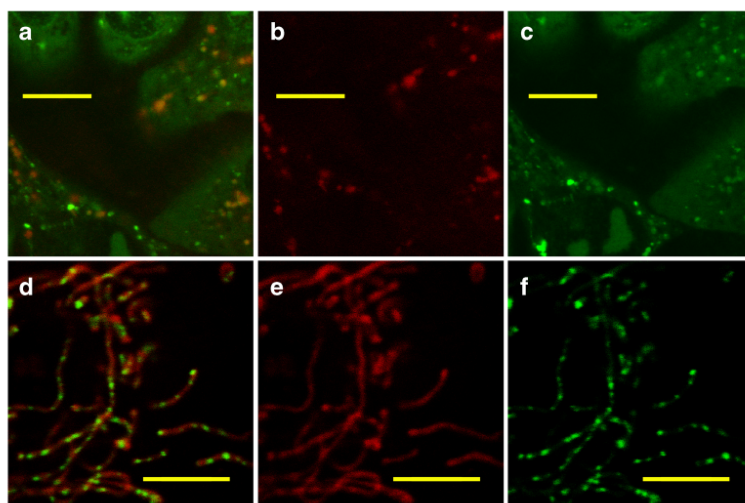


Fig. 6 Mitochondrial fluorescent in vivo hybridization (mtFIVH) by ribonucleoid probes bearing a short antisense NDS annealing portion enables nucleoid visualization. **a, b, c** Confocal images: **a** overlay of **b** and **c** after 24 h of DQA-some facilitated delivery of a MAM-L-NDS Alexa Fluor 647 ribonucleoid probe, which is individually imaged in the red channel (**b**); **c**

SYTO 9 DNA staining of mitochondrial nucleoids (green). Scale bars 10 μ m. Pearson's correlation coefficient was 0.36 while Manders' colocalization coefficient was 0.89 for panel **a**. **d, e, f** Confocal images of mtKEIMA transfected-mitochondria (**e**) and SYTO 9 DNA stained-nucleoids (**f**) in cells untreated with DQA-somes; **d** overlay of **e** and **f**. Scale bars 5 μ m

To provide efficient mitochondrial fluorescent in vivo hybridization, one needs not only to ensure the uptake of such probe into the cell, but also to deliver such nucleic acid cargo to the proximity of mitochondria. Moreover, conditions for probe annealing and hence accumulation must be fulfilled, otherwise the probe is most probably degraded. As we demonstrated, when all these conditions are achieved, the natural TOM-TIM import system, can subsequently take up this cargo and import it into the mitochondrial matrix. Other tested systems such as MitoPorter (Yamada et al. 2008; Nakamura et al. 2012) were not efficient. Our attempts with MitoPorter did not lead to staining either of the cell cytosol even at concentrations showing significant cytotoxicity. Thus the DQAsomes, the dequalinium chloride micelles seems to be optimum for in vitro experiments with cultured cells (D'Souza et al. 2003). However, due to their detergent-like properties, DQAsome dosage should be exactly titrated not to interfere with studied phenomena. We observed frequently that DQAsomes fragmented mitochondrial network, and that could be interfering with numerous studies, unless recovery is achieved after withdrawal from media containing DQAsomes later after successful import.

The fluorophore-conjugated recombinant RNA delivery into mitochondria of living cells may become versatile for research on various components of mt genetic machinery as the annealing RNA portion hybridizes to complementary nucleic acids in the nearly intact situation in living cultured cells. Facilitation of nucleic acid import by using a proper ribonucleoide address

derived from mt 5S RNA and employing of its native nucleic acid import system (Comte et al. 2013; Smirnov et al. 2008a, b, 2010, 2011; Tarasov et al. 2007; Vedrenne et al. 2012; Wang et al. 2010, 2012a, b) was a successful combination with the ability to pass also the desired nucleic acid cargo even with attached fluorophores. Focusing onto such mitochondrial fluorescent in vivo hybridization, mtFIVH, should be further developed in order to distinguish various entities of mtRNAs (Fig. 1e). Note that theoretically a H-NDS probe, that was not used here, should anneal to the H-strand of ds mtDNA, i.e., to the "true" *nd5* gene, (Alán et al. 2010). It might anneal also to the corresponding mRNA transcribed from the L-strand of mtDNA, however, we can speculate that this L-strand DNA transcribed RNA could be degraded much faster than the "real" mt-mRNA transcribed from the H-strand and existing either as polycistronic mt-mRNA or sole NDS mRNA (Kasiviswanathan et al. 2012; Holt 2009; Falkenberg et al. 2007; Alán et al. 2010)(Fig. 1d). Thus L-NDS probe can hybridize to nucleoids on either L-strand mtDNA and attached polycistronic mt-mRNA plus to NDS mRNA diffused out or bound to mitochondrial ribosomes (Fig. 1e). In other words, mitochondrial mRNAs can be structural parts of nucleoids, at least transiently, or may be dispersed in the mitochondrial matrix, and finally may locate on mt ribosomes. Currently,

employing conventional confocal microscopy [e.g.34], we are limited by ~250 nm resolution, which does not allow to distinguish two objects in proximity closer than 250 nm, hence it is unable to distinct objects within the tubules of the mitochondrial network nor inside nucleoids. Consequently, mtFIVH stains the whole mitochondrial network (although mostly fragmented) and rarely mt nucleoids (Alán et al. 2010; Tauber et al. 2013), e.g., when visualized by colocalization with fluorescently labeled nucleoid protein TFAM (Fig. 6). Therefore, proper resolution within a single nucleoid cannot be achieved. In the future combination with dSTORM providing superresolution (Linde et al. 2011) and with the ability to resolve nucleoids in their true dimensions (Brown et al. 2011) and inside, one could distinct (using such future advanced mtFIVH) for example the D-loop region location or the gene, such as NDS coding region, from mt ribosomes and from the complex mtDNA/mtRNA structures (Pohjoismäki et al. 2010), such as RITOLS (RNA incorporations throughout of lagging strand) and R-loops. RITOLS are formed by double strand mtDNA and double strand mtRNA interrupted by single strand mtDNA and single strand mtRNA and represent the extended mtRNA segments encompassing the entire lagging strand of mtDNA which are remnants or intermediates of strand-coupled bidirectional replication of mtDNA. In turn, frequently found R-loops are transcription intermediates. Thus mtFIVH probes properly designed may be developed into specific tools for studies of mitochondrial transcription and replication in the future.

In conclusion, we have employed mt 5S rRNA mitochondria-addressing domain sequence and developed an import system as expressed either from the vector transfected to the cell or artificial DQAsome-facilitating import system for fluorescently labeled probes for mt fluorescent in vivo hybridization (mtFIVH). Depending on the target choice, one may use both systems for targeting the accessible complementary mtDNA strand plus all types of relevant mtRNAs containing the targeted sequence (polycistronic mt-RNA, its cleaved portions, and mt-poly-A-mRNAs), including mRNA attached to ribosomes.

Acknowledgment Supported by grants of the Grant Agency of the Czech Republic No. P305/12/1247 to MJ and P305/12/P388 to JZ; and by the research projects RVO67985823 and BIOCEV—Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University (CZ.1.05/1.1.00/02.0/109), from the European Regional Development Fund.

References

- Alán L, Zelenka J, Ježek J, Dlasková A, Ježek P (2010) Fluorescent in situ hybridization of mitochondrial DNA and RNA. *Acta Biochimica Polonica* 57(4):403–408
- Andrews RM, Kubacka I, Chinnery PF, Lightowlers RN, Turnbull DM, Howell N (1999) Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. *Nature Genetics* 23(2):147
- Bogenhagen DF (2012) Mitochondrial DNA nucleoid structure. *Biochimica et Biophysica Acta* 1819(9–10):914–920
- Bogenhagen DF, Rousseau D, Burke S (2008) The layered structure of human mitochondrial DNA nucleoids. *Journal of Biological Chemistry* 283(6):3665–3675
- Brown TA, Tkachuk AN, Shtengel G, Koepke BG, Bogenhagen DF, Hess HF et al (2011) Superresolution fluorescence imaging of mitochondrial nucleoids reveals their spatial range, limits, and membrane interaction. *Molecular and Cellular Biology* 31(24):4994–5010
- Comte C, Tonin Y, Heckel-Mager AM, Boucheham A, Smimov A, Auré K et al (2013) Mitochondrial targeting of recombinant RNAs modulates the level of a heteroplasmic mutation in human mitochondrial DNA associated with Kearns Sayre Syndrome. *Nucleic Acids Research* 41(1):418–433
- D'Souza GG, Rammohan R, Cheng SM, Torchilin VP, Weissig V (2003) DQAsome-mediated delivery of plasmid DNA toward mitochondria in living cells. *Journal of Controlled Release* 92(1–2):189–197
- Durazo SA, Kompella UB (2012) Functionalized nanosystems for targeted mitochondrial delivery. *Mitochondrion* 12(2):190–201
- Falkenberg M, Larsson NG, Gustafsson CM (2007) DNA replication and transcription in mammalian mitochondria. *Annual Reviews in Biochemistry* 76:679–699
- Holt IJ (2009) Mitochondrial DNA replication and repair: all a flap. *Trends in Biochemical Sciences* 34(7):358–365
- Jash S, Adhya S (2012) Induction of muscle regeneration by RNA-mediated mitochondrial restoration. *FASEB Journal* 26(10):4187–4197
- Kasiviswanathan R, Collins TR, Copeland WC (2012) The interface of transcription and DNA replication in the mitochondria. *Biochimica et Biophysica Acta* 1819(9–10):970–978
- Kogure T, Kawano H, Abe Y, Miyawaki A (2008) Fluorescence imaging using a fluorescent protein with a large Stokes shift. *Methods* 45(3):223–226
- Kolesnikova O, Kazakova H, Comte C, Steinberg S, Kaminski P, Martin RP et al (2010) Selection of RNA aptamers imported into yeast and human mitochondria. *RNA* 16(5):926–941
- Li H, Liu D, Lu J, Bai Y (2012) Physiology and pathophysiology of mitochondrial DNA. In: Scatena R, Bottoni P, Giardina B (eds) *Advances in mitochondrial medicine, advances in experimental medicine and biology* vol. 942. New York, Springer, pp 39–51
- Linde S, Löschberger A, Klein T, Heidebreder H, Wolter S, Heilemann M, Sauer M (2011) Direct stochastic optical reconstruction microscopy with standard fluorescent probes. *Nature Protocols* 6(7):991–1009
- Magalhães PJ, Andreu AL, Schon EA (1998) Evidence for the presence of 5S rRNA in mammalian mitochondria. *Molecular Biology of the Cell* 9(9):2375–2382
- Mahata B, Mukherjee S, Mishra S, Bandyopadhyay A, Adhya S (2006) Functional delivery of a cytosolic tRNA into mutant mitochondria of human cells. *Science* 314(5798):471–474
- Muratovska A, Lightowlers RN, Taylor RW, Turnbull DM, Smith RA, Wilce JA et al (2001) Targeting peptide nucleic acid (PNA) oligomers to mitochondria within cells by conjugation to lipophilic cations: implications for mitochondrial DNA replication, expression and disease. *Nucleic Acids Research* 29(9):1852–1863
- Nakamura T, Akita H, Yamada Y, Hatakeyama H, Harashima HA (2012) Multifunctional envelope-type nanodevice for use in nanomedicine: concept and applications. *Accounts of Chemical Research* 45(7):1113–1121
- Niazi AK, Mileshina D, Cosset A, Val R, Weber-Lotfi F, Dietrich A (2013) Targeting nucleic acids into mitochondria: progress and prospects. *Mitochondrion* 13(5):548–558
- Pohjoismäki JL, Holmes JB, Wood SR, Yang MY, Yasukawa T, Reyes A et al (2010) Mammalian mitochondrial DNA replication intermediates are essentially duplex but contain extensive tracts of RNA/DNA hybrid. *Journal of Molecular Biology* 397(5):1144–1155

- Rorbach J, Minczuk M (2012) The post-transcriptional life of mammalian mitochondrial RNA. *Biochemical Journal* 444(3):357–373
- Sieber F, Duchene AM, Marechal-Drouard L (2011a) Mitochondrial RNA import: from diversity of natural mechanisms to potential applications. *International Review of Cell and Molecular Biology* 287:145–190
- Sieber F, Placido A, El Farouk-Ameqrane S, Duchene AM, Marechal-Drouard L (2011b) *Nucleic Acids Research* 39(14):e96
- Smirnov AV, Entelis NS, Krasheninnikov IA, Martin R, Tarassov IA (2008a) Specific features of 5S rRNA structure—its interactions with macromolecules and possible functions. *Biochemistry (Moscow)* 73(13):1418–1437
- Smirnov A, Tarassov I, Mager-Heckel AM, Letzelier M, Martin RP, Krasheninnikov IA, Entelis N (2008b) Two distinct structural elements of 5S rRNA are needed for its import into human mitochondria. *RNA* 14(4):749–759
- Smirnov A, Comte C, Mager-Heckel AM, Addis V, Krasheninnikov IA, Martin RP et al (2010) Mitochondrial enzyme rhodanese is essential for 5S ribosomal RNA import into human mitochondria. *Journal of Biological Chemistry* 285(40):30792–30803
- Smirnov A, Entelis N, Martin RP, Tarassov I (2011) Biological significance of 5S rRNA import into human mitochondria: role of ribosomal protein MRP-L18. *Genes and Development* 25(12):1289–1305
- Tarassov I, Kamenski P, Kolesnikova O, Karicheva O, Martin RP, Krasheninnikov IA, Entelis N (2007) Import of nuclear DNA-encoded RNAs into mitochondria and mitochondrial translation. *Cell Cycle* 6(20):2473–2477
- Tauber J, Dasková A, Šantorová J, Smolková K, Alán L, Špaček T et al (2013) Distribution of mitochondrial nucleoids upon mitochondrial network fragmentation and network reintegration in HEPG2 cells. *International Journal of Biochemistry and Cell Biology* 45(3):593–603
- Vedrenne V, Gowher A, De Lonlay P, Nitschke P, Serre V, Boddaert N et al (2012) Mutation in PNPT1, which encodes a polynucleotide nucleotidyltransferase, impairs RNA import into mitochondria and causes respiratory-chain deficiency. *American Journal of Human Genetics* 91(5):912–918
- Wang G, Chen HW, Oktay Y, Zhang J, Allen EL, Smith GM et al (2010) PNPASE regulates RNA import into mitochondria. *Cell* 142(3):456–467
- Wang G, Shimada E, Koehler CM, Teitell MA (2012a) PNPASE and RNA trafficking into mitochondria. *Biochimica et Biophysica Acta* 1819(9–10):998–1007
- Wang G, Shimada E, Zhang J, Hong JS, Smith GM, Teitell MA et al (2012b) Correcting human mitochondrial mutations with targeted RNA import. *Proceedings of National Academy of Sciences U S A* 109(13):4840–4845
- Weber-Lotfi F, Ibrahim N, Boesch P, Cosset A, Konstantinov Y, Lightowlers N, Dietrich RNA (2009) Developing a genetic approach to investigate the mechanism of mitochondrial competence for DNA import. *Biochimica et Biophysica Acta* 1787:320–327
- Yamada Y, Akita H, Kamiya H, Kogure K, Yamamoto T, Shinohara Y et al (2008) MITO-Porter: a liposome-based carrier system for delivery of macromolecules into mitochondria via membrane fusion. *Biochimica et Biophysica Acta* 1778(2):423–432
- Zhadanov SI, Grechanina EY, Grechanina YB, Gusar VA, Fedoseeva NP, Lebon S et al (2007) Fatal manifestation of a de novo NDS mutation: insights into the pathogenetic mechanisms of mtDNA ND5 gene defects. *Mitochondrion* 7(4):260–266



Assessment of Mitochondrial DNA as an Indicator of Islet Quality: An Example in Goto Kakizaki Rats

L. Alán, T. Špaček, J. Zelenka, J. Tauber, Z. Berková, K. Zacharovová, F. Saudek, and P. Ježek

ABSTRACT

Background. Diabetic Goto Kakizaki (GK) rats represent an established model of type 2 diabetes that exhibit an onset of pancreatic islet (PI) pathology characterized by islet hypertrophy with a decreased number of insulin-secreting β -cells. Among the remaining β -cells, oxidative phosphorylation (OXPHOS) and consequently glucose-stimulated insulin secretion (GSIS) are impaired, perhaps owing to a deficit in mitochondrial DNA (mtDNA). We sought to identify this abnormality.

Methods. β -Cells were obtained from Accutase-dissolved PI isolated from GK or Wistar rats and sorted based on the positive Zn^{2+} signal of Newport Green. The mtDNA copy number per cell was quantified as the amplicon ratio by polymerase chain reaction using specific primers against the rat ND5 mt gene and UCP2 nuclear gene.

Results. The 12-month-old GK rats exhibited drastically reduced copy numbers per remaining β -cell, from $7,400 \pm 600$ in 12-month old Wistar rats (100%) to $24 \pm 4\%$; mtDNA content in heart and liver was $70 \pm 25\%$ and $60 \pm 20\%$, respectively. Versus age-paired Wistar rats, 6- and 4-month-old GK rats showed reductions to $60 \pm 15\%$ and $50 \pm 20\%$, respectively.

Conclusions. OXPHOS of remnant β -cells in diabetic GK was drastically impaired due to the lack of sufficient mtDNA levels. We suggest the use of mtDNA quantification to quickly assess PI quality before transplantation.

THE TYPE 2 diabetes mellitus (T2DM) model GK rats show islet hypertrophy with a progressive decrease in the insulin-secreting β -cell mass.^{1,2} Multiple gene defects may be responsible. For example, GK rats exhibit down-regulation of Snare family membrane proteins, such as Syntaxin-1A, Snap-25, Vamp-2, and nSec1/munc18 proteins, which prevent exocytosis of insulin-containing vesicles.² These rats show a possible origins of the diabetic process to include mitochondrial dysfunction.² Because oxidative phosphorylation (OXPHOS) provides an intimate glucose sensor, its impairments lead to immediate glucose-stimulated insulin secretion (GSIS) dysfunction. OXPHOS decrease may be due to various gene defects, oxidative stress,³ dysregulated mitochondrial specific autophagy,⁴ termed mitophagy, and excessive apoptosis. However, the most direct way to produce dysfunctional OXPHOS is insufficient or mutated mitochondrial DNA (mtDNA) that might result from all of the above-described events. Thus, a lower mtDNA content, as indicated by a reduced 12S ribosomal RNA and cytochrome B mRNA, has been re-

ported among adult GK rat islets.⁵ One may speculate that increased mitophagy explains the lower mtDNA content. It has been suggested that mitochondrial degradation, including mtDNA degradation, proceeds specifically in small objects disintegrated from the main mitochondrial reticulum.⁴ Therefore, the hypothetically increased mitophagy in diabetic GK rat β -cells fits well with our recent finding of a fragmented mitochondrial reticulum that is disintegrated into numerous small objects.⁶ Also, smaller mitochondrial volume and a decreased number of mitochondria per unit

From Department No. 75, Institute of Physiology, Academy of Sciences of the Czech Republic (L.A., T.S., J.Z., J.T., P.J.), and Institute of Clinical and Experimental Medicine (Z.B., K.Z., F.S.), Prague, Czech Republic.

Supported by grant no. NS10219-3/2009 (P.J.) from the Internal Grant Agency of the Czech Ministry of Health.

Address reprint requests to Dr Petr Ježek, PhD, DSc, Dept. No. 75, Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague 4, Czech Republic. E-mail: jezek@biomed.cas.cz

© 2011 by Elsevier Inc. All rights reserved.
360 Park Avenue South, New York, NY 10010-1710

0041-1345/-see front matter
doi:10.1016/j.transproceed.2011.09.055

Transplantation Proceedings, 43, 3281-3284 (2011)

3281

tissue volume have been reported in β -cells of GK rats,⁵ which matches our observations.⁶

METHODS

Islet Isolation

Wistar rats and Goto Kakizaki rats (Taconic, Ejby, Denmark) were bred, housed, and killed in accordance with the European Guidelines on Laboratory Animal Care and according to the Institute of Clinical and Experimental Medicine licensing committee approval. GK rats were derived by embryo transfer from the Danish (Aarhus) colony established in 1994 from the original Tohoku University (Japan) colony initiated in 1975. We used 4-, 6-, and 12-month old Goto Kakizaki rats. Pancreatic islet (PI) samples were isolated from 1 Wistar rat and always from 2 Goto Kakizaki rats, weighing >420 g at 12 months (>360 g at 4 months) and exhibiting hyperglycemia usually between 16 and 17 mmol/L glucose and impaired intravenous glucose tolerance test (~15–20 mmol/L glucose remaining 60 minutes after glucose intake). Wistar rats were nondiabetic (normoglycemic and normal intravenous glucose tolerance test), weighing >600 g at 12 months (>490 g at 4 months).

PI isolation was performed according to a standard protocol.⁶ Pancreata were distended by infusion of 15 mL collagenase (1 mg/mL; Sevapharma, Prague, Czech Republic) with islets separated from exocrine tissue by centrifugation in a Ficoll discontinuous gradient (Sigma, St Louis, MO, USA). Purified islets were cultivated in RPMI 1640 medium without glucose, supplemented with 10% fetal calf serum, 1% penicillin/streptomycin/L-glutamine, 1% HEPES, and 5 mmol/L glucose, in a humidified CO₂ incubator at 37°C and 5% CO₂. Islet viability was checked by propidium iodide and acridine orange methods.

β -Cell Isolation

β -Cells were isolated after PI treatment with StemPro Accutase (Gibco; Invitrogen, San Diego, CA, USA) and cell dissociation reagent (1 mL per 300–1000 islets, while waiting for islet disintegration). They were washed with glucose-containing RPMI 1640 medium. A FACS Vantage SE cell sorter (BD Biosciences, Franklin Lakes, NJ, USA) was used to separate Zn²⁺ signal positive cells with Newport Green DCF (Invitrogen) using alkaline lysis buffer as previously described.⁷

mtDNA Copy Number Estimation

The mtDNA from heart and liver tissues was isolated by phenol-chloroform extraction. Sybr Green qPCR amplification used primers annealing on the UCP2 nuclear gene (intron 2 and exon 3) and ND5 mitochondrial gene (bp 11092 to 11191 according to Genbank sequences from The National Center for Biotechnology Information, USA). The ratio between ND5 amplicon and half of nuclear amplicon amounts was taken as the mtDNA copy number per cell. In the case of β -cells, the mtDNA copy number was determined by 10 cycles of PCR preamplification, followed by qPCR with Sybr Green detection. The nuclear gene concentration before the preamplification step was not sufficient to perform qPCR. Owing to the preamplification step, it was not possible to derive a copy number just by dividing the amount of amplicon by the number of cells.

Islet Respiration Measurements

Oxygen consumption of 100 PI used in a chamber was calculated from changes in oxygen concentration, as measured by HIOXY probe and NeoFox Phase Measurement system (Ocean Optics, Dunedin, FL, USA), in a 175- μ L tempered chamber.

Statistical Analysis

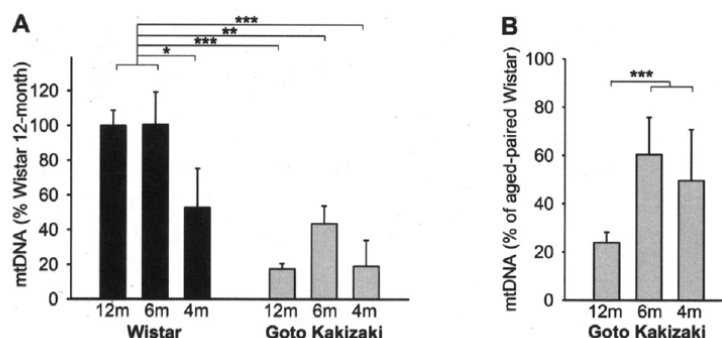
Statistical analysis was performed using Student *t* test. All data are presented as mean $\pm$ SD. *P* values of <.02 were deemed to be significant.

RESULTS

mtDNA Copy Number

The 12-month old Goto Kakizaki rats exhibited on average a reduced mtDNA copy number of $24 \pm 4\%$ versus the average copy number in 12-month old Wistar rats, which was estimated to be $7,400 \pm 600$ (100%; Fig 1A). Six- and 4-month-old GK rats showed reductions to $60 \pm 15\%$ and $50 \pm 20\%$, respectively, versus the age-paired Wistar rats (Fig 1). The mtDNA copy number increased with maturation up to 6 months in both Wistar and GK rats (Fig 1A). Similar intermediate mtDNA reduction in the heart muscle and liver was observed also for 12-month-old GK rats (Fig 2), exhibiting on average $70 \pm 25\%$ and $60 \pm 20\%$, respectively, of the levels in the heart and liver of age-

Fig 1. mtDNA copy number in PI β -cells of 12-, 6-, and 4-month old Wistar and Goto Kakizaki rats. (A) The relative scale was set such that an average copy number for 12-month old Wistar rats was ascribed as 100%. (B) The relative scale was set vs age-paired Wistar rats. **P* < .05; ***P* < .02; ****P* < .001.



paired Wistar rats. Note that the latter weighed about twice as much as compared with Goto Kakizaki rats.

Islet Respiration

We also confirmed that the respiration test indicated a slightly impaired response to glucose that may predict insufficient GSIS (although insulin release was not measured) in the 4 PI samples collected from 6-month-old GK rat pairs exhibited hyperglycemia and impaired intravenous glucose tolerance tests (~15–20 mmol/L glucose remaining 60 minutes after glucose intake). In contrast, Wistar rat PI respired ~34% and 50% more at 5 mmol/L and 25 mmol/L glucose, respectively, than PI from age-paired 6-month-old GK rats (Fig 3). The typical respiration increase simulated by a 20 mmol/L higher glucose was 1.30 ± 0.05 in Wistar PI and 1.45 ± 0.07 in GK PI. Impaired OXPHOS among GK PI was clearly indicated by a much greater state-3/state-4 ratio of 2.0 ± 0.5 in Wistar PI versus only 1.5 ± 0.2 in GK PI.

DISCUSSION

The number of pancreatic β -cells is substantially reduced in hypertrophied islets of GK rats. Moreover, in the remaining β -cells, the mtDNA copy number per cell is drastically reduced, down to one-fourth in 12-month-old GK rats, and to 50% and 60%, respectively in 4- and 6-month-old GK rats. As a consequence, OXPHOS capacity may be considered to be adequately diminished. Indeed, the results of our respiratory tests fit the expected correlation. There was a ~42% reduction of state-3/state-4 ratio in 6-month-old GK rats compared with age-paired Wistar rats. (Note, at 0 adenosine triphosphate (ATP) synthesis, the state-3/state-4 ratio is 1). This observation might be interpreted as a similar 42% reduction of ATP synthesis. From our estimates of the glucose response to 5 mmol/L and 25 mmol/L, the simulated GSIS was reduced by ~35%, which fit our calculations and matched the mtDNA copy number reduction of 40%. Therefore, we suggest that evaluation of

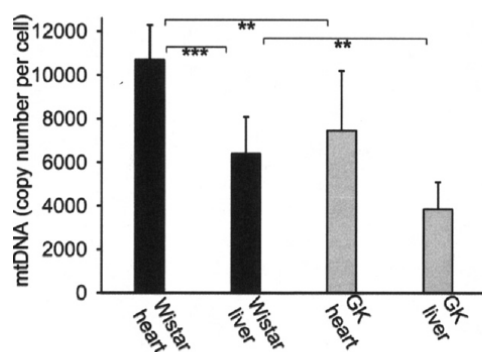


Fig 2. mtDNA copy number in hearts and liver of 6-month-old Wistar and Goto Kakizaki rats. ** $P < .02$; *** $P < .001$.

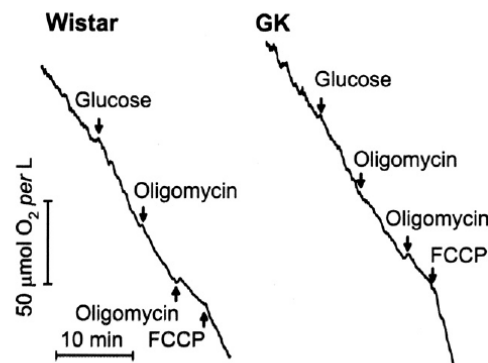


Fig 3. Respiration of pancreatic islets of 6-month-old Wistar and Goto Kakizaki rats. Typical traces of oxygen consumption are shown. Where indicated, 20 mmol/L incremental glucose was added (raising 5 mmol/L to 25 mmol/L glucose), 0.2 μ g/mL oligomycin and 3 μ mol/L FCCP, an uncoupler. GK rats exhibited hyperglycemia at 12 mmol/L glucose.

mtDNA in pancreatic β -cells may serve a diagnostic purpose, because the respiration test simulating GSIS (measured via respiration raise or other bioenergetics parameters) requires quite high quantities of PI.

The T2DM onset in GK rats was indicated in our evaluation by the finding that 4- and 6-month-old GK rats possessed mtDNA copy number reductions of 50% and 60%, respectively, whereas a greater decline to 25% was evident at 1 year. Moreover, we observed a growth-related aspect even in Wistar rat β -cells, indicating an increased mtDNA copy number during the rat's lifetime between 4 and 6 months. Because this was identified also in GK rats, the static figure of mtDNA reduction by 75% means that an enormous amount of mtDNA is degraded in their remaining β -cells during GK rat maturation. Evidently, the absolute numbers or amounts *per* pancreas are even higher, because the β -cell number was reduced as well. Hypothetically, increased mitochondrial-specific autophagy,^{4,6} mitophagy, may cause events leading to the reduced mtDNA copy number. The absolute mtDNA copy numbers observed in β -cells and other tissues of Wistar rats, such as heart and liver, were slightly higher than the data reported elsewhere,⁸ possibly owing to our choice of mitochondrial genes with a high incidence in nuclear DNA.

In conclusion, independent of the mechanism of origin, the decreased mtDNA copy number in this T2DM model correlated with impaired respiration responses to glucose. The reduced GSIS might be clinically relevant to the design of quality tests for PI preparations.

REFERENCES

1. Mizukami H, Wada R, Koyama M, et al: Augmented β cell loss and mitochondrial abnormalities in sucrose-fed GK rats. *Virchows Arch* 452:383, 2008

2. Ostenson C-G, Efendic S: Islet gene expression and function in type 2 diabetes; studies in the Goto-Kakizaki rat and humans. *Diabetes Obes Metab* 9(Suppl):180, 2009
3. Ježek P, Hlavatá L: Mitochondria in homeostasis of reactive oxygen species in cell, tissues, and organism. *Int J Biochem Cell Biol* 37:2478, 2005
4. Twig G, Elorza A, Molina AJ, et al: Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. *EMBO J* 27:433, 2008
5. Serradas P, Giroix MH, Saulnier C, et al: Mitochondrial deoxyribonucleic acid content is specifically decreased in adult, but not fetal, pancreatic islets of the Goto-Kakizaki rat, a genetic model of noninsulin-dependent diabetes. *Endocrinology* 136:5623, 1995
6. Dlasková A, Špaček T, Šantorová J, et al: 4Pi microscopy reveals an impaired three-dimensional mitochondrial network of pancreatic islet beta-cells, an experimental model of type-2 diabetes. *Biochim Biophys Acta* 1797:1327, 2010
7. Tsuchiya S, Sueoka K, Matsuda N, et al: The "spanning protocol": A new DNA extraction method for efficient single-cell genetic diagnosis. *J Assist Reprod Genet* 22:407, 2005
8. Walters MW, Bjork JA, Wallace KB: Perfluorooctanoic acid stimulated mitochondrial biogenesis and gene transcription in rats. *Toxicology* 264:10, 2009



Contents lists available at SciVerse ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocyte



Distribution of mitochondrial nucleoids upon mitochondrial network fragmentation and network reintegration in HEPG2 cells

Jan Tauber, Andrea Dlasková, Jitka Šantorová, Katarína Smolková, Lukáš Alán, Tomáš Špaček, Lydie Plecíť-Hlavatá, Martin Jabůrek, Petr Ježek*

Department of Membrane Transport Biophysics, No. 75, Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic

ARTICLE INFO

Article history:

Received 17 July 2012

Received in revised form 2 November 2012

Accepted 28 November 2012

Available online xxx

Keywords:

Mitochondrial DNA nucleoids

Mitochondrial fission

Mitochondrial network fragmentation

Mitochondrial network reintegration

ABSTRACT

Mitochondrial DNA (mtDNA) is organized in nucleoids in complex with accessory proteins, proteins of mtDNA replication and gene expression machinery. A robust mtDNA genome is represented by hundreds to thousands of nucleoids in cell mitochondrion. Detailed information is lacking about the dynamics of nucleoid distribution within the mitochondrial network upon physiological and pathological events. Therefore, we used confocal microscopy to study mitochondrial nucleoid redistribution upon mitochondrial fission and following reintegration of the mitochondrial network. Fission was induced by oxidative stress at respiration inhibition by rotenone or upon elimination of the protonmotive force by uncoupling or upon canceling its electrical component, $\Delta\psi_m$, by valinomycin; and by silencing of mitofusin MFN2. Agent withdrawal resulted in concomitant mitochondrial network reintegration. We found two major principal morphological states: (i) a tubular state of the mitochondrial network with equidistant nucleoid spacing, 1.10 ± 0.2 nucleoids per μm , and (ii) a fragmented state of solitary spheroid objects in which several nucleoids were clustered. We rarely observed singular mitochondrial fragments with a single nucleoid inside and very seldom we observed empty fragments. Reintegration of fragments into the mitochondrial network re-established the tubular state with equidistant nucleoid spacing. The two major morphological states coexisted at intermediate stages. These observations suggest that both mitochondrial network fission and reconnection of the disintegrated network are nucleoid-centric, i.e., fission and new mitochondrial tubule formation are initiated around nucleoids. Analyses of combinations of these morphological icons thus provide a basis for a future mitochondrial morphology diagnostics.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Within the mitochondrial network, mitochondrial DNA (mtDNA) is organized in so-called nucleoids that contain accessory proteins and proteins of mitochondrial replication/transcription machinery (Alán et al., 2010; Bogenhagen, 2012; Cameron et al., 2011; Elachouri et al., 2011; Holt, 2009, 2010; Holt et al., 2007; Kienhöfer et al., 2009; Spelbrink, 2010), hypothetically recruited during replication and transcription

(Bogenhagen et al., 2008; Spelbrink, 2010). Several groups have reported that mtDNA does not mix between individual nucleoids (Gilkerson and Schon, 2008; Gilkerson et al., 2008; Poe et al., 2010), a conclusion that was later interpreted as the presence of only a single copy of mtDNA per nucleoid (Kukat et al., 2011). Other data, however, suggest even six to ten of mtDNA copies per nucleoid (Bogenhagen, 2012; Spelbrink, 2010) and a size range of nucleoids between 50 and 300 nm (Bogenhagen, 2012; Brown et al., 2011; Prachar, 2010). Moreover, nucleoids fit into cristae-free cavities separated by cristae-rich regions of the matrix/intracristal space (Kopek et al., 2012).

No precise data exist regarding nucleoid distribution within the mitochondrial network upon network morphological changes such as fission at the G2 phase of the cell cycle (Taguchi et al., 2007) or under pathological conditions. Because, in addition to ATAD3B (Bogenhagen, 2012), also the pro-fusion protein OPA1 may connect the inner mitochondrial membrane (IMM) to nucleoids (Elachouri et al., 2011), nucleoids may be specifically redistributed upon fission. It is also not known how nucleoids divide upon mtDNA replication or whether their structure changes when

Abbreviations: FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; IMM, inner mitochondrial membrane; mt, mitochondrial; mtDNA, mitochondrial DNA; MTS, mitochondrial matrix targeting sequence; mtSSB, mitochondrial single-stranded DNA binding protein; OMM, inner mitochondrial membrane; OXPHOS, oxidative phosphorylation; SIM, structured illumination microscopy; TFAM, mitochondrial transcription factor-A; TMRE, tetramethylrhodamine, ethyl ester; $\mu\text{m}^2\text{cbv}$, μm^2 of cell excluding nucleus.

* Corresponding author at: Department of Membrane Transport Biophysics, No. 75, Institute of Physiology, Academy of Sciences of the Czech Republic, Videňská 1083, 14220 Prague 4, Czech Republic. Tel.: +420 296442285; fax: +420 296442488. E-mail address: jezek@biomed.cas.cz (P. Ježek).

1357-2725/\$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
<http://dx.doi.org/10.1016/j.biocyte.2012.11.019>

mtDNA transcription is initiated. Nevertheless, it is reasonable to assume that, during periods of increased mitochondrial biogenesis, mtDNA replication increases as does the number of nucleoids.

Studies of nucleoids are complicated because mitochondria are organized into a highly connected reticular network that undergoes constant fission and fusion cycles (Chan, 2012; Chen and Chan, 2010; Ježek and Pleštilá-Hlavatá, 2009; Lackner and Nunnari, 2009; Suen et al., 2008; Twig et al., 2010; Zorzano et al., 2010). In intact cells with predominant oxidative phosphorylation, the dynamics of fission and fusion within each cell is so that essentially all mitochondria are connected in a continuous network, forming a nearly single mitochondrion within the cell (Pleštilá-Hlavatá et al., 2008). Various pathological states (e.g., Dlasková et al., 2010) or the action of numerous agents lead, however, to prevailing fission and the fragmented mitochondrial reticulum predominates. Consequently, an individual cell appears to contain a relatively larger number of what we have traditionally called “mitochondria”. Recently, our studies using high-resolution 3D microscopy have shown that small reticula plus solitary small objects (mostly spheres and short tubular segments) may coexist in such a fragmented mitochondrial reticulum (Pleštilá-Hlavatá et al., 2008; Dlasková et al., 2010). A specific morphology has been observed in cells in which mitochondrial fission was induced by rotenone, when only short segments and small spheres of diameter equal to the original tubule diameter coexist. Similar morphology was observed with FCCP, where also small rings (toroids) were observed (De Vos et al., 2005; Pleštilá-Hlavatá et al., 2008). Pancreatic β -cells of isolated Langerhans islets from diabetic Goto Kakizaki rats frequently contain a fragmented mitochondrial reticulum, perhaps as a result of impaired mitochondria-specific autophagy, termed mitophagy (Dlasková et al., 2010). The morphology of these fragmented reticula ranges from that of the rotenone-induced pattern to the coexistence of several reticula and small fragmented mitochondrial objects. These small mitochondrial structures, when separated from the main reticulum, may be more susceptible to mitophagy (Gomes and Scorrano, 2011; Gomes et al., 2011; Twig et al., 2008). We therefore studied nucleoid redistribution upon fission and following reintegration of the mitochondrial network.

2. Materials and methods

2.1. Fluorescent protein constructs and fusion protein expression

The vector for the mitochondrion-matrix-targeting peptide sequence (MTS) conjugated with redox-sensitive GFP (mtRoGFP, a modified EGFP vector of “type 1” containing the MTS of the pyruvate dehydrogenase E1 α subunit) was a kind gift of Dr. Rossignol, Univ. Bordeaux 2, France (Rossignol et al., 2004). The mtRoGFP redox-sensing property was not utilized. The mtRoGFP sequence was inserted into the Viral Power system vector, pLenti 6.3/V5-DEST (Invitrogen), and ligated by T4 DNA ligase (Fermentas).

The MTS conjugate of fluorescent protein Keima (mtKeima) was prepared using a pRSETB vector containing a tandem dimer Keima sequence (a kind gift from A. Miyawaki, see Kogure et al., 2008). The monomeric Keima open reading frame was inserted into pLenti 6.3/V5-DEST using *SaI* and *MluI* restriction enzymes and T4 ligase. The MTS of cytochrome c oxidase subunit 8, COX8A, was obtained from pcDNA3-mito-EosFPwt (MoBiTec, Germany), amplified using PCR with *EcoRV* restriction site-containing primers, and inserted into the intermediate using T4 ligase so that the ATTATT sequence of the vector was removed and the MTS was fused to the amino-terminal end of the monomeric Keima.

2.2. Lentiviral particle production

The pLenti 6.3/V5-DEST-based constructs were overproduced, purified, and used for lentiviral particle production according to the manufacturer (Invitrogen). Thus, the lentiviral stock (containing the packaged pLenti expression construct) was produced by co-transfecting the optimized packaging plasmid mix (packaging plasmids pLP1, pLP2, and pLP/VSVG, which supplies the helper functions as well as structural and replication proteins required to produce the lentiviruses) and our expression constructs into the 293LTV cell line (Invitrogen), which stably and constitutively expresses the SV40 large T antigen, facilitating optimum lentivirus production. Lipofectamine 2000 (Invitrogen) was used as the transfection reagent. The lentiviral stock was filtered and concentrated by PEG-it Virus Precipitation Solution (System Biosciences).

2.3. Cell cultures, transfections, and immunocytochemistry

The human hepatocellular carcinoma HEPG2 cell line (ECACC 85011430) was cultivated at 37°C in humidified air with 5% CO₂ in DMEM medium (Invitrogen; contains no glucose) supplemented with 3 mM glutamine, 1 mM sodium pyruvate, 10% (v/v) fetal calf serum (Biochrome), 10 mM HEPES, 100 IU/ml penicillin, and 100 μ g/ml streptomycin. The supplemented carbon source was 25 mM glucose (Glc25 cells), 5 mM glucose (Glc5 cells), or 10 mM galactose (Oxphos cells); for the latter, glucose-free dialyzed fetal bovine serum was used (PAA Laboratories, Pasching, Austria, catalog no. A15-107). For confocal microscopy, HEPG2 cells were cultured for 2 days on glass coverslips coated with poly-L-lysine. Generally, lentiviral-transfected cell lines permanently expressing mtKeima were created using blasticidin selection, whereas a second transfection was performed with baculoviral particles (a kind gift of Dr. Werner Koopman, Radboud University Medical Centre, Nijmegen, Netherlands) coding for EGFP conjugated with mitochondrial transcription factor-A (TFAM), and the double-transfected HEPG2 cells were used to visualize nucleoids within the mitochondrial network. The third transfection was superimposed, when Glc5 cells were incubated with three different pre-designed siRNA against human MFN2 (Entrez gene ID 9927, Stealth siRNA, cat. no. 1299001, Invitrogen), added once for 24 h incubation or twice (0 and 12 h) for 48 and 64 h incubation.

Similarly, the mtRoGFP cell line was established by lentiviral expression. Also, baculoviral particles coding for MTS-(COX8)-conjugated EGFP (mtEGFP) were used to transiently transfect cells. Cells transfected with mtRoGFP or mtEGFP were used for immunocytochemistry. Baculoviral particles were prepared from a modified *Baculovirus* vector (Koopman et al., 2007). The culture medium contained 1.5 mM sodium butyrate for proper expression of EGFP-TFAM. Transfection was performed by incubating cells with baculoviral particles for 20–24 h. Then the cells were washed with phosphate-buffered saline (PBS). Immunostaining with primary anti-mt single-stranded DNA binding protein (mtSSB) or anti-TFAM or alternatively anti-DNA was followed with secondary Alexa-647- or Alexa-568-conjugated antibodies. Samples were fixed for 20 min with warm 2.5% paraformaldehyde and 0.025% glutaraldehyde in PBS, washed three times with washing buffer containing 0.1% (w/v) Tween 20 and 0.3 M glycine. Then samples were permeabilized for 10 min with PBS+0.1% (w/v) Triton X-100 and washed. Coverslips were blocked with 5% donkey serum for 45 min and incubated for 2 h with the appropriate primary antibody. Primary calf smooth muscle cells and fibroblasts were obtained from Prof. Kurt Stenmark (Univ. Colorado, Denver, CO, USA) and were cultivated in DMEM supplemented with 1% (v/v) non-essential amino acids, 2% (v/v)

L-glutamine, 10% (v/v) bovine calf serum and 1% (v/v) streptomycin/penicillin. Their lentiviral or baculoviral transfection was similar as above.

2.4. Confocal microscopy

An inverted confocal fluorescent Leica TCS SP2 AOBS microscope was used with a PL APO 100 $\times$ /1.40 – 0.70 oil immersion objective (a pinhole of 1 Airy unit). SYTO 16, RoGFP, EGFP, and tdKEIMA were excited at 488 nm with a 20 mW argon laser, whereas tetramethylrhodamine, ethyl ester (TMRE), and Alexa-568-conjugated antibodies were excited at 543 nm with a 1.2-mW HeNe laser. Alexa-647-conjugated antibodies were excited at 633 nm by a 10 mW HeNe laser.

2.5. Structured illumination microscopy (SIM)

Custom imaging at the Department of Cell Biology, School of Medicine, Yale University, New Haven, CT, USA, was done by a structured illumination microscope OMX (Applied Precision, Issaquah, WA) with an Olympus 60 $\times$, 1.42NA, Plan Apo N oil immersion objective and a CoolSNAP HQ2 camera (Photometrics, Tucson, AZ) and used a 488 nm laser for excitation. Series of 20–50 z stacks were taken with a step size of 125 nm, hence the total of z depths was 2.5–6.75 μ m.

2.6. Image analysis

The total number of nucleoids was counted as follows: after basic contrast and brightness adjustment, images or their overlays were manually thresholded, and nucleoids were counted by the “Analyze Particles” function of Image J software (National Institutes of Health, USA). Cell size and tubule/object length were measured after choosing the appropriate scale using the “Measure” function of the software.

2.7. mtDNA copy number

The number of mtDNA copies per cell was derived from qPCR as described by Alán et al. (2011).

2.8. Cellular ATP content

The ATP content during the agent treatments was assayed using the Luciferase ATP assay kit (Roche).

3. Results

3.1. Mitochondrial nucleoids in glycolytic vs. Oxphos cells

To estimate the spatial distribution of mtDNA nucleoids within the mitochondrial (mt) network, we first visualized mtDNA nucleoids using SYTO16 DNA stain, whereas the mt network reticulum was visualized by the IMM potential ($\Delta\Psi_m$) fluorescent indicator TMRE (Fig. 1). HEPG2 cells were first cultivated with 25 mM glucose (Glc25 cells), hence exhibiting a partial glycolytic (Warburg) phenotype where a substantial portion of ATP is formed by glycolysis disconnected from oxidative phosphorylation (Oxphos) (Rossignol et al., 2004). In the selected confocal plane sections, Glc25 cells exhibited a substantial number of mtDNA nucleoids, as spheres along the entire tubule length and apparently filling nearly the whole diameter of mitochondrial tubules (Fig. 1A and B). To simulate the second metabolic mode, HEPG2 cells were cultivated with 5 mM glucose (Glc5 cells). Now, mtRoGFP was used to visualize the mt network,

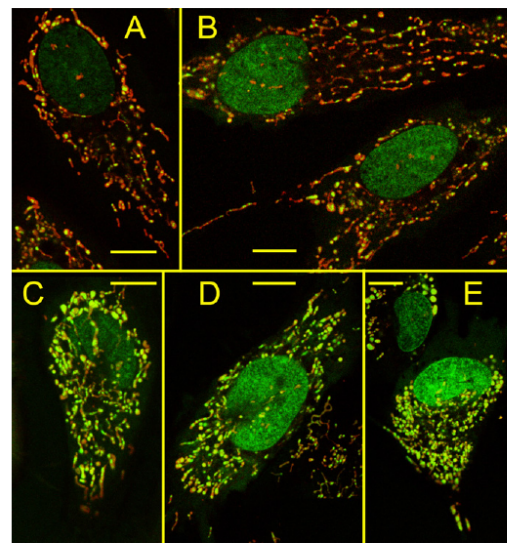


Fig. 1. Dye-stained mtDNA nucleoids and mitochondrial network in live cells. Confocal overlay images are shown for (A and B) intact HEPG2 cells cultivated at 25 mM glucose (Glc25 cells); (C–E) Glc25 cells treated with 1.5 μ M rotenone for 24 h (ATP dropped by 4% only). On average $1.02 \pm$ nucleoids were visualized using SYTO16 stain (green), whereas the mitochondrial network is partly visualized by the $\Delta\Psi_m$ -fluorescent indicator TMRE (red, fading out at reduced $\Delta\Psi_m$ owing to rotenone treatment). On average 1.02 ± 0.17 and 1.02 ± 0.08 nucleoids per μ m of tubule (0.2 and 0.27 per μ m² of cell excluding nucleus, i.e., μ m²_{cyt}) were counted in panels A and B, respectively. Scale bars: 10 μ m.

whereas nucleoids were immunostained for mtSSB along with an Alexa-568-conjugated secondary antibody (Fig. 2A). Unlike with TMRE, a perfect contour of the mt network was obtained with fluorescent proteins such as mtRoGFP (Figs. 2, 3 and 9), mtEGFP (Fig. 4), or mtKEIMA (Figs. 5, 6 and 10). To establish Oxphos cells, HEPG2 cells were cultivated with a non-fermentable sugar (galactose) and relied on glutaminolysis (Smolková et al., 2011). Their basic nucleoid distribution within the mt network (Figs. 3Ab, Ac; 4A, B and 5A, B) did not substantially differ from that of glycolytic cells (Figs. 1A, B; 6A; 9A) (Table 1) or primary cells (see below).

Oxphos cells exhibited similar numbers of nucleoids in selected continuous tubule segments (1.08 ± 0.18 nucleoids per μ m of mt tubules), whereas Glc5 cells had slightly more nucleoids (1.21 ± 0.23 nucleoids per μ m of mt tubules). Glc25 cells exhibited 1.05 ± 0.08 nucleoids per μ m of mt tubules. Altogether, independent of the metabolic mode, HEPG2 cells exhibited 1.10 ± 0.19 nucleoids per μ m of mt tubules in 95 estimates from confocal microscopy or 1.1 ± 0.5 nucleoids per μ m of mt tubules in 25 estimates from 3D structured illumination microscopy (SIM). The above pattern complied with 0.41 ± 0.07 nucleoids per μ m² (excluding the area of the nucleus, 10 images) found in Oxphos cells and 0.43 ± 0.18 (20 images) and 0.36 ± 0.05 (20 images) in Glc5 and Glc25 cells, respectively; and with nearly equal mtDNA copy numbers in Oxphos, Glc5, and Glc25 cells (Table 1). Nucleoid numbers were principally equal when visualized immunocytochemically or by EGFP-TFAM overexpression (altogether 1.11 ± 0.15 vs. 1.12 ± 0.17 nucleoids per μ m of mt tubules, respectively; numbers sorted by metabolic modes see Table 1), indicating that the latter does not trigger a substantial

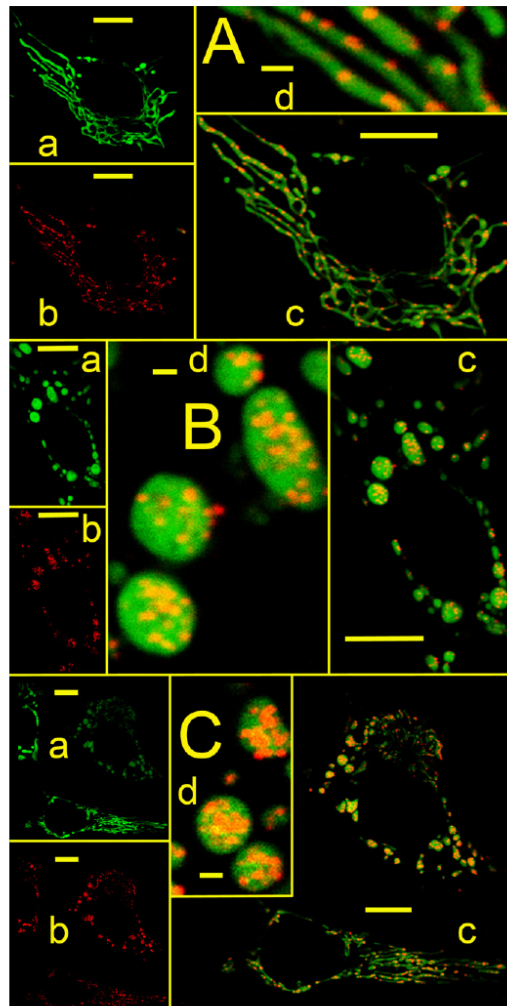


Fig. 2. Rotenone-induced fission and redistribution of nucleoids immunostained by mtSSB in mtRoGFP-visualized mitochondrial network. In panels A–C, four subdivisions (a–d) illustrate confocal images of the green (mtRoGFP) channel (a), red (mtSSB/Alexa-568) channel (b), and their overlays (c), enlargements of overlays are also shown (d). Images show HEPG2 cells cultivated with 5 mM glucose (Glc5 cells) transfected with mtRoGFP and immunostained with mtSSB/Alexa-568 and demonstrate rotenone-induced fission and its recovery for (A) control, no treatment (1.13 ± 0.15 nucleoids per μm of tubule; 0.36 per $\mu\text{m}^2_{\text{CN}}$); (B) 20-min incubation with $10 \mu\text{M}$ rotenone ($4\text{--}22$ nucleoids per fragment; 0.37 per $\mu\text{m}^2_{\text{CN}}$); (C) 60-min incubation with $10 \mu\text{M}$ rotenone (ATP dropped down to 40%; $2\text{--}16$ nucleoids per fragment; 0.38 per $\mu\text{m}^2_{\text{CN}}$). Note the spontaneous recovery of the second cell, exhibiting 1.05 ± 0.18 nucleoids per μm of tubule (0.3 per $\mu\text{m}^2_{\text{CN}}$). Scale bars: $10 \mu\text{m}$ in a–c; $1 \mu\text{m}$ in d.

increase in nucleoids. Volume density of nucleoids in HEPG2 cells corresponded to 0.15 ± 0.1 per μm^3 of cell as derived from SIM. Primary calf smooth muscle cells contained 1.12 ± 0.22 nucleoids per μm of mt tubules (0.25 ± 0.07 nucleoids per μm^2 excluding nuclei, 8 images) and calf fibroblasts 1.16 ± 0.24 nucleoids per μm

Table 1
Viability, mtDNA copy number and number of nucleoids in glycolytic and OxPhos HEPG2 cells.

	OxPhos cells	Glc5 cells	Glc25 cells
Viability			
Untreated	$98\% \pm 0.6\%$	$97\% \pm 0.5\%$	$97\% \pm 1\%$
Rotenone	$96\% \pm 0.8\%$	$88\% \pm 0.8\%$	$97\% \pm 0.8\%$
mtDNA copy No.			
Untreated	870 ± 100	920 ± 240	920 ± 270
FCCP	980 ± 120	1080 ± 70	990 ± 120
Rotenone	850 ± 50	870 ± 200	790 ± 130
Valinomycin	n.d.	980 ± 100	1050 ± 200
No. of nucleoids per μm of tubule			
Fluorescence	1.12 ± 0.18	1.19 ± 0.23	1.05 ± 0.09
Immunocytochemistry	1.13 ± 0.16	1.16 ± 0.11	1.09 ± 0.13
No. of nucleoids per μm^2 (excl. area of the nucleus)			
Fluorescence	0.39 ± 0.18	0.39 ± 0.16	0.36 ± 0.04
Immunocytochemistry	0.33 ± 0.07	0.33 ± 0.05	0.34 ± 0.09

Data $\pm$ SDs are listed for intact ("Untreated") HEPG2 cells cultivated with galactose/glutamine (OxPhos cells) or at 5 mM glucose (Glc5 cells) or 25 mM glucose (Glc25 cells). Viability in quadruplicates was assayed by trypan blue in the absence and presence of 35 nM, 100 nM and 1.5 μM rotenone for OxPhos, Glc5, and Glc25 cells, respectively, after 48 h of incubation. The copy number of mtDNA per cell was assayed for untreated cells (10–16 estimates) and 4–7 estimates of copy number for each treatment as follows: for 0.5 μM FCCP or 0.05 μM valinomycin after 48 h of incubation; or for rotenone assays distinguished by concentration for metabolic modes (as for the viability). The nucleoid numbers (from three to nine cells) were counted for EGFP-TFAM co-expression with mtKEIMA ("Fluorescence") or in mtSSB- or antiDNA-immunostained mtRoGFP-transfected cells ("Immunocytochemistry").

of mt tubules (0.24 ± 0.04 nucleoids per μm^2 excluding nuclei, 10 images).

3.2. Mitochondrial nucleoids after fission of the mitochondrial network induced by an uncoupler and after subsequent recovery

Agents that affect bioenergetics and namely respiration instantly induce fission of the mitochondrial network (De Vos et al., 2005). For example, an uncoupler, such as FCCP, at optimized dose (6 μM for OxPhos cells) ensures maximum respiration but ultimately disrupts mitochondrial ATP synthesis – cells survive only because of ATP formed in glycolysis (Rossignol et al., 2004). Nucleoids were tracked after FCCP-induced fission of both glycolytic and OxPhos cells, and images of partial and complete fission of the mtRoGFP-visualized mt network were analyzed while cells, fixed at particular times, were immuno-stained with anti-mtSSB/Alexa-568-secondary antibody (Fig. 3) or a primary antibody against DNA (in mtEGFP-transfected cells, Fig. 4). All FCCP-treated OxPhos cells had a completely disintegrated mt reticulum network and almost no tubular structures, even during the first 20 min of incubation with 1 μM FCCP. Fig. 4C shows this situation after 50 min of incubation of OxPhos cells, when ATP content dropped down only to 80%. Lower FCCP doses (and situation in Glc5 cells) led to fission at later times, but most cells exhibited (retained) this morphology after 48 h (though multidrug resistance pumps might diminish its concentration), and nucleoids filled each of the small mitochondrial fragments in these cases (Fig. 3B, C and E).

A certain fraction of cells with a completely fragmented mt reticulum, however, exhibited larger fission objects in which nucleoids clustered in groups (Figs. 3C, E and 4C, E). On average, we found 3.5 ± 1.0 nucleoids per 1 μm -diameter circular mt fragment (mean of two dimensions of objects; Fig. 7B). Using this counting method a 2 μm circle contained 7 ± 2 nucleoids. The method is justified by a narrow aspect ratio (1.2 ± 0.2 ; independent of treatment) of fragmented mitochondrial objects, which ranged mostly up to 1.67 (5% over 1.67; 13% of 1.4 to 1.67; 18% of 1.25 to 1.4; 35% of 1.1 to 1.25; and 29% 1 to 1.1). However, cells recovered from fission

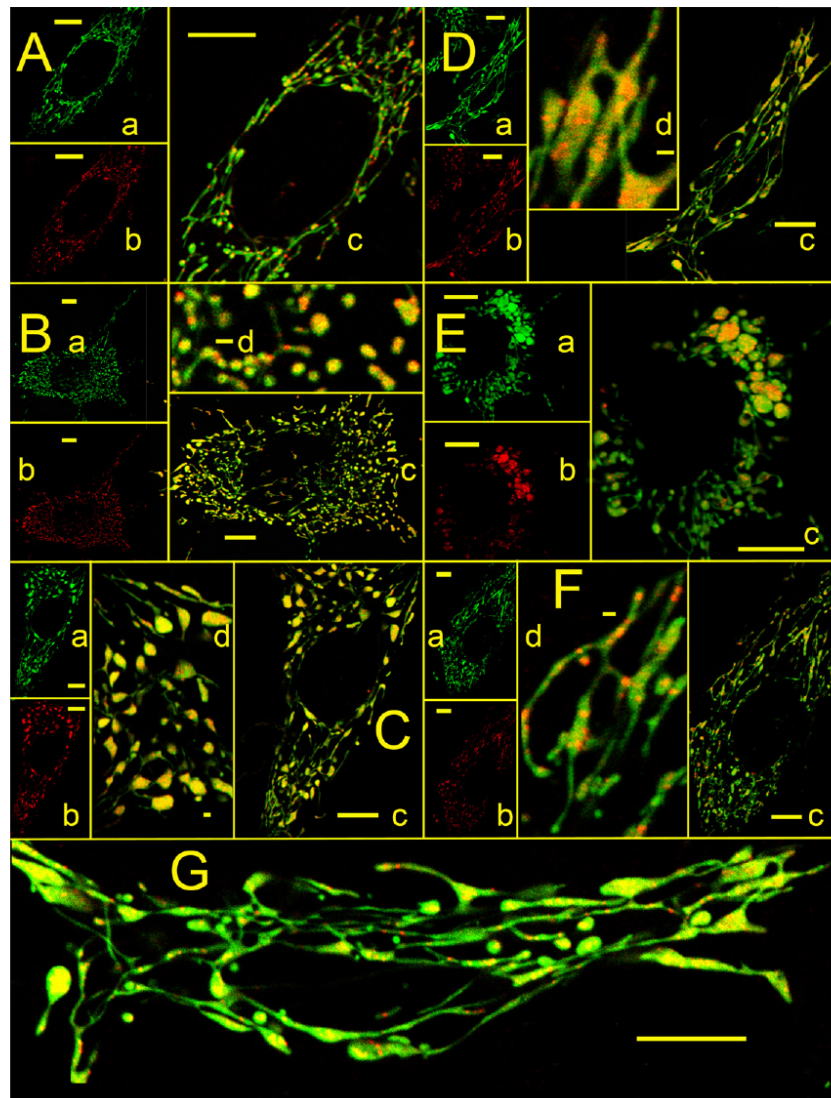


Fig. 3. FCCP-induced fission and redistribution of nucleoids immunostained by mtSSB in mtRoGFP-visualized mitochondrial network. In panels A–F, three or four subdivisions (a–d) illustrate confocal images of the green (mtRoGFP) channel (a), red (mtSSB/Alexa-568) channel (b), and their overlays (c), in some cases, enlargements of overlays are displayed (d). Images show OxiPHos cells (A) transfected with mtRoGFP and immunostained with mtSSB/Alexa-568 (1.17 ± 0.08 nucleoids per μm of tubule; 0.37 per $\mu\text{m}^2_{\text{CEN}}$) and demonstrate FCCP-induced fission and its recovery after FCCP withdrawal, as follows: (B) a 48-h incubation with $0.1 \mu\text{M}$ FCCP (1–6 nucleoids per fragment; 0.22 per $\mu\text{m}^2_{\text{CEN}}$); (C) 48-h incubation with $0.5 \mu\text{M}$ FCCP (6–10 nucleoids per fragment); (D, G) 48-h incubation with $0.5 \mu\text{M}$ FCCP, after which FCCP was washed out and cells fixed 2 h later; (E, F) 48-h incubation with $1 \mu\text{M}$ FCCP. Nucleoids per μm of tubule, per fragment and per $\mu\text{m}^2_{\text{CEN}}$, respectively: (D–G) 1.14 ± 0.27 ; 2–6; 0.25 ; (E) N/A; 5–19; 0.38 ; (F) 1.28 ± 0.17 ; 1–6; 0.36 . Scale bars: $10 \mu\text{m}$ in a–c, $1 \mu\text{m}$ in d.

within 2 h after FCCP removal (ATP content returned back to 100%) and exhibited newly re-fused mitoreticula, yet nucleoid clusters were still present in bulkier portions of the mt reticulum (Fig. 3D and G). Fig. 4D illustrates a 24-h lasting recovery after incubation with FCCP for 50 min. A similar recovery after FCCP removal is

illustrated in Fig. 3F. The same pattern (not shown) was observed with EGFP-TFAM expression in the Glc5 cell line stably transfected with mtKEIMA. No excessive reduction in the number of nucleoids or mtDNA copy number was observed after treatment with FCCP, rotenone, or valinomycin (Table 1, see below).

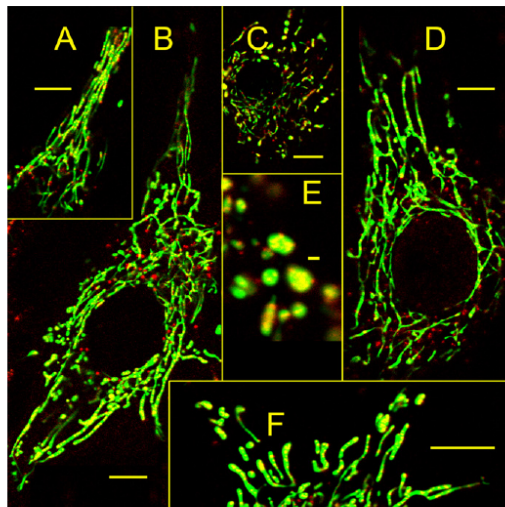


Fig. 4. FCCP-induced fission and redistribution of nucleoids immunostained for DNA in the mtEGFP-visualized mitochondrial network. Confocal colocalization (yellow) images show OXPHOS cells (A) for detail and (B) for the whole cell, transfected with mtEGFP and immunostained with anti-DNA primary and Alexa-568-conjugated secondary antibodies (red channel) and demonstrate FCCP-induced fission by 50-min incubation with 1 μ M FCCP (C); (E) for detail; and its recovery by washing out FCCP and fixation 2 h later (D); (F) for detail. Nucleoids per μ m of tubule, per fragment and per μ m²_{CV}, respectively: (A) 1.16 ± 0.19 ; N/A; 0.27; (B) 1.07 ± 0.1 ; N/A; 0.2; (C) N/A; 4 to 6; 0.21; (D) 1.04 ± 0.04 ; N/A; 0.26. Scale bars: 10 μ m except of E, where scale is 1 μ m.

3.3. Mitochondrial nucleoids after fission of the mitochondrial network induced by rotenone

Cultivation with rotenone also led to immediate fission of the mt network (Figs. 2B; 5C, D, E and 6D), which persisted up to 48 h in Glc5 cells with 100 nM rotenone (Fig. 6D), but OXPHOS cells died at concentrations higher than 50 nM up to 48 h, nevertheless at 35 nM were viable (Table 1). More intensive rotenone treatment led again to bulky fission objects of the originally interconnected network as visualized by EGFP-TFAM co-expression with mtKEIMA in OXPHOS cells (Fig. 5C, D, E) and in mtSSB-immunostained Glc5 cells (Figs. 2B and 7B). Like with FCCP treatment, these large fission objects also contained clusters of 4.1 ± 1.0 nucleoids per 1 μ m-diameter circularized mt fragment (Fig. 7B). The cells individually responded to the oxidative stress combined with respiration inhibition, which is exerted by rotenone (Dlasková et al., 2008). Interestingly, resistant cells were observed proximal to those containing clustered nucleoids (Figs. 2Ca, Cb, Cc and 5D). The clusters were not apparent when stained by SYTO16 because individual nucleoids were smoothed into one spot (Fig. 1C, D, E). SIM, a high-resolution technique, was able to resolve all individual nucleoids in clusters of rotenone-disbursed spherical fragments (Fig. 8).

The Glc5 cell ATP content dropped down to 10% after 20 min with 1 μ M rotenone and stayed low. With 100 nM rotenone Glc5 cells were still viable after 48 h (Table 1), while ATP dropped down to 4%. Glc25 cells withstand with 1.5 μ M rotenone for 48 h. Since the amount of control growing Glc5 cells tripled after 48 h incubations in contrast to almost no cell growth with rotenone, one could consider that upon recovery by washing, the effect of rotenone is reversible on the initial cell population.

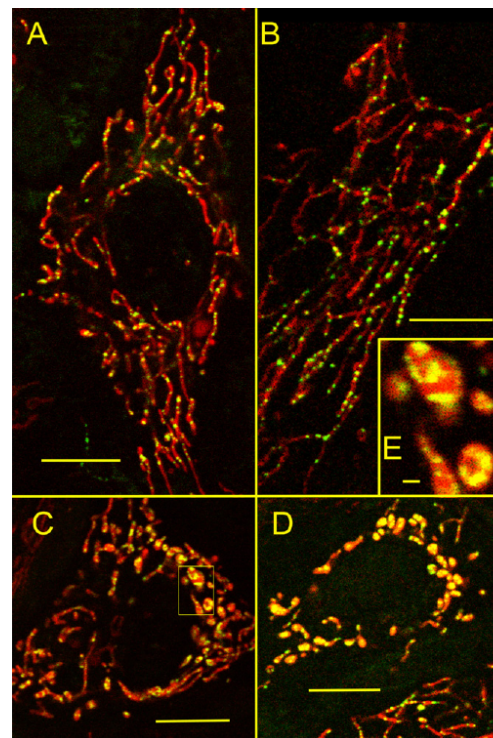


Fig. 5. Rotenone-induced fission and redistribution of TFAM-EGFP-visualized nucleoids within mtKEIMA-visualized mitochondrial network in live cells. Rotenone-induced fission of mitochondrial network is shown in OXPHOS cells and its recovery. The nucleoids in living cells were visualized by baculoviral TFAM-EGFP (originally green, but yellow in perfect colocalization) in OXPHOS cell line stably transfected with mtKEIMA (red). (A) Intact cell (1.15 ± 0.18 nucleoids per μ m of tubule); (B) detail of another intact cell (1.07 ± 0.14 nucleoids per μ m of tubule; 0.25 per μ m²_{CV}). (C and D) 4-h incubation with 1 μ M rotenone (ATP content dropped down to 8%; 3–5 and 2–7 nucleoids per fragment; 0.5 and 0.4 per μ m²_{CV} respectively). (E) Detail of boxed region in C. Scale bars: 10 μ m in A–D; 1 μ m in E.

3.4. Mitochondrial nucleoids after fission of the mitochondrial network induced by valinomycin or nigericin

We further tested a K⁺ uniporter, valinomycin, which cancels the electrical component ($\Delta\Psi_m$) of the protonmotive force while preserving the Δ pH component. Valinomycin caused fragmentation of the mt network (as seen in Glc25 cells, Fig. 9A), yielding large fragmented objects (Fig. 9B, C) that coexisted in some cells with small fission objects. On average, 2.7 ± 0.6 nucleoids per 1 μ m-diameter circularized mt fragment were found in sections of these fragmented mt objects (Fig. 7B). In the case of the K⁺/H⁺ antiporter, nigericin, no substantial disintegration of the mt network occurred (Fig. 6B), showing the absence of fission stimulus under conditions when $\Delta\Psi_m$ is maintained while the Δ pH component is canceled. This may suggest that the valinomycin-induced fission of mt network is mediated by $\Delta\Psi_m$ disruption. However, such interpretation must be taken with caution, because rotenone-induced fission was potentiated by nigericin (Fig. 6C, E, F).

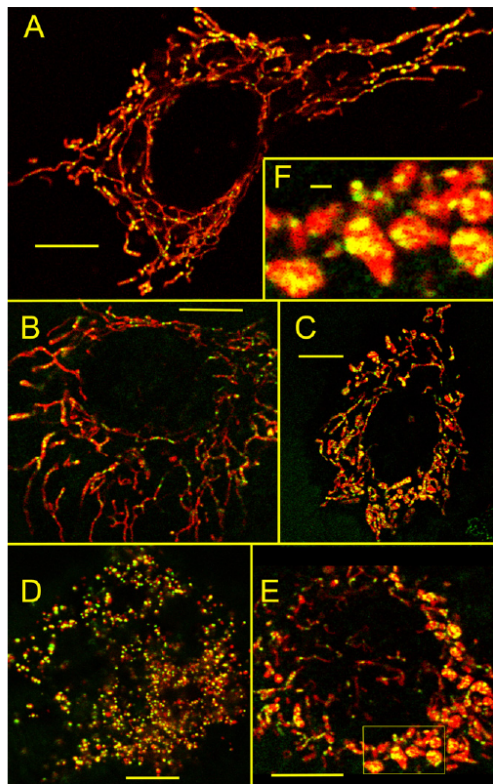


Fig. 6. Rotenone-induced fission and redistribution of TFAM-EGFP-visualized nucleoids within the mtKEIMA-visualized mitochondrial network in live cells. Rotenone-induced fission of mitochondrial network is shown in glycolytic Glc5 cells and its recovery. The nucleoids were visualized by baculoviral TFAM-EGFP (originally green, but yellow in perfect colocalization) in Glc5 cells stably transfected with mtKEIMA (red). (A) Intact cell; (B) 24-h incubation with 1 μ M nigericin; (C) 4 h incubation with 0.5 μ M rotenone plus 1 μ M nigericin; (D) 24 h incubation with 0.1 μ M rotenone; (E) 24-h incubation with 0.1 μ M rotenone plus 1 μ M nigericin. (F) Detail of boxed region in E. Nucleoids per μ m of tubule, per fragment and per μ m² cell, respectively: (A) 1.09 ± 0.10 ; N/A; 0.5; (B) 1.24 ± 0.21 ; N/A; 0.3; (C) 1.24 ± 0.22 ; 5–7; 0.34; (D) N/A; 1; 0.36; (E) N/A; 4–10; 0.3. Scale bars: 10 μ m in A–E; 1 μ m in F.

3.5. Mitochondrial nucleoids after fission resulting from silencing of mitofusin MFN2

Glc5 cells silenced for mitofusin MFN2 also exhibited nucleoid clustering in disintegrated mt network into spheroids visualized either by TMRE or mtKeima while baculoviral EGFP-TFAM visualized nucleoids (Fig. 10). MFN2 silencing usually yielded mixed morphology of tubules and spheroids, but prolonged incubations with three corresponding siRNAs up to 48 h resulted in the complete mt network disintegration into spheroids (Fig. 10D).

4. Discussion

In this work we demonstrate the existence of two principal morphology states of the mitochondrial network in relation to nucleoids. The term “state” is meant as the most probable snapshot or integrated dynamics of the network over a sufficient time. The first state, a basic morphology state of mitochondrion undergoing

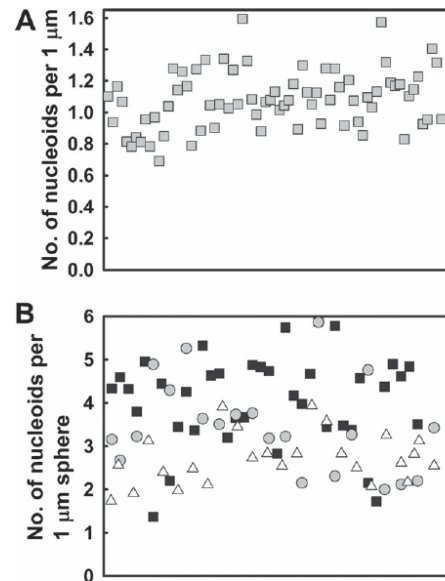


Fig. 7. Number of nucleoids per tubule length (A) and per 1 μ m diameter of circularized ovals of disintegrated objects (B). (A) Distribution of 73 estimates for number of nucleoids per 1 μ m in OXPHOS cells. (B) Distribution of estimates for number of nucleoids (N) per 1 μ m diameter of idealized circular objects (averages from both dimensions of ellipses or ovals, d_{avg} , were taken into account to calculate d_{avg}/N) after mitochondrial network disintegration by FCCP (gray circles), rotenone (black squares), or valinomycin (white triangles).

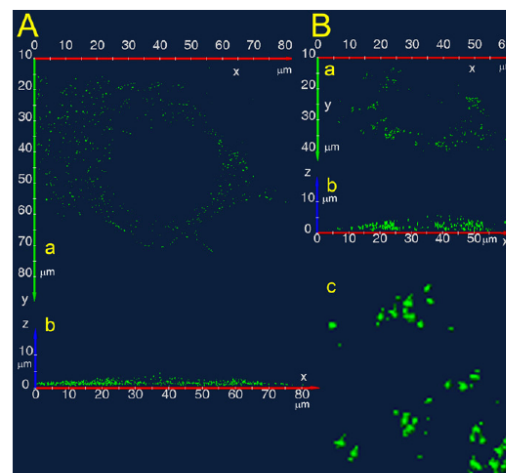


Fig. 8. Rotenone-induced fission and redistribution of mtSSB-immunostained nucleoids as observed by 3D SIM. Nucleoids without the network are visualized by 3D SIM after Alexa-488/mtSSB immunostaining. (A) An intact OXPHOS cell (774 nucleoids counted, an average apparent nucleoid diameter 374 nm). (B) Rotenone-induced fission of the mitochondrial network in an OXPHOS cell led to clustering of individual nucleoids within the invisible spheroid fragments (into which the network is supposed to be transformed) after a 12-h incubation with 80 nM rotenone. Series of z stacks are shown in xy (a) and xz projections (b). (C) Shows detail of nucleoid clusters. Volume density of nucleoids corresponded to 0.15 ± 0.1 per μ m³ of cell.

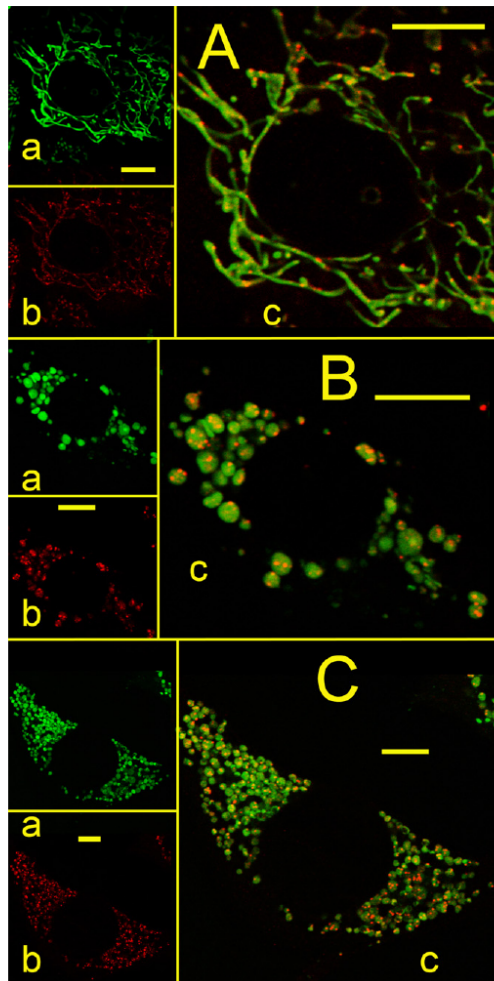


Fig. 9. Valinomycin-induced fission and redistribution of nucleoids immunostained by mtSSB in the mtRoGFP-visualized mitochondrial network. In panels A–C, three subdivisions (a,b,c) illustrate confocal images of the green (mtRoGFP) channel (a), red (mtSSB/Alexa-568) channel (b), and their overlays (c). Images show Guc25 cells transfected with mtRoGFP and immunostained with mtSSB/Alexa-568 and demonstrate valinomycin-induced fission: (A) control, no treatment (1.05 ± 0.14 nucleoids per μm of tubule; 0.39 per $\mu\text{m}^2_{\text{CEN}}$); (B) 48-h incubation with $0.05 \mu\text{M}$ valinomycin ($3\text{--}7$ nucleoids per fragment; 0.34 per $\mu\text{m}^2_{\text{CEN}}$); (C) 48-h incubation with $0.1 \mu\text{M}$ valinomycin (ATP dropped down to 50%; $2\text{--}5$ nucleoids per fragment; 0.38 per $\mu\text{m}^2_{\text{CEN}}$). Scale bars: $10 \mu\text{m}$.

OxPhos, is characterized by a network comprised of highly interconnected tubules of almost equivalent diameter that contain evenly spaced nucleoids. In turn, the second principal state represents a nearly completely fragmented mt reticulum, i.e., solitary objects (~ 1 to $3 \mu\text{m}$, spherical or ellipsoidal sections) with predominantly several nucleoids clustered in these small mitoreticulum remnants. The recovery from such a complete fission is reflected by the mixed morphology, when bulky solitary objects coexist with the recovered tubular mt reticulum morphology. Importantly, we

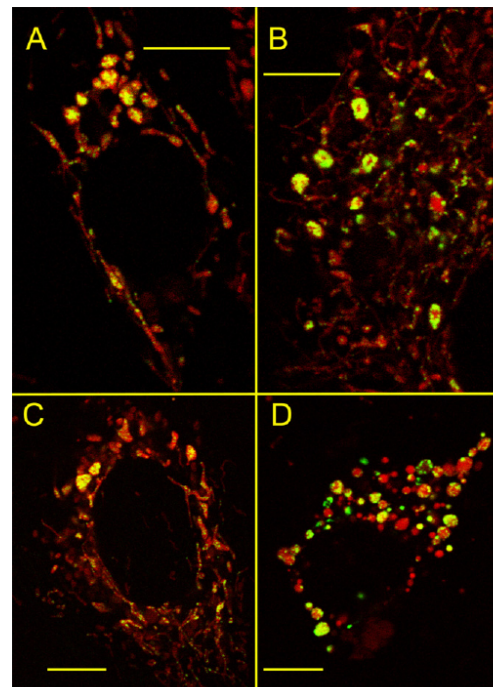


Fig. 10. Clustering of nucleoids upon silencing of mitofusin MFN2. Nucleoids are visualized by baculoviral EGFP-TFAM with mt network visualized by TMRE (panels A and C) or mtKeima (panels B and D) in Guc5 cells incubated with three siRNAs selected for mitofusin MFN2 silencing for 64 h (A), 48 h (C and D) and 24 h (B). Nucleoids per fragment and per $\mu\text{m}^2_{\text{CEN}}$, respectively: (A) $4\text{--}10$; 0.23 ; (B) 4 to 9 ; 0.15 ; (C) $3\text{--}13$; 0.4 ; (D) $4\text{--}11$; 0.27 . Scale bars: $10 \mu\text{m}$.

only occasionally observed individual mt fragments with a single nucleoid inside and we very seldom observed empty fragments. Consequently, we developed the following new model of four-stage fission/recovery of the mitochondrial reticulum network in relation to nucleoid redistribution (Fig. 11):

Stage 1 – prevalent fission. The fission after treatment with FCCP, rotenone, or valinomycin prevails due to a sudden depletion of matrix and peri-mitochondrial ATP (and hence GTP), in spite of the fact that both pro-fusion mitofusins MFN1 or MFN2 and OPA1, but also pro-fission proteins such as DRP1 require GTP (Chan, 2012). The dysbalance results from OPA1 cleavage (Chan, 2012) and GTP shortage for mitofusins, because DRP1 can still be recruited due to the local cytosolic ATP supplied by glycolysis (distant from the mitochondrion, Ježek and Plečtitá-Hlavatá, 2009). When two potential fission sites on a larger mt tubule become competent to undergo fission, the resultant post-fission small cylinder/tubule subsequently takes the shape of a sphere containing a single nucleoid. If this were to happen in the entire mt network, one would exclusively observe single objects with single nucleoids, but this was only observed as a mix with multi-nucleoid-containing network fragments after treatment with FCCP (Fig. 3B), rotenone (Fig. 6D), or valinomycin (Fig. 9C). Because we rarely observed nucleoid-free fragmented objects, we speculate that when two fission sites occur between the two nucleoids, the excised small nucleoid-free empty cylinder must either immediately fuse with the neighbor nucleoid-containing object or be instantly degraded.

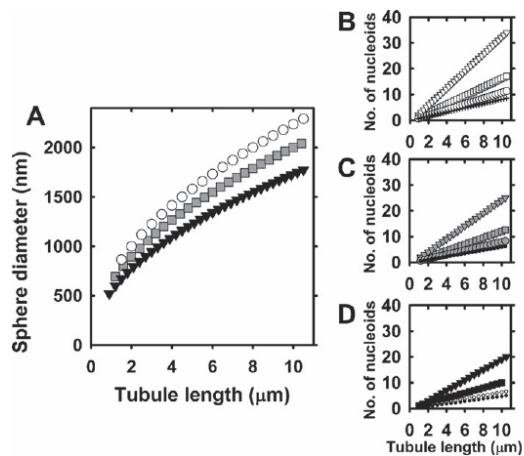


Fig. 11. Simulations of nucleoid redistribution upon fission/reintegration of the mitochondrial network. (A) Simple geometry considerations provide estimates of the size of a sphere, the surface of which is equal to the surface of the cylindrical segment (excluding the top and bottom of the cylinder) for a cylinder (tubule) with a diameter of 300 nm (open circles), 400 nm (gray squares), and 500 nm (black triangles). (B–D) Number of nucleoids contained in a model tubule (cylinder) when the distance between nucleoids is equal to one (triangles), two (squares), three (circles), or four tubule diameters (bottom traces for a tubule diameter of 300 nm (B), 400 nm (C), and 500 nm (D)).

Under conditions of a sudden ATP decline, however, even DRP1 cannot create fission sites between each of the two nucleoids because of ATP shortage. As a result, longer, up to $\sim 10 \mu\text{m}$, tubule fragments containing several nucleoids must be initially formed. A mixture of short fragments has been previously observed for rotenone-induced fission by 4Pi microscopy (Plečić-Hlavatá et al., 2008). Ultimately, these fragments turn into spheres or ellipsoids with clustered nucleoids. The outer mitochondrial membrane (OMM) surface of the final sphere is predicted to be equal to the surface of the original cylinder tubule, but the matrix volume may become greater.

Stage II – swelling of matrix volume in disintegrated objects. We also speculate that IMM swelling and thus cristae disassembly participates in this morphology transfer. Nucleoids are supramolecular objects and since matrix is interrupted by rich cristae, which hinder nucleoid movement (Kopeck et al., 2012), only a gross reshaping of a mt tubule into a sphere, in which cristae are reshaped due to IMM swelling, would allow nucleoid clustering. Mitochondrial matrix swelling has long been known for the case of valinomycin, which allows K^+ influx and hence anion entry into the matrix through various IMM carriers that usually require H^+ symport (Ježek et al., 1990). Protons for this symport are pumped out of the matrix by the respiratory chain, and hence the anion readily enters the matrix accompanying valinomycin-facilitated K^+ entry. As a result, water enters the matrix, causing IMM swelling. Such a swelling might participate in the reshaping of a short tubular segment to the sphere, which traps all the nucleoids of the original segment inside and IMM swelling that causes cristae rearrangement then allows their clusters due to less folded cristae. The situation with FCCP is similar; its protonophoric activity allows the K^+/H^+ antiporter (Ježek et al., 1990) to assure K^+ influx, and anions again enter the matrix via various carriers. We may expect IMM swelling to occur even after rotenone treatment, when more bulky objects with clustered nucleoids were formed, reflecting a slower swelling.

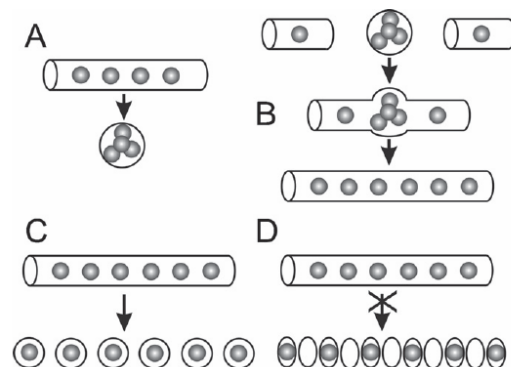


Fig. 12. Schematic of nucleoid redistribution upon fission/reintegration of the mitochondrial network. Allowed (A and C) and not allowed (D) redistributions of mitochondrial nucleoids upon fission of mitochondrial tubules. (B) Reintegration of bulky fragmented objects back into the network. For explanation, see Section 4.

Simple geometry considerations provide an estimate of the size of such a bulky sphere if its surface is equal to the surface of the original cylindrical segments (excluding the top and bottom of a cylinder, Fig. 12A). Assuming a tubule diameter of 300 nm (Plečić-Hlavatá et al., 2008) and an equal 900 nm distance between nucleoids, the $10.2 \mu\text{m}$ tubular segment would provide a sphere with a $1.75 \mu\text{m}$ diameter (Fig. 12A). Both the tubular segment and the sphere would contain 8 nucleoids in this case (Fig. 12B). Similar considerations can be made for a tubule with a diameter of 400 nm (Fig. 12A, C) or 500 nm (Fig. 12A, D). Note that for the experimentally observed distance of $\sim 1 \mu\text{m}$ between nucleoids yields for 500 nm thick tubules, just 10 nucleoids in a $2.29 \mu\text{m}$ sphere. The rotenone-fragmented network for example contained on average 8 nucleoids in a $\sim 2 \mu\text{m}$ circular section of spheroids (Fig. 7B). Hence, our model is in accordance with our experiments, bearing in mind that average numbers of nucleoids were counted for sections and not for the full spheroid volumes. The actual nucleoid number in a sphere might be up to twice as high because a confocal plane thickness at 1 Airy unit was $\sim 1.4 \mu\text{m}$ in our microscope. Therefore, 2D confocal microscopic images are imperfect for exact model fitting because they represent sections of the spheres, and therefore the actual number of nucleoids in our images was underestimated.

Stage III – partial reintegration of disintegrated objects: following the initial decline in ATP concentration after agent addition, ATP may start to increase above that decline during several following hours of incubation owing to glycolysis, which then may allow even spontaneous (and frequently partial) reintegration of fragmented mitoreticulum objects, such as observed for rotenone treatment. Trivial situation occurs after the agent withdrawal, since the glycolytic ATP source is surpassed by the reestablished Oxphos source, such as in the case of rotenone and FCCP withdrawal. The ATP abundance induces both, the IMM shrinkage and hence condensation of matrix space and cristae, and the tendency for fusion. As a result, a bulky sphere will fuse with two neighboring mitochondrial tubules (Fig. 11B) and an intermediate morphology of an inflated tubule reintegrated into the whole mt reticulum is established (Fig. 3D, G). This event may be regarded as “elongation” reported by Gomes and Scorrano (2011) and Gomes et al. (2011).

Stage IV – fusion accompanied by continuing shrinkage of the matrix space: the continuing IMM shrinkage inside the mt tubule acts in synergy with continuing prevailing fusion. This process causes mt tubule elongation (cf. Gomes et al., 2011) and thinning of the

inflated part of the tubule within a network. Consequently, the regular network with a unified diameter is re-established.

Overall, we can describe the entire process (fission and fusion of the mt network) as nucleoid-centric, i.e., determined by the nucleoid positions. Only a gross transition of mt tubule into a sphere, in which cristae are reshaped due to swelling, allows nucleoid clustering. Nucleoid positions within cristae-empty matrix space may also be ensured by the recently revealed attachment to IMM via the pro-fusion OPA1 protein (Elachouri et al., 2011) or ATAD3B (Bogenhagen, 2012). One may speculate that de novo formation of new mitochondrial mass (expansion of existing tubules or solitary objects) is also initiated around the new nucleoid formed by mtDNA replication. In turn, when fragmented objects do not contain nucleoids, their fast degradation may occur. The alternative reason, however, stems from the fact that there is not enough material in the OMM to form a sphere. Thus a 900 nm long tubular segment (300 nm in diameter) can form only a 520-nm sphere. Only a single 300 nm-sized nucleoid could fit into such a single sphere. Regardless of these considerations, a search for empty small mitochondria and the definitive exclusion of their existence requires further effort by super-resolution and electron microscopy.

The outlined mechanism may also act in concert with ongoing mitophagy (Chan and Chan, 2011; Chan et al., 2011; Gomes and Scorrano, 2011; Gomes et al., 2011; Kim and Lemasters, 2011; Springer and Kahle, 2011; Tanaka et al., 2010; Twig et al., 2008; Wang and Klionsky, 2011; Youle and Narendra, 2011). The nucleoid population engulfed during mitophagy, if existed in our experiments, had to be replaced by mtDNA replication in the long-segment tubules of the mitoreticulum. This is because no excessive reduction in the number of nucleoids or mtDNA copy number was observed.

The obtained confocal 2D images represent only a fraction of all mtDNA nucleoids within the cell because their complete number could be determined exclusively by 3D high-resolution (Fig. 8) or super-resolution microscopy. In contrast to 3D 4Pi and 2D STED microscopy, the conventional confocal images of both mitochondrial tubules (Dlasková et al., 2010; Plečtitá-Hlavatá et al., 2008) and nucleoids (Kukat et al., 2011; Prachar, 2010) are optically inflated owing to the diffraction-limited resolution of ~250 nm and hence appear thicker than in reality. Moreover, clustered nucleoids may be mistaken for a single, larger one. Consequently, one may obtain neither precise dimensions of nucleoids nor their number from conventional confocal images (Kukat et al., 2011). The uniform diameter of nucleoids in fibroblasts and other flat cells has been claimed as 100 nm (Kukat et al., 2011). Other reports, however, show a range of nucleoid sizes between 50 nm and 300 nm may exist (Bogenhagen, 2012; Brown et al., 2011; Kopek et al., 2012). Our SIM data (Fig. 8) and preliminary data from 3D super-resolution BiplaneFPALM microscopy confirmed this range (Młodzianoski et al., 2011). Also the precise number of nucleoids should be counted by super-resolution 2D microscopy for flat cells, in which no more than a single layer of mitochondrial tubules exists (Kukat et al., 2011), or by 3D SIM (Fig. 8). Otherwise 3D super-resolution microscopy must be used for normal cells.

Reports exist claiming an increase in size of nucleoids with e.g. ablation of AAA⁺ protease ClpX (Kasashima et al., 2012), changes in TFAM to mtDNA ratio within the nucleoid (Picca et al., 2012), and also changes in mtDNA molecule content in a single nucleoid may vary (specifically upon mtDNA replication). Confocal microscopy is unable to visualize these changes in details, nor size and numbers of nucleoids in the cell. However, it appears that primary cells exhibit nucleoids of similar size range as cultured cancer cells such as HEPG2 used in this study, as exemplified by our imaging of smooth muscle cells and fibroblasts or other cell types (Kukat et al., 2011).

5. Conclusions

The “mitochondrial nucleoid code” (see graphical abstract) predicts that with an average distance between nucleoids of 1 μm , nine nucleoids in a 10 μm mitochondrial tubule segment will turn after fission into a 2 μm spheroid containing those nine nucleoids. If a lower number of nucleoids is observed, degradation of mtDNA must have occurred. The inspection of such a “mitochondrial nucleoid code”, i.e., the pattern of the two basic morphological icons, may provide a basis for novel mitochondrial morphology diagnostics.

Acknowledgement

SIM was performed under the supervision of Dr. Joerg Bewersdorf at the Dept. Cell Biology, School of Medicine, Yale University, New Haven, CT, USA. We gratefully acknowledge the vectors and baculoviral particles supplied by Dr. Werner Koopman, Center for Molecular Life Sciences, Radboud University Nijmegen Medical Center, Nijmegen, Netherlands. The project was supported by grants of the Grant Agency of the Czech Republic (No. 302/10/0346 to PJ; No. P304/10/P204 to AD; No. P305/12/1247 to MJ) and the research projects AV0Z50110509 and RVO67985823.

References

- Alán L, Zelenka J, Ježek J, Dlasková A, Ježek P. Fluorescent in situ hybridization of mitochondrial DNA and RNA. *Acta Biochimica Polonica* 2010;57:403–8.
- Alán L, Špaček T, Zelenka J, Tauber J, Berková Z, Zacharovová K, Saudek F, Ježek P. Assessment of mitochondrial DNA as an indicator of islet quality: an example in Goto Kakizaki Rats. *Transplantation Proceedings* 2011;43:3281–4.
- Bogenhagen DF. Mitochondrial DNA nucleoid structure. *Biochimica et Biophysica Acta* 2012;1819:914–20.
- Bogenhagen DF, Rousseau D, Burke S. The layered structure of human mitochondrial DNA nucleoids. *Journal of Biological Chemistry* 2008;283:3665–75.
- Brown TA, Tkachuk AN, Shtengel G, Kopek BG, Bogenhagen DF, Hess HF, et al. Superresolution fluorescence imaging of mitochondrial nucleoids reveals their spatial range, limits, and membrane interaction. *Molecular and Cellular Biology* 2011;31:4994–5010.
- Cameron JM, Levandovsky V, Mackay N, Ackerley C, Chitayat D, Raiman J, et al. Complex V TMEM70 deficiency results in mitochondrial nucleoid disorganization. *Mitochondrion* 2011;11:191–9.
- Chan DC. Fusion and fission interlinked processes critical for mitochondrial health. *Annual Review of Genetics* 2012.
- Chan NC, Chan DC. Parkin uses the UPS to ship off dysfunctional mitochondria. *Autophagy* 2011;7:771–2.
- Chan NC, Salazar AM, Pham AH, Sweredoski MJ, Kolawa NJ, Graham RL, et al. Broad activation of the ubiquitin-proteasome system by Parkin is critical for mitophagy. *Human Molecular Genetics* 2011;20:1726–37.
- Chen H, Chan DC. Physiological functions of mitochondrial fusion. *Annals of the New York Academy of Sciences* 2010;1201:21–5.
- De Vos KJ, Allan VJ, Grierson AJ, Sheetz MP. Mitochondrial function and actin regulate dynamin-related protein 1-dependent mitochondrial fission. *Current Biology* 2005;15:678–83.
- Dlasková A, Hlavatá L, Ježek P. Mitochondrial complex I superoxide production is attenuated by uncoupling. *International Journal of Biochemistry and Cell Biology* 2008;40:1792–805.
- Dlasková A, Špaček T, Šantorová J, Plečtitá-Hlavatá L, Berková Z, Saudek F, et al. 4Pi microscopy reveals an impaired three-dimensional mitochondrial network of pancreatic islet β -cells in an experimental model of type-2 diabetes. *Biochimica et Biophysica Acta* 2010;1797:1327–41.
- Elachouri G, Vidoni S, Zanna C, Pattyn A, Boukhaddaoui H, Gaget K, et al. OPA1 links human mitochondrial genome maintenance to mtDNA replication and distribution. *Genome Research* 2011;21:12–20.
- Gilkerson RW, Schon EA. Nucleoid autonomy: an underlying mechanism of mitochondrial genetics with therapeutic potential. *Communicative and Integrative Biology* 2008;1:34–6.
- Gilkerson RW, Schon EA, Hernandez E, Davidson MM. Mitochondrial nucleoids maintain genetic autonomy but allow for functional complementation. *Journal of Cell Biology* 2008;181:1117–28.
- Gomes LC, Scorrano L. Mitochondrial elongation during autophagy: a stereotypical response to survive in difficult times. *Autophagy* 2011;7:1251–3.
- Gomes LC, Di Benedetto G, Scorrano L. During autophagy mitochondria elongate, are spared from degradation and sustain cell viability. *Nature Cell Biology* 2011;13:589–98.
- Holt IJ. Mitochondrial DNA replication and repair: all a flap. *Trends in Biochemical Sciences* 2009;34:358–65.
- Holt IJ. Zen and the art of mitochondrial DNA maintenance. *Trends in Genetics* 2010;26:103–9.

- Holt IJ, He J, Mao C-C, Boyd-Kirkup JD, Martinsson P, Sembongi H, et al. Mammalian mitochondrial nucleoids: organizing an independently minded genome. *Mitochondrion* 2007;7:311–21.
- Ježek P, Plečičá-Hlavatá L. Mitochondrial reticulum network dynamics in relation to oxidative stress, redox regulation, and hypoxia. *The International Journal of Biochemistry and Cell Biology* 2009;41:1790–804.
- Ježek P, Mahdi F, Garlid KD. Reconstitution of the beef heart and rat liver mitochondrial K^+ / H^+ (Na^+ / H^+) antiporter. Quantification of K^+ transport with the novel fluorescent probe PBFI. *Journal of Biological Chemistry* 1990;265:10522–6.
- Kasashima K, Sumitani M, Endo H. Maintenance of mitochondrial genome distribution by mitochondrial AAA+ protein ClpX. *Experimental Cell Research* 2012;318:2335–43.
- Kienhöfer J, Häussler DJ, Ruckelshausen F, Muessig E, Weber K, Pimentel D, et al. Association of mitochondrial antioxidant enzymes with mitochondrial DNA as integral nucleoid constituents. *FASEB Journal* 2009;23:2034–44.
- Kim I, Lemasters JJ. Mitochondrial degradation by autophagy (mitophagy) in GFP-LC3 transgenic hepatocytes during nutrient deprivation. *American Journal of Physiology Cell Physiology* 2011;300:C308–17.
- Kogure T, Kawano H, Abe Y, Miyawaki A. Fluorescence imaging using a fluorescent protein with a large Stokes shift. *Methods* 2008;45:223–6.
- Koopman WJH, Hink MA, Verkaart S, Visch HJ, Smeitink JAM, Willems PHGM. Partial complex I inhibition decreases mitochondrial motility and increases matrix protein diffusion as revealed by fluorescence correlation spectroscopy. *Biochimica et Biophysica Acta* 2007;1767:940–7.
- Kopeck BG, Shtengel G, Xu CS, Clayton DA, Hess HF. Correlative 3D superresolution fluorescence and electron microscopy reveal the relationship of mitochondrial nucleoids to membranes. *Proceedings of the National Academy of Sciences of the United States of America* 2012;109:6136–41.
- Kukat C, Wurm CA, Spahr H, Falkenberg M, Larsson N-G, Jacobs S. Super-resolution microscopy reveals that mammalian mitochondrial nucleoids have a uniform size and frequently contain a single copy of mtDNA. *Proceedings of the National Academy of Sciences of the United States of America* 2011;108:13534–9.
- Lackner LL, Nunnari JM. The molecular mechanism and cellular functions of mitochondrial division. *Biochimica et Biophysica Acta* 2009;1792:1138–44.
- Młodzianowski MJ, Schreiner JM, Callahan SP, Smolková K, Dlásková A, Santorová J, Ježek P, Bewersdorf J. Sample drift correction in 3D fluorescence photoactivation localization microscopy. *Optics Express* 2011;19:15009–19.
- Picca A, Fracasso F, Pesce V, Cantore P, Joseph AM, Leeuwenburgh C, et al. Age- and calorie restriction-related changes in rat brain mitochondrial DNA and TFAM binding. *Age (Dordrecht)* 2012(September (4)) (Epub ahead of print).
- Plečičá-Hlavatá L, Lessard M, Santorová J, Bewersdorf J, Ježek P. Mitochondrial oxidative phosphorylation and energetic status are reflected by morphology of mitochondrial network in INS-1E and HEP-G2 cells viewed by 4Pi microscopy. *Biochimica et Biophysica Acta* 2008;1777:834–46.
- Poe 3rd BG, Duffy CF, Greminger MA, Nelson BJ, Arriaga EA. Detection of heteroplasmy in individual mitochondrial particles. *Analytical and Bioanalytical Chemistry* 2010;397:3397–407.
- Prachar J. Mouse and human mitochondrial nucleoid – detailed structure in relation to function. *General Physiology and Biophysics* 2010;29:160–74.
- Rosignol R, Gilkerson R, Aggeler R, Yamagata K, Remington SJ, Capaldi RA. Energy substrate modulates mitochondrial structure and oxidative capacity in cancer cells. *Cancer Research* 2004;64:985–93.
- Smolková K, Plečičá-Hlavatá L, Bellance N, Benard G, Rosignol R, Ježek P. Waves of gene regulation suppress and then restore oxidative phosphorylation in cancer cells. *The International Journal of Biochemistry and Cell Biology* 2011;43, 950–68.
- Spelbrink JN. Functional organization of mammalian mitochondrial DNA in nucleoids: history, recent developments, and future challenges. *IUBMB Life* 2010;62:19–32.
- Springer W, Kahle PJ. Regulation of PINK1-Parkin-mediated mitophagy. *Autophagy* 2011;7:266–78.
- Suen DF, Norris KL, Youle RJ. Mitochondrial dynamics and apoptosis. *Genes and Development* 2008;22:1577–90.
- Tanaka A, Cleland MM, Xu S, Narendra DP, Suen DF, Karbowski M, et al. Proteasome and p97 mediate mitophagy and degradation of mitofusins induced by Parkin. *Journal of Cell Biology* 2010;191:1367–80.
- Taguchi N, Ishihara N, Jofuku A, Oka T, Mihara K. Mitotic phosphorylation of dynamin-related GTPase Drp1 participates in mitochondrial fission. *Journal of Biological Chemistry* 2007;282:11521–9.
- Twig G, Elorza A, Molina AJ, Mohamed H, Wikstrom JD, Walzer G, et al. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. *EMBO Journal* 2008;27:433–46.
- Twig G, Liu X, Liesa M, Wikstrom JD, Molina AJ, Las G, et al. Biophysical properties of mitochondrial fusion events in pancreatic beta-cells and cardiac cells unravel potential control mechanisms of its selectivity. *The American Journal of Physiology* 2010;299:C477–87.
- Wang K, Klionsky DJ. Mitochondria removal by autophagy. *Autophagy* 2011;7:297–300.
- Youle RJ, Narendra DP. Mechanisms of mitophagy. *Nature Reviews Molecular Cell Biology* 2011;12:9–14.
- Zorzano A, Liesa M, Sebastián D, Segalés J, Palacin M. Mitochondrial fusion proteins: dual regulators of morphology and metabolism. *Seminars in Cell and Developmental Biology* 2010;21(August (6)):566–74.