DISSERTATIONES
TECHNOLOGIAE
UNIVERSITATIS
TARTUENSIS
64

KOIT AASUMETS

The dynamics of human mitochondrial nucleoids within the mitochondrial network

DISSERTATIONES TECHNOLOGIAE UNIVERSITATIS TARTUENSIS
64

KOIT AASUMETS

The dynamics of human mitochondrial nucleoids within the mitochondrial network

UNIVERSITY OF TARTU
Press
1632

Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia

This dissertation was accepted for the commencement of the degree of Doctor of Philosophy in biomedical technology on November 22th, 2021 by the Council of the Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia.

Supervisors: Joachim Gerhold, Ph.D,
Head of the Immunological Development Laboratory
Icosagen Cell Factory OÜ, Tartu, Estonia

Hannes Kollist, Ph.D,
Professor of Molecular Plant Biology
Institute of Technology, University of Tartu, Estonia

Reviewer: Allen Kaasik, Ph.D,
Professor of Pharmacology Molecular Toxicology
Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia

Opponent: Luca Scorrano, M.D., Ph.D,
Professor of Biochemistry
Department of Biology, University of Padua, Italy

Commencement: January 25, 2022

Publication of this thesis is granted by the Institute of Technology, University of Tartu

ISSN 2228-0855
ISBN 978-9949-03-791-9 (print)
ISBN 978-9949-03-792-6 (pdf)

University of Tartu Press
www.tyk.ee

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to by the Roman Numerals I–III.

I **Aasumets, K.**, Basikhina, Y., Pohjoismäki, J. L., Goffart, S., Gerhold, J. (2021). TFAM knockdown-triggered mtDNA-nucleoid aggregation and a decrease in mtDNA copy number induce the reorganization of nucleoid populations and mitochondria-associated ER-membrane contacts. Biochemistry and Biophysics Reports *28:* 1–8.
DOI: https://doi.org/10.1016/j.bbrep.2021.101142

II Hangas, A., **Aasumets, K.**, Kekäläinen, N. J., Paloheinä, M., Pohjoismäki, J. L., Gerhold, J. M., Goffart, S. (2018). Ciprofloxacin impairs mitochondrial DNA replication initiation through inhibition of Topoisomerase 2. Nucleic Acids Research *46*: 9625–9636. DOI: https://doi.org/10.1093/nar/gky793

III Wagner, A., Hofmeister, O., Rolland, S. G., Maiser, A., **Aasumets, K.**, Schmitt, S., Schorpp, K., Feuchtinger, A., Hadian, K., Schneider, S., Zischka, H., Leonhardt, H., Conradt, B., Gerhold, J. M., Wolf, A. (2019). Mitochondrial Alkbh1 localizes to mtRNA granules and its knockdown induces the mitochondrial UPR in humans and *C. elegans*. Journal of Cell Science *132*: 1–12. DOI: https://doi.org/10.1242/jcs.223891

The author's contribution to each article is as follows:

I The author participated in designing the study, performed all the experiments, analyzed the data, and participated in the writing of the manuscript as a corresponding author.

II The author performed the floatation analysis of human mitochondrial nucleoids participated in the writing of the manuscript.

III The author performed some of the experiments and also contributed to writing the manuscript.

Other publications:

IV Ianevski, A., Kulesskiy, E., Krpina, K., Lou, G., Aman, Y., Bugai, A., **Aasumets, K.**, Akimov, Y., Bulanova, D., Gildemann, K., Arutyunyan, A.F., Susova, O. Y., Zhuze, A. L., Ji, P., Wang, W., Holien, T., Bugge, M., Zusinaite, E., Oksenych, V., Lysvand, H., Gerhold, J. M., Bjørås, M., Johansen, P., Waage, A., Heckman, C. A., Fang, E. F., Kainov, D. E. (2020). Chemical, Physical and Biological Triggers of Evolutionary Conserved Bcl-xL-Mediated Apoptosis. Cancers *12*: 1–21.
DOI: https://doi.org/10.3390/cancers12061694

ABBREVIATIONS

ABH1 (also AlkBh1)	–	Fe^{+2} and 2-oxoglutarate dependent oxygenase
ATAD3	–	ATPase family AAA domain-containing protein 3
APP	–	amyloid-beta precursor protein
BiP (also Grp78)	–	immunoglobulin heavy chain-binding protein, an ER chaperone
CCCP	–	carbonyl cyanide 3-chlorophenylhydrazone
CMT2A	–	Charcot-Marie-Tooth disease subtype 2A
CPEO	–	Chronic progressive external ophthalmoplegia
Drp1	–	dynamin-related protein 1, GTPase
ETC	–	electron transport chain
FASTKD2	–	FAST kinase domain-containing protein 2
Grp75	–	glucose-regulated protein 75
HSP	–	heavy strand promoter
HSP60	–	heat shock protein 60 kDa
INF2	–	inverted formin 2
IP3R	–	inositol-1,4,5-trisphosphate receptor
KSS	–	Kearns–Sayre syndrome
LHON	–	Leber's hereditary optic neuropathy
LSP	–	light strand promoter
MAM	–	mitochondria-associated ER membranes
MCU	–	mitochondrial calcium uniporter
MEF	–	mouse embryonic fibroblasts
MELAS	–	mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
MERRF	–	myoclonic epilepsy with ragged red fibers
Mff	–	mitochondrial fission factor
Mfn1/Mfn2	–	mitofusins 1 and 2
MiD49/51	–	mitochondrial dynamics proteins of 49 kDa and 51 kDa
MTS	–	mitochondrial targeting sequence
NCR	–	non-coding region
OPA1	–	optic atrophy 1
OXPHOS	–	oxidative phosphorylation
PACS2	–	phosphofurin acidic cluster sorting protein 2
POLG	–	mitochondrial polymerase γ
POLRMT	–	mitochondrial RNA polymerase
PS1/2	–	presenilins 1 and 2
PTPIP51	–	protein tyrosine phosphatase interacting protein-51
ROS	–	reactive oxygen species
SSB	–	single stranded DNA binding protein

STED	–	stimulated emission depletion microscopy
TCA	–	tricarboxylic acid cycle
TFAM	–	mitochondrial transcription factor A
TOMM20/40	–	translocase of outer mitochondrial membrane 20 kDa and 40 kDa
TUFM	–	mitochondrial translation elongation factor Tu
TWNK	–	Twinkle helicase
UCP	–	uncoupling protein
VAPB	–	vesicle-associated membrane protein-associated protein B
VDAC	–	voltage-dependent anion channel

INTRODUCTION

Mitochondria are eukaryotic cell organelles primarily specialized for the synthesis of ATP, which is an energy currency obtained from the catabolism of various organic compounds. Mitochondria are also essential in ion homeostasis, apoptosis, ROS production and in various metabolic pathways.

Besides nuclear genome (nDNA) almost all eukaryotes possess a small and compact mitochondrial genome (mtDNA) that is packaged into complexes with the help of proteins (nucleoids), which are associated with the inner mitochondrial membrane (Rajala et al., 2014). MtDNA packaging into nucleoprotein complexes and inner membrane association are essential for its proper maintenance and portioning into daughter mitochondria, i.e., segregation.

It has been demonstrated that actively replicating nucleoids are transiently associated with a subset of ER-mitochondria contact sites (mitochondria associated ER-membranes or MAMs) leading to mitochondrial fission, thereby directly regulating the correct distribution of mtDNA to the newly generated daughter mitochondria (Friedman et al., 2011; Lewis et al., 2016). This innovative unravelling of interconnectedness of mtDNA synthesis and ER-mitochondria structures has provided evidence that mtDNA synthesis could be one of the first steps of mitochondrial division event. However, despite of these novel findings it remains to be elucidated how exactly mitochondrial DNA replication is coupled to the formation (and dissipation) of ER-mitochondria tethers. The aim of this thesis was to first study the impact of impaired nucleoid integrity on the replication microdomain, which supposedly couples the mtDNA replication and subsequent distribution of nucleoids via mitochondrial fission to membrane connections between ER and mitochondria. We further aimed to shed light onto the composition of nucleoids (protein factors) that contributes to the understanding of mtDNA maintenance in the dynamic mitochondrial network. As a multitude of mtDNA maintenance proteins are associated with various human diseases the study of nucleoids is important to gain new knowledge that could have a potential to lead to improved treatment of many diseases in the future.

1. REVIEW OF LITERATURE

1.1. Mitochondria – hallmarks of eukaryotic cells

Mitochondria originate from a far-reaching endosymbiosis more than 1,4 billion years ago (Margulis, 1970). This endosymbiotic theory posits that a prokaryotic host acquired an ancestral mitochondrion that itself was a metabolically flexible facultative anaerobe able to produce ATP by aerobic respiration if oxygen is present, but capable of diverting from respiration to fermentation if oxygen is absent (Gray, 1989; Margulis, 1981, 1970). The endosymbiosis theory of mitochondrial origin appears to be most widely accepted, since eukaryotes harbor genetic material most likely obtained vertically from the endosymbiont (Andersson et al., 1998; Gray et al., 2001). However, it is being speculated that eventually the mitochondrial DNA becomes integrated into nuclear DNA, since a large proportion of mitochondrial proteins is already encoded by the nuclear DNA, and majority of these are endosymbiotically acquired from the mitochondrial predecessor. During the evolution process the genesis of mitochondria has occurred only once, as the clearest support for this is the presence of conserved genes across all eukaryotes, making for instance studies in nematodes, fruit flies or mice highly relevant to mammalian mitochondria (Polyak et al., 2012; Vergano et al., 2014).

1.2. Mammalian mitochondria

Architecturally mitochondria have two bounding membranes – inner and outer membrane (IMM and OMM, respectively) with different permeabilities, dividing them into 4 distinguishable compartments. IMM is convoluted and folded into cristae that greatly increase its surface area. Both membranes define two mitochondrial sub-compartments – intermembrane space (IMS) and matrix (enclosed by the IMM). Mitochondria are morphologically highly dynamic, ceaselessly undergoing fusion and fission, which are intimately linked with cellular physiological conditions. Mitochondria are best known for possessing metabolic pathways, such as tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) that ensure the synthesis of ATP. Flexibility and productivity of such pathways are intimately linked with the structure and function of mitochondria. The TCA cycle is a series of enzymatic reactions that produces energy (i.e., electrons) during the catabolism of six-carbon sugar glucose, fatty acids and amino acids. This energy released from TCA cycle is harvested *via* reduction of electron shuttles, coenzymes NAD^+ and FAD, and further transported to electron transfer chain (ETC), where NADH and $FADH_2$ are oxidized. ETC complexes I–IV (cI-cV) establish the formation of an electrochemical gradient across IMM *via* proton pumping into the IMS by cI, cIII and cIV (Figure 1). The energy of the electrochemical proton gradient enables complex V (F_0F_1-ATP synthase) to release protons back into the matrix and use the energy of the electrochemical gradient to produce ATP by ADP phosphorylation (Figure 1). However, as mitochondrial

oxidative phosphorylation is not immaculately coupled to ATP production, the energy of the proton gradient (so-called proton-leak) can also be consumed by the uncoupling proteins (UCPs), the mitochondrial anion carriers anchored in the IMM that compete with the F_0F_1-ATP synthase.

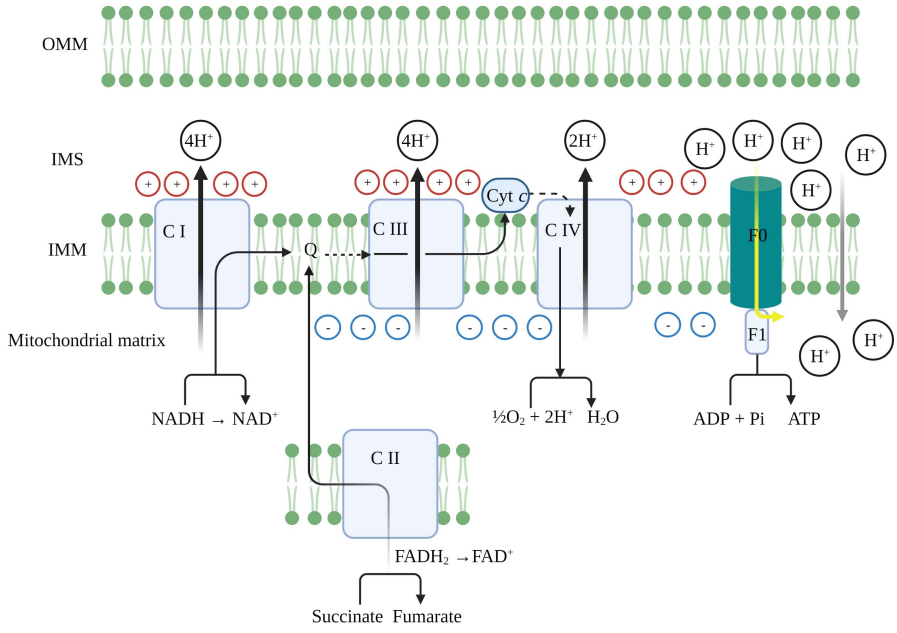

Figure 1. A scheme of mitochondrial oxidative phosphorylation. ATP as an energy currency of the cell is generated mainly in mitochondria *via* oxidative phosphorylation (OXPHOS) process, in which electrons produced by TCA cycle are transferred down the macromolecular electron transfer chain complexes. OXPHOS is fueled by the consumption of oxygen at cytochrome c oxidase (complex IV, cIV). High energy electron carriers NADH and $FADH_2$ generated from the TCA cycle are recruited into the ETC by their oxidation at NADH dehydrogenase (complex I, cI) and succinate dehydrogenase (complex II, cII). The energy in the form of donated electrons drives the proton pumping across the inner mitochondrial membrane (IMM) into the intermembrane space (IMS) thereby creating a membrane potential (~170 mV) and proton concentration gradient (10–30 mV). Oxidation of NADH and subsequent donation of electrons to cI enables the pumping of 4 protons across the IMS, whereas the oxidation of $FADH_2$ does not contribute to the proton pumping, instead it is used to reduce Q (also known as coenzyme Q) to produce QH_2. Electrons produced at cI and cII and picked up by the Q are transferred to cytochrome c reductase (complex III, cIII), which releases 4 protons into the IMS (at the end of a full Q cycle). Finally, cytochrome c oxidase (cIV) oxidizes cytochrome c and transfer the produced electrons to oxygen (which *inter alia* is the final electron carrier in aerobic cellular respiration) that is converted into water. Also, 4 protons are pumped into the IMS. Ultimately, the established electrochemical proton gradient across the IMM empowers ATP production by ATP synthase (F_1F_0-ATP synthase, also referred to as complex V), which drives the synthesis of 1 ATP for every 4 H^+ ions. The amount of final yield of ATP produced is eventually dependent on the organic molecules broken down. Created with Biorender.com.

However, it is well established that mitochondria are not only involved in the synthesis of ATP but harbor a plethora of other activities. The production of fatty acids, amino acids, nucleotides, and heme groups (i.e., ferric iron reduced to ferrous iron, $Fe^{3+} \rightarrow Fe^{2+}$) is essential to ensure the sufficient storage of building blocks for biosynthetic pathways.

A diverse signaling platform that is critical for the maintenance of cellular signaling involves calcium homeostasis. The trafficking of Ca^{2+} ions is regulated by multiple ion channels, such as voltage dependent anion channel (VDAC) (Gincel et al., 2001; Schein et al., 1976; Tan and Colombini, 2007) and mitochondrial inner membrane calcium uniporter (MCU) complex (Oxenoid et al., 2016). Mitochondria are capable of accumulating calcium at significantly higher concentrations than the cytosol, having a powerful Ca^{2+} buffering capacity. Calcium surges can initiate the release of neurotransmitters, muscle fiber contraction, transcriptional regulation and even cell death (Ca^{2+} overload) *via* apoptosis. For example, in heart the muscle contraction is linked to an enhanced ATP production that results from Ca^{2+}-increased activity of TCA cycle enzymes. (Cortassa et al., 2003; McCormack and Denton, 1984)

Hence, mitochondria are metabolically very flexible, being able to rapidly coordinate their energetics and biosynthetic capacity in response to cell status. This is crucial for cell proliferation and differentiation, and cellular signaling that coordinate functional content exchanges between mitochondria and other cellular components (Valm et al., 2017).

1.3. Mitochondrial genome

Mitochondrial form and function are under control of dual genomes – nuclear (nDNA) and mitochondrial DNA (mtDNA). While the majority of mitochondrial proteins are encoded by the nDNA, 13 proteins are still encoded by the mtDNA. Human mtDNA is a 16 569-bp long double-stranded circular molecule (Anderson et al., 1981; Andrews et al., 1999; Radloff et al., 1967) that is not merely a monomeric circular molecule, but is found to exist in various complex structures like catenanes (inter-connected mtDNA molecules) or supercoils and open circles (Pohjoismäki and Goffart, 2011) (Figure 2). The distinction of the two strands of mtDNA lies in the difference in their nucleotide composition and subsequently in their densities (Berk and Clayton, 1974; Radloff et al., 1967). The heavy (H) strand, which also encodes the majority of genes is guanine-rich and the light (L) strand is cytosine-rich (Berk and Clayton, 1974; Nass, 1969).

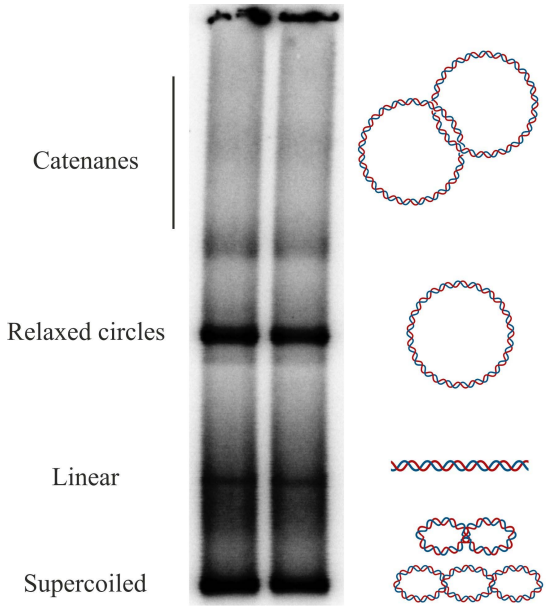

Figure 2. Topological structures of mitochondrial DNA (mtDNA) of human embryonic kidney cells (HEK293). Mammalian mtDNA is usually circular and covalently closed except in some stages of replication and can be distinguished from open circles based on their different electrophoretic mobility in agarose gels. However, more complex forms of double-stranded circular mtDNA, including a range of supercoiled molecules, nicked circles and multiple catenated species are present in mammalian mitochondria. (Illustration created by the author of this thesis)

Human mtDNA encodes for 22 tRNAs, 2 ribosomal RNAs (12S and 16S rRNA) and 13 proteins that correspond to the subunits of oxidative phosphorylation system (Anderson et al., 1981). Being very compact (without introns) and highly coding the 37 genes of mitochondrial genome are mainly disposed end to end. Furthermore, mtDNA also contains the non-coding region (NCR) sequence of about 1,1 kb in length that is located between the $tRNA^{Phe}$ and $tRNA^{Pro}$ genes. This region contains important conserved elements necessary for mtDNA replication and transcription, such as the origin of replication for heavy strand (O_H) and promoters for polycistronic transcription (LSP and HSP). MtDNA possesses a structure at the origin of replication referred to as D-loop, which contains the 7S DNA. The 7S DNA forms at the region of the origin of H-strand by synthesis of a short (approx. 650 bps) complementary piece of DNA (incorporation of a third strand) (Kasamatsu et al., 1971). Studies have shown that the synthesis of this structure is supported by mitochondrial transcription factor A (TFAM), as 7S DNA is produced downstream of transcription (Gensler, 2001). Notwithstanding its poorly understood existence it is believed to be important in mtDNA replication according to the strand-displacement (also strand-asynchronous) model, which was described in the 1972 in rodent mitochondria (Robberson et al., 1972). According to this model the D-loop is formed upon the unidirectional displace-

ment replication initiation of the heavy strand and accounts for the synthesis of about 60% of the heavy strand (~11 kb), until passing the origin of the light strand (Martens and Clayton, 1979) (Figure 3). After the primer synthesis by POLRMT the synthesis of the light strand commences and proceeds by POLG (counter-clockwise until full circle).

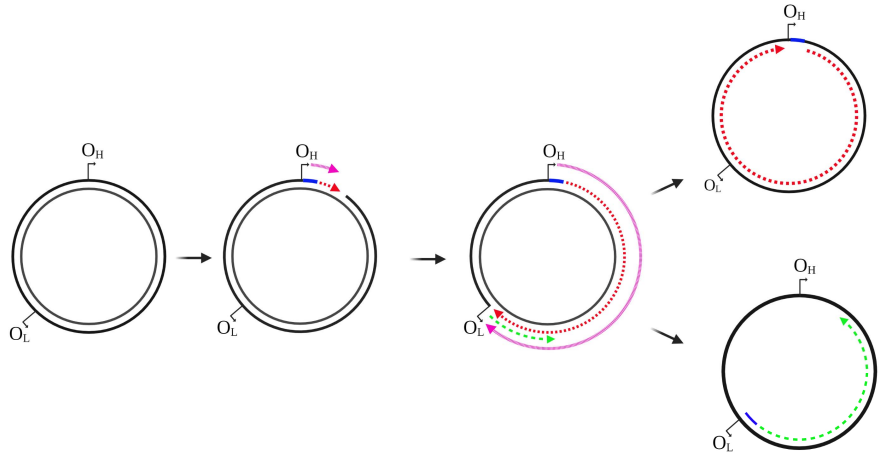

Figure 3. Strand-displacement model of mtDNA replication already descried in 1972 by Vinograd and colleagues (Robberson et al., 1972). The synthesis of the leading or H-strand is initiated within the non-coding D-loop region at O_H site. H-strand synthesis proceeds continuously and unidirectionally (shown in purple). Lagging or L-strand synthesis does not initiate until the newly synthesized H-strand is nearly 70% complete and passes the L-strand replication origin (O_L) (~11 kb away from OH). L-strand replication is likewise continuous and unidirectional, but counterclockwise until full circle (green dashed line). Modified from (Brown, 2005; Robberson et al., 1972).

1.3.1 Mitochondrial nucleoids

It was long thought that mtDNA is a naked molecule within the mitochondrial matrix, but it is now evident that mtDNA together with proteins is organized into complexes called mitochondrial nucleoids (Holt et al., 2007; Spelbrink, 2009) (Figure 4). Formerly it was suggested that each nucleoid contains ~3–6 mitochondrial genomes (even up to 10 mtDNA molecules) per nucleoid (Legros et al., 2004), but this has recently changed due to an exertion of high-resolution techniques. Using STED microscopy, with a resolution of 40–50 nm (230–260 nm in confocal) it was shown that the nucleoid has a diameter of approximately 100 nm, which would not enable the packaging of more than 2 mtDNA molecules by TFAM (Kukat et al., 2011). As the most stable protein component of nucleoids, TFAM is capable of covering and packing the entire mtDNA molecule, similarly to the role of histones in the nucleus. The ability of TFAM to pack mtDNA into nucleoids is attributed to its affinity to mtDNA, inducing unwinding and bending of DNA without sequence specificity (Alam et al., 2003; Kaufman et al., 2007;

Kukat et al., 2015). It is well established that the expression levels of TFAM protein and mtDNA are tightly linked, as the knockdown of TFAM results in mtDNA depletion and decreased mtDNA copy number leads to the degradation of TFAM (Ekstrand, 2004; Larsson et al., 1998; Maniura-Weber et al., 2004; Pohjoismäki et al., 2006). Hence, this TFAM contributes to mtDNA maintenance *via* nucleoid distribution and segregation (Nicholls and Gustafsson, 2018; Sasaki et al., 2017).

Besides TFAM many other proteins have been co-purified with nucleoids or seen to co-localize with mitochondrial nucleoids (also called mitochondrial nucleoid associated proteins; Table 1), such as mitochondrial replicative helicase Twinkle (TWNK) (Spelbrink et al., 2001), DNA polymerase γ (POLG) (Carrodeguas et al., 2002; Fridlender et al., 1972), single-stranded DNA binding protein (mtSSB) (Hoke et al., 1990; Tiranti et al., 1993), ATPase family AAA domain-containing protein 3 (ATAD3) (Gilquin et al., 2010), DNA topoisomerases 2α and 2β (Paper II in this thesis) *et cetera* (for a more thorough list see Hensen et al., 2014).

Table 1. A noninclusive list of the core nucleoid proteins and nucleoid-associated proteins identified in various biochemical purification assays.

Protein	**Identified roles**	**Publication(s)**
TFAM	Packaging of mtDNA; transcription; mtDNA segregation	(He et al., 2007; Wang and Bogenhagen, 2006)
Twinkle	mtDNA replication; formation of mtRNA granules	(Bogenhagen et al., 2008; Wang and Bogenhagen, 2006)
PolγA and B	mtDNA replication; mtDNA repair	(Garrido et al., 2003; Wang and Bogenhagen, 2006)
mtSSB	7S DNA synthesis; mtDNA replication	(Bogenhagen et al., 2008; Wang and Bogenhagen, 2006)
ATAD3	Nucleoid organization; mitochondrial biogenesis	(He et al., 2012; Wang and Bogenhagen, 2006)
Top1mt	mtDNA replication; mtDNA transcription	(Bogenhagen et al., 2008; Zhang and Pommier, 2008)
POLRMT	mtDNA transcription	(He et al., 2012; Wang and Bogenhagen, 2006)
TFB1M and TFB2M	mtDNA transcription	(Bogenhagen et al., 2008; Wang and Bogenhagen, 2006)
mTERF1 and 2	mtDNA transcription	(Bogenhagen et al., 2008; Pellegrini et al., 2009)
Prohibitin 1 and 2	Functional integrity of mitochondria via prohibitin complexes	(Wang and Bogenhagen, 2006)
Lon protease	Maintenance of mitochondrial proteostasis	(Bogenhagen et al., 2008; Lu et al., 2007)

Protein	Identified roles	Publication(s)
ClpX protease	Maintenance of mitochondrial proteostasis; stress signaling via UPR^{mt}	(Bogenhagen et al., 2008; Wang and Bogenhagen, 2006)
Suv3-like helicase	Mitochondrial metabolism	(Bogenhagen et al., 2008; Wang and Bogenhagen, 2006)
EF-Tu	Mitochondrial translation elongation	(He et al., 2007; Wang and Bogenhagen, 2006)
ANT2 and ANT3	Energy metabolism; maintenance of mitochondrial membrane potential	(Bogenhagen et al., 2008, 2003; Wang and Bogenhagen, 2006)
LRP130	Activation of mitochondrial transcription	(Bogenhagen et al., 2003; Wang and Bogenhagen, 2006)
Top2α	mtDNA maintenance in a cell and tissue specific manner	(Bogenhagen et al., 2008)
β-actin	mtDNA maintenance	(Reyes et al., 2011; Xie et al., 2018)
Dna2	mtDNA maintenance	(Duxin et al., 2009)
HSP60 and 70	Mitochondrial chaperones important for mitochondrial proteostasis	(Wang and Bogenhagen, 2006)

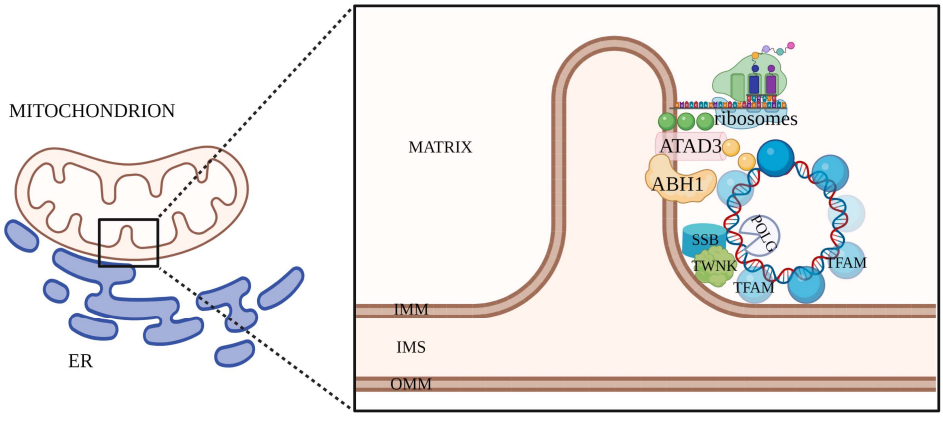

Figure 4. Mitochondrial DNA is packaged into nucleoids that are associated with the inner mitochondrial membrane. TFAM is considered as the most abundant and most stable nucleoid component, that packages mitochondrial DNA into nucleoprotein complexes. Inner membrane associated nucleoids are active in replication containing e.g., TWNK, POLG and mtSSB, and are also found in close association with mtRNA granules and ribosomes. Replicating mtDNA nucleoids are spatially linked to ER-mitochondria contact sites, where mitochondrial division events take place. Created with Biorender.com.

TWNK, mtSSB and POLG are shown to form a so called minimal replisome capable of synthesizing a full mtDNA molecule *in vitro* (Korhonen et al., 2004) (Figure 5). TWNK is a nuclear encoded hexameric replicative 5'→ 3' DNA helicase structurally similar to phage T7 gene 4 primase/helicase (Spelbrink et al., 2001). The primase domain of TWNK, however, is nonfunctional and primers for replication are generated by mitochondrial RNA polymerase (POLRMT) (Fusté et al., 2010; Wanrooij et al., 2008). As part of the replication machinery TWNK increases the processivity and fidelity of POLG that is specifically stimulated by mtSSB (Korhonen et al., 2003). MtDNA synthesis is catalyzed by POLG, a highly accurate (error frequency of less than $1^{x}10^{-6}$ per nucleotide) polymerase composed of one catalytic subunit and two accessory subunits (Longley et al., 2001). The POLG catalytic subunit possesses 3'→ 5' exonuclease activity important for DNA proofreading (Longley et al., 2001; Macao et al., 2015). MtSSB, initially identified in *Xenopus laevis* oocytes, is dynamically incorporated into nucleoids during replication and is required for protecting the single stranded DNA (ssDNA) and also by helping to prevent the formation of secondary structures in ssDNA (Mignotte et al., 1988). It is evident that mtSSB has an important role in mtDNA maintenance, as the knock-down of mtSSB causes the loss of mtDNA and has also been shown to impair the synthesis of the 7S DNA (Ruhanen et al., 2010).

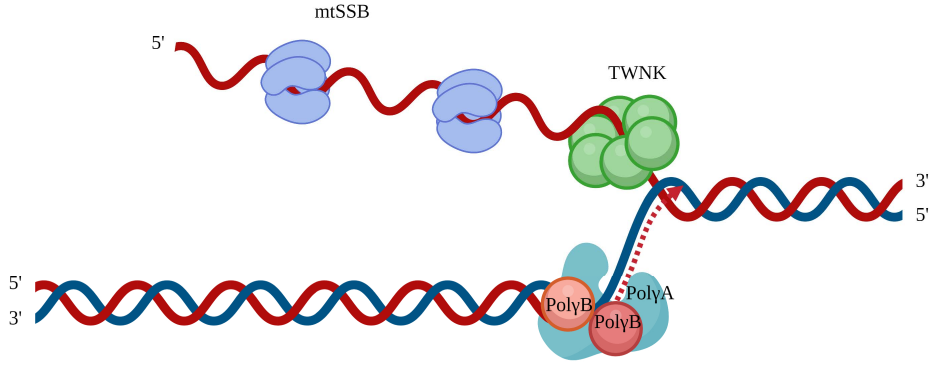

Figure 5. Minimal replisome in human mitochondria. Multimeric helicase TWNK (green), homotetrameric single stranded DNA binding protein mtSSB (dark blue) and DNA polymerase POLG are able to synthesize full length mtDNA molecules *in vitro*. TWNK unwinds the DNA duplex (ATP-dependent process) in 5′ → 3′ direction. Generated ssDNA is bound and protected against degradation and formation of secondary structures by mtSSB. The final step, i.e., mtDNA synthesis is driven by polymerase γ, composed of a catalytic subunit (POLGA), and two DNA binding accessory subunits (POLGB). Modified from (Wanrooij and Falkenberg, 2010). Created with Biorender.com.

A study of Rajala et al. in 2014 elucidated that a specific actively replicating nucleoid pool exists in human mitochondria (Rajala et al., 2014). In this study a tight inner membrane-association of TWNK helicase was described and further biochemical fractionations showed the presence of other mtDNA replication factors (e.g. mtSSB, POLG) together with TWNK and mtDNA in a discrete fraction of density gradients (Gerhold et al., 2015; Rajala et al., 2014). A later study further demonstrated that this membrane-associated TWNK containing replicating nucleoid population was also enriched in mitochondrial cholesterol (Gerhold et al., 2015). A subsequent disruption of cholesterol metabolism by the loss of ATAD3, a protein regulating mitochondrial inner membrane structure and cholesterol metabolism, led to a modified distribution of mitochondrial cholesterol (later also found in mouse models and in ATAD3 patients) (Desai et al., 2017; Gerhold et al., 2015). An observation that also TWNK containing nucleoids were not anymore found in one discrete fraction indicated an existence of a specialized membrane platform, where mitochondrial cholesterol and replicating nucleoids coexist (Gerhold et al., 2015).

However, not all nucleoids have been found to harbor TWNK or other replisome proteins, instead, components distinctive for transcription (POLRMT, TFAM, Top1mt and mtRNA granules) and the absence of TWNK (and the bulk of mtDNA) has led to an understanding that more than one nucleoid population exist (Hensen et al., 2019; Rajala et al., 2014). Biochemical fractionations have clearly shown that, for example, mitochondrial ribosomal proteins (e.g. MRPL48, MRPS35) and mtRNA granules (e.g. mitochondrial elongation factor Tu, FAST kinase domain-containing protein 2 or Fe(II) and 2-oxoglutarate-dependent oxygenase Alkb homologue 1) are either found in the soluble or less mtDNA-associated fractions of specific bottom-up density (Paper III in this thesis).

1.4. Mitochondrial network dynamics

Mitochondria exist as a very active network continuously undergoing fusion and division to ensure correct distribution and segregation of mtDNA (Bereiter-Hahn, 1990). Mitochondrial morphology is maintained by fusion and fission events mediated by conserved GTPases mitofusins (Mfn1 and Mfn2) (Santel and Fuller, 2001), optic atrophy 1 (OPA1) (Olichon et al., 2002; Satoh et al., 2003) and dynamin-related protein 1 (Drp1) (Smirnova et al., 1998).

In the cells Drp1 activation and inhibition are mainly regulated by its phosphorylation and coordinated with cellular conditions. Inhibitory phosphorylation by cyclic AMP-activated PKA at Ser637 of Drp1 allows to maintain its cytosolic localization. However, for example changes in cytosolic calcium level activate Ca^{2+}-dependent phosphatase calcineurin, that subsequently dephosphorylates Drp1 at Ser637 allowing its translocation to mitochondria. (Cereghetti et al., 2008; Cribbs and Strack, 2007)

Before Drp1 recruitment and subsequent oligomerization it is necessary that actin polymerizes at ER-mitochondria interface. Actin polymerization serves as

an initiating driving force for the upcoming constriction and its assembly is regulated by the interconnected binding of ER-associated inverted-formin 2 (INF2) and mitochondria-anchored actin- and formin-binding protein Spire1C (Manor et al., 2015).

Further mitochondrial constriction involves primarily Drp1, that is translocated to mitochondrial outer membrane *via* interactions with mitochondrial fission factor (Mff) and mitochondrial dynamics proteins 49/51 (MiD49/51) (binding of Fis1 protein remains to be elucidated) that promote Drp1 recruitment to the prospective fission sites (Otera et al., 2010; Palmer et al., 2011). Further polymerization of Drp1 is driven by the GTP hydrolysis leading to a formation of constrictive multimers around mitochondria and subsequently to the final scission. Mitochondrial fission directly influences mitochondrial morphology and according to recent findings it is essential for mtDNA segregation to the newly generated daughter cells. Initial findings revealed that ER defines the prospective division sites prior to the recruitment of Drp1 (Friedman et al., 2011). Further studies showed that the mitochondrial fission process occurs in the vicinity of nucleoids, which exist adjacent to ER (Ban-Ishihara et al., 2013; Lewis et al., 2016). Finally, by employing live cell imaging and high-resolution microscopy it was shown that specifically replicating nucleoids are associated with ER-mitochondria contact sites that represent future mitochondrial fission sites and lead to correct mtDNA segregation (Lewis et al., 2016).

Mitochondrial membrane fusion can be divided into two consecutive steps. Fusion of the outer mitochondrial membrane is dependent on two large nuclear encoded GTPases – Mfn1 and Mfn2 (Mfn1 and Mfn2 share nearly 80% sequence similarity) that possess an N-terminal GTPase domain required for their oligomerization (Zorzano and Pich, 2006). Briefly, Mfn1 dimerization induces conformational changes that in turn result in GTP hydrolysis by Mfn1 and subsequently tether outer membrane of adjacent mitochondria (Ishihara, 2004). Mfn1 is shown to possess much higher GTPase activity and tethering capacity of mitochondrial membranes than Mfn2 (Ishihara, 2004). However, while Mfn1 is exclusively found only in mitochondria its close homolog Mfn2 is also present in ER membranes (de Brito and Scorrano, 2008). Mfn1 and Mfn2 can either form homo- or heterodimers to promote mitochondrial joining (Detmer and Chan, 2007). Outer membrane fusion is followed by the inner membrane fusion, which is mediated by a unique dynamin family GTPase Opa1. Opa1 is proteolytically processed by e.g. ATP-dependent metalloprotease YME1L1 or zinc metalloprotease OMA1 to generate a short soluble form, which together with a long membrane-anchored form is required for the inner-membrane fusion (Song et al., 2007), although it has been proposed that Opa1 short isoform is likely associated with mitochondrial fission (Anand et al., 2014). It also has been shown that Opa1 mediated inner-membrane fusion is dependent on Mfn1 and not Mfn2, since Opa1 alone is unable to tubulate and fuse mitochondria lacking only Mfn1 (Cipolat et al., 2004). Nevertheless, mutated Mfn2 and Opa1 are shown to be responsible for Charcot-Marie-Tooth type 2A (CMT2A) (Züchner et al., 2004) and dominant optic

atrophy (DOA) (Delettre et al., 2000), respectively, demonstrating their great importance in maintaining the normal physiology of the cell.

Similar to mitochondrial fission, mitochondrial fusion is also important for the mtDNA maintenance and its distribution (Chen et al., 2010). The segregation is partly explained by the fact that mitofusins are required for the formation of a mtDNA replication platform needed for the subsequent distribution of mtDNA nucleoids. In Mfn1 and Mfn2 double knockout mice a depletion of mtDNA has been reported, which is followed by a reduced respiratory activity and impaired cardiac function (Chen et al., 2011; Papanicolaou et al., 2012). A strong decrease in mtDNA copy number and nucleoid clustering was shown to cause impaired mtDNA replication, which has been suggested to be due to altered expression of replisome proteins accompanied by mtDNA depletion (Silva Ramos et al., 2019). While Opa1 downregulation results in mtDNA depletion and is required for the nucleoid distribution, it does not cause nucleoid clustering (Elachouri et al., 2011).

Hence, mitochondrial dynamics are essential for maintenance of the mitochondrial genome, mitochondrial network and cellular homeostasis, as several studies have confirmed that the nature of mitochondrial dynamics is well-orchestrated with the cellular signaling pathways in both normal and pathological conditions.

1.5. Mitochondria-associated ER-membranes (MAM)

Already in 1956 a group of researchers revealed a study showing unique compact structures of ergastoplasm (now termed endoplasmic reticulum) in rat liver cells that were in close contact with mitochondria (Bernhard and Rouiller, 1956). It was elucidated that formation of these ergastoplasmic membranes along other membranes is merely a mechano-physical phenomenon, but further observations revealed a dynamic re-appearance of ergastoplasm in the close approximation of mitochondria in response to different stimuli (fasting, intoxication with carbo tetrachloride or partial hepatectomy), indicating a physiological role of this inter-organellar communication (Bernhard and Rouiller, 1956).

A later study focusing on phospholipid biosynthesis in rat liver showed that a crude mitochondrial preparation contained biosynthetic activities of phosphatidylserine (PtdSer), phosphatidylethanolamine (PtdEtn), and phosphatidylcholine (PtdCho), as confirmed by thin layer chromatography (Vance, 1990a; Vance and Vance, 1986). Enzymes, like PtdSer decarboxylase, PtdSer synthase and PtdEtn methyltransferase showed apparent existence in crude mitochondria, but considerably less in purified mitochondria (Vance, 1990a; Voelker, 1989). Percoll fractionation of crude mitochondria showed a presence of a clear band just above mitochondria, that was analyzed for various enzymatic activities (endoplasmic marker enzyme NADPH:cytochrome c reductase), Golgi (UDP-galactose:N-acetylglucosamine galactosyltransferase), mitochondria (cytochrome c oxidase), and plasma membrane (5'-nucleotidase) showing no phospholipid biosynthetic enzyme activities of the ER (Vance, 1990a). However, the specific activity of a

second endoplasmic reticulum marker enzyme, glucose-6-phosphate phosphatase in fraction X was almost twice as high in microsomes possessing clearly different properties than the mitochondria, ER, Golgi, plasma membrane, peroxisomes or lysosomes (Vance, 1990a). Hence, based on the previous findings that mitochondria are held together by strands of ER associated with mitochondrial outer membrane (Baranska, 1980), it was proposed that a specific subfraction of ER exists. This subfraction associated with mitochondria, initially fraction X, but later termed mitochondria-associated ER membranes (or MAM) could serve as an important platform for lipid transfer to and from mitochondria (Rusiñol et al., 1994; Vance, 1990b). The close proximity of the two adjacent membranes (ER and mitochondria) in terms of the distance is heterogenous, ranging from ~10 nm at the smooth and ~30–40 nm at the rough ER, as showed by electron microscopy (EM) (Csordás et al., 2006). The surfaces of the two membranes, however, never touch each other and remain adjoined by physical (protein) tethers. Based on high-resolution 3D imaging and high-magnification cryo-EM it has been estimated that ~5–20% of the mitochondrial network is in close proximity to the ER in HeLa cells (Rizzuto, 1998) and ~4–11% in mouse liver (depending on the metabolic changes) (Sood et al., 2014).

On the present day this dynamic specialized subdomain of the ER associated with mitochondria is under thorough investigation due to its involvement in the pathogenesis of, for example, neurological diseases (see paragraph below). The synthesis of cellular lipids and their dynamic trafficking between ER and mitochondria due to the presence of long-chain-fatty-acid-CoA ligase and phosphatidylserine synthase enzymes is only one of the roles of MAM. It has been shown that MAM are also involved in the Ca^{2+} homeostasis (Patergnani et al., 2011) that influences cell proliferation, differentiation, apoptosis (Simmen et al., 2005) and even gene expression, ROS signaling (Zhou et al., 2011) and mitochondrial DNA maintenance *via* its segregation (Lewis et al., 2016).

MAM tethers between two adjacent organelles are mediated by various proteins, such as IP3R, Grp75 and VDAC1 (Szabadkai et al., 2006). IP3R and VDAC1 proteins are both associated with intracellular calcium homeostasis, IP3R being a calcium channel residing in the ER membrane that controls the release of Ca^{2+} and VDAC1 locating in mitochondrial outer membrane and mediating the mitochondrial uptake of Ca^{2+}. Mitochondrial outer membrane chaperone Grp75 is a member of a heat shock protein family 70 (Szabadkai et al., 2006) that regulates the interaction between VDAC1 and IP3R thereby coordinating the transfer and exchange of Ca^{2+} and maintaining the crosstalk between these organelles. Grp75 participation in the formation of MAM tether has been confirmed by *in situ* proximity ligation assay (Honrath et al., 2017; Tubbs and Rieusset, 2016).

Another protein complex modulating ER-mitochondria coupling is formed by mitofusins 1 and 2, which assemble into homo-or heterodimeric complexes, and mediate coupling between organelles (Naon et al., 2016). However, the direct role of Mfn2 in ER-mitochondria interface formation is still under investigation, as the results have been controversial with opposite outcomes. For example, a

visualization of ER-mitochondria apposition in Mfn2 knockout mouse embryonic fibroblasts (Mfn2-KO MEFs) by confocal microscopy showed a decrease in the co-localization of the two adjacent organelles (de Brito and Scorrano, 2008). In the same study a biochemical analysis of the induced ER Ca^{2+} bursts revealed that the respective mitochondrial Ca^{2+} peaks were considerably lower than that in control cells, indicating an impaired mitochondrial Ca^{2+} uptake (de Brito and Scorrano, 2008). However, a quantitative analysis of the ER-mitochondria coupling by electron microscopy and further quantification of confocal microscopy data in Mfn2-KO MEFs showed instead increased apposition of the two organelles (Cosson et al., 2012; Filadi et al., 2015).

Furthermore, a more recent study using the same knockout cell line and novel split green fluorescent protein-based contact site sensors for measuring the close and long range juxtapositions of the two organelles also reported an increased ER-mitochondria coupling (specifically narrow, 8–10 nm contacts) (Cieri et al., 2018).

Ultimately, an alternative negative regulation hypothesis proposes that Mfn2 may not be essential in the formation and/or maintenance of ER mitochondria tethering, but rather might possess yet undefined tethering subunits to interact with and thereby limiting the formation of functional ER-mitochondria conjugation (Filadi et al., 2018).

Besides the aforementioned interactions a connection involving vesicle-associated membrane protein-associated protein B (VAPB) and protein tyrosine phosphatase-interacting protein 51 (PTPIP51) has been recently described (De Vos et al., 2012; Lee and Min, 2018). The association of an ER membrane-anchored protein VAPB and a microtubule-associated protein PTPIP51 is important for the modulation Ca^{2+} transport and autophagy (De Vos et al., 2012).

An important protein shown to be responsible for MAM integrity is phosphofurin acidic cluster sorting 2 protein (PACS2), since a downregulation of PACS2 disrupts the integrity of MAM by separating the two organelles (Herrera-Cruz and Simmen, 2017; Simmen et al., 2005). A proposed mechanism for destruction of MAM integrity upon the depletion of PACS2 is associated with mitochondrial fragmentation *via* Drp1-mediated fission. Furthermore, the disruption of uncoupling of the organelles induces increase in ER calcium levels and ER stress *via* upregulation of BiP (also known as Grp78) levels, an ER chaperone that is necessary to maintaining proper protein folding and subsequent export (Rutkowski and Kaufman, 2004). Although it is evident that PACS2 participates in the maintenance of ER homeostasis and ER-mitochondria interplay, this complex pathway definitely warrants future research.

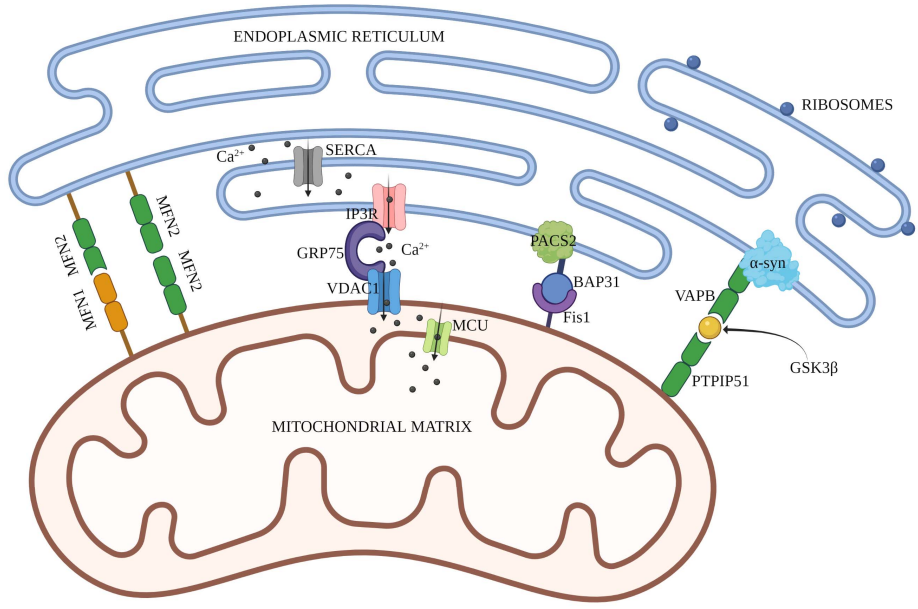

Figure 6. Communication between ER and mitochondria via protein bridges and various regulators. Close apposition of mitochondria to ER is mediated by protein tethers, which form specialized membrane regions termed mitochondria-associated ER-membranes (MAM). These interactions are modulated by different protein complexes, such as mitofusins (Mfn1-Mfn2 or Mfn2-Mfn2), VDAC1-Grp75-IP3R, PACS2 and VAPB-PTPIP51. The reversible linkages between ER and mitochondria are essential for Ca^{2+} transfer and signaling, synthesis and nonvesicular transport of lipids, ROS signaling and many other. VDAC1 – mitochondrial voltage-dependent anion channel, GRP75 – mitochondrial outer membrane chaperone glucose-regulated protein 75, IP3R – ER membrane located inositol 1,4,5-trisphosphate receptor, PACS2 – cytosolic phosphofurin acidic cluster sorting protein 2, BAP31 – ER located cell receptor–associated protein 31, VAPB – ER vesicle-associated membrane protein-associated protein B, PTPIP51 – protein tyrosine phosphatase interacting protein 51 on mitochondrial outer membrane, GSK3β – glycogen synthase kinase 3β, MCU – mitochondrial calcium uniporter, SERCA – sarcoendoplasmic reticulum (SR) calcium transport ATPase. Modified from (Sharma et al., 2020). Created with Biorender.com.

Of great importance is the MAM involvement in mitochondrial DNA maintenance. It is established that in the budding yeast *Saccharomyces cerevisiae*, a multiprotein complex called ER-mitochondria Encounter Structure (ERMES) physically tethers ER and mitochondria thereby contributing to mitochondrial distribution (Boldogh et al., 2003). ERMES tethering complex formation is dependent on integral mitochondrial outer membrane proteins Mdm10 and Mdm34, cytosolic protein Mdm12 and ER transmembrane protein Mmm1 (Kornmann et al., 2009; Stroud et al., 2011). Live cell imaging revealed that the bulk of mitochondrial division events were spatially linked to ER-mitochondria contact points and in these foci mitochondrial constriction occurs leading to division (Friedman et al., 2011). Furthermore, studies have demonstrated that ERMES complexes are located in the vicinity of replicating nucleoids and are important for mito-

chondrial nucleoid distribution (Hanekamp et al., 2002; Hobbs et al., 2001; Meeusen and Nunnari, 2003; Murley et al., 2013). Of note, as ER-mitochondria connection exists in yeast cells it led to an assumption that something similar might occur in mammalian and human cells. Time lapse imaging of Cos-7 cells (African green monkey kidney fibroblast-like cells) showed that mitochondrial division events chiefly occurred at ER-mitochondria contact sites, where ER tubules crossover mitochondria and constrict the latter before Drp1 recruitment (Friedman et al., 2011). Thus, it was proposed that in ER specifically marks the foci where subsequent mitochondrial division events occur (Friedman et al., 2011).

Further studies with human cells investigating mtDNA maintenance in dynamic mitochondrial network shed light into an interplay between mitochondrial division and nucleoid segregation (Ban-Ishihara et al., 2013, p.). Again, using live cell imaging of HeLa cells it was reported that mitochondrial division events occurred in the proximity of nucleoids. A disruption of nucleoid integrity *via* mtDNA replication inhibition altered the distribution and levels of mitochondrial fission factors (Mff and Drp1). Interestingly, treatment of cells with carbonyl cyanide m-chlorophenylhydrazone (CCCP), which is a lipid-soluble weak acid and a potent mitochondrial uncoupler (causes dissipation of membrane potential and mitochondrial depolarization) did not induce significant changes in Mff or Drp1 (Ban-Ishihara et al., 2013). A further manipulation of cells using depletion of Mff and Drp1 and division inhibitor Mdivi-1, a cell-permeable quinazolinone derivative that selectively inhibits mitochondrial division proteins Drp1 and Dnm1, resulted in the clustering of nucleoids and the occurrence of highly dense cristae, indicating an important relationship between nucleoids and mitochondrial division machinery (Ban-Ishihara et al., 2013). However, nucleoid and inner membrane clustering in cells with impaired division was proposed to be a yet unknown protective response to prevent mitochondrial degradation and needs further elucidation.

Biochemical studies have later on shown that replicating nucleoids characterized by the presence of the replicative helicase TWNK and mtDNA are spatially and dynamically associated with mitochondrial inner membrane (Rajala et al., 2014). The further demonstration that also mitochondrial cholesterol co-exists specifically with replicating nucleoids at inner membrane led to an understanding that a cholesterol rich membrane structure that tethers actively replicating nucleoids to the inner mitochondrial membrane *via* ATAD3 (mitochondrial inner membrane ATPase) and is associated with ER mitochondria contacts exists (Gerhold et al., 2015). ATAD3 involvement in this interplay has been extensively investigated (Gilquin et al., 2010; Issop et al., 2015; Liu et al., 2006) and having a significant role in cholesterol metabolism it likely acts as a stabilizing scaffolding structure for mitochondrial replicating nucleoids (Desai et al., 2017; Gerhold et al., 2015; Peralta et al., 2018).

Hence, along with the improvement of technology over the past 10 years a discovery of several mechanisms by which mitochondria regulate their dynamics and genome maintenance has come to pass. It will only be a matter of time when a thorough insight into a molecular mechanism by which the signals from the mitochondrial matrix are conveyed to the ER will be provided.

1.6. Diseases associated with mitochondria and mitochondrial DNA

Unlike nuclear DNA, mitochondrial DNA generally exists as multiple copies per cells, ranging from a few thousand in human fibroblasts up to ~300 000 in human oocytes (Reynier et al., 2001; Steuerwald et al., 2000). The reason for this variability is mainly due to different oxidative capacity of cells, which is undoubtedly highest in muscle cells, such as skeletal or cardiac muscle, and in the brain. By and large mitochondrial diseases are heterogenous, progressive, early-onset and frequently embryonically lethal, and with an estimated prevalence of 1 in 4300, of which mtDNA accounts for up to 40% of the cases (Schaefer et al., 2008, Tan et al., 2020). For example, in 2012 it was reported that a live-birth prevalence of early onset mitochondrial disorders in Estonia is ~1 in 20 000 (Joost et al., 2012). By applying an improved novel diagnostic algorithm in 2003, 36 mitochondrial disease cases were diagnosed from 2003 to 2017 in Estonia (before this period only once case was diagnosed), which accounted for nearly 20% of all inborn metabolic diseases (Tiivoja et al., 2020).

One peculiarity that makes the study of mitochondrial diseases so intricate is the coexistence of different mtDNA genomes in a single cell – a phenomenon referred to as heteroplasmy (Holt et al., 1988). Of note, on the contrary to heteroplasmy there can be a situation where all mtDNA molecules within the cell are identical called homoplasmy (Wallace et al., 1988). The occurrence of the pathological phenotypes in this case is dependent on the greater number of mtDNA molecules having partial deletions or duplications, although wild-type mtDNA molecules are still present in disease cells – generally considered as threshold effect (Wallace, 1993, 1986).

It is now widely accepted that any kind of damage to mitochondrial DNA can potentially have a detrimental impact on the main source of cellular energy currency. Various mutations in mtDNA are shown to be directly associated with several human pathologies, such as Leber's hereditary optic neuropathy or LHON (Wallace et al., 1988) (mtDNA point mutation leading to a missense substitution of a histidine for arginine in subunit 4 of NADH dehydrogenase (complex I)), mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes or MELAS (Goto et al., 1990) (point mutations in mtDNA-encoded tRNA genes), Kearns–Sayre syndrome or KSS (Lestienne and Ponsot, 1988, Zeviani et al., 1988), myoclonic epilepsy with ragged-red fibers or MERFF (Shoffner et al., 1990), chronic progressive external ophthalmoplegia or CPEO (Hutchinson, 1879; von Graefe, 1856) with mutations in the *POLG* (Lamantea et al., 2002) and *TWNK* (Spelbrink et al., 2001) genes also associated with this condition.

However, besides directly mtDNA sequence-derived pathologies there have been several studies showing that also mtDNA copy number is associated with human diseases, such as cardiomyopathy or neurodegenerative disorders. A large number of studies addressing these disorders have been focusing on investigating the role of TFAM. In *in vivo* mouse models the heterozygous mutation of TFAM

causes ~40% decline in mtDNA copy number, while on the contrary, the homozygous mutation is embryonically lethal (Larsson et al., 1998). Both decreased mtDNA and TFAM levels have been described in Alzheimer's (Oka et al., 2016; Rice et al., 2014), Parkinson's (Grünewald et al., 2016) and Huntington's (Kim et al., 2010) diseases in numerous patients.

A broad range of diseases are further associated with altered mitochondrial dynamics and impaired ER-mitochondria interplay. As intercommunication between mitochondria and ER is involved in regulating various physiological processes, such as maintaining the stable state of Ca^{2+} equilibrium and lipid homeostasis, and also mtDNA segregation, it has gained remarkable attention in epidemiology, especially in neurology. For example, alterations in ER-mitochondria communication through disruption of VAPB and PTPIP51 tether have been reported in ALS, a severe and rapidly progressive motor neuron disorder (Stoica et al., 2014). Disturbed ER-mitochondria interplay *via* dysregulated mitochondrial proteins, such as PINK, parkin or α-synuclein, has been further associated with familial form of Parkinson's disease (Vila et al., 2008).

One of the hallmarks of Alzheimer's diseases (AD) is an accumulation of amyloid beta peptides in amyloid plaques (Thal et al., 2019). In familiar AD autosomal dominant gene mutations in PS1, PS2 and APP have been described (Area-Gomez et al., 2012). APP processing by β-and γ-secretases generates Aβ and a fundamental role in this process lies on PS1 and PS2, both proteins also enriched in MAMs (Area-Gomez et al., 2009). The discovery that also APP is found at MAM sites (Pera et al., 2017) has contributed to the understanding that AD and changes in MAM are associated and the precise mechanism/pathway definitely requires further elucidation.

Aberrant ER-mitochondria interplay in Huntington's disease has also been under intense investigation, since the association between IP3R, huntingtin-associated protein 1A (HAP1A) and mutated huntingtin was shown to promote Ca^{2+} release from the ER (Naia et al., 2017). During formation of a physical tether between ER and mitochondria, IP3R interacts with VDAC1, allowing more Ca^{2+} to be taken up by mitochondria, resulting in mitochondrial depolarization and disrupted Ca^{2+} homeostasis (Naia et al., 2017).

The list of disorders with primary and secondary mitochondrial dysfunction described above is merely the tip of the iceberg. There are more than 400 pathogenic gene variants alone that have been found to cause mitochondriopathies (Frazier et al., 2019). The complexity of all defects with either direct or indirect mitochondrial contribution is still beyond the scope of our understanding, thus continuous research is needed to resolve the missing pieces that could lead to an efficient treatment. Hence, being so small, yet having such a fundamental role in maintaining its own genome and at the same time regulating inter-organelle communication mitochondria influence life and death of an entire organism.

2. AIMS OF THE STUDY

The aim of this study was to investigate protein composition and maintenance of human mitochondrial DNA-protein complexes nucleoids.

The specific objectives were to:

a) Study how does the nucleoid composition influence the replication micro-domain.

b) Elucidate proteins interacting with mitochondrial nucleoids.

c) Study the functional signaling hubs between mitochondria and ER (MAM) in terms of mtDNA maintenance.

3. MATERIAL AND METHODS

3.1. Cell culture

HEK293 and U2OS cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. Transfection of cells was performed using DharmaFECT 1 transfection reagent (Dharmacon) according to the provided guidelines. Shortly, cells were seeded and grown for 24 hours prior transfection using either siGENOME Non-Targeting siRNA (Dharmacon) or specific siGENOME Human TWNK siRNA SMARTpool (#M-017815-01-0020) and siGENOME Human TFAM siRNA SMARTpool (#M-019734-00-0020) at a concentration of 25 nM. Three days post-transfection cells were harvested for subsequent analyses.

3.2. Mitochondrial isolation and subsequent sub-fractionation

Mitochondria from HEK293 cells were isolated according to (Gaines and Attardi, 1984) with modifications. Briefly, cells were collected by centrifugation at 500 *g* and washed once with PBS. Pelleted cells were resuspended in a hypotonic buffer (4 mM Tris-HCl, pH 7.8, 2.5 mM NaCl, 0.5 mM $MgCl_2$ supplemented with protease inhibitor cocktail), swelled for 6 minutes and stroked in a Teflon-glass Elvehjem homogenizer 20 times. Upon disruption the tonicity was brought to isotonic, and suspension was transferred to a polypropylene tube for subsequent centrifugation at 1200 *g* for 5 minutes at 4 °C. The collected supernatant was finally centrifuged at 14 000 *g* for 10 minutes at 4 °C to obtain crude mitochondria.

Crude mitochondrial preparation was further sub-fractioned to isolate mitochondrial membrane associated (insoluble) and matrix (soluble) fractions. For that an equal amount of mitochondrial protein (determined by Bradford assay) was lysed with a non-ionic mild detergent digitonin at a ratio of 1:2 (µg digitonin:µg protein) for 10 minutes on ice, centrifuged at 14 000 *g* for 10 minutes at 4 °C and supernatant transferred to a separate tube. Insoluble pellet fraction was resuspended in an equal volume of PBS and aliquoted for further analyses.

Crude mitochondria for floatation fractionation assay (Rajala et al., 2014) (summarized on Figure 7) were further purified over sucrose gradient (1M and 1.5 M sucrose in 20 mM HEPES pH 7.6, 10 mM EDTA). Pure mitochondrial layer was recovered and re-suspended in PBS. 1 mg of total mitochondrial protein was lysed with digitonin and membrane and matrix fractions were separated by centrifugation at 14 000 *g* for 10 minutes at 4 °C. The obtained fractions were mixed with Optiprep™ (Cosmo Bio Usa Inc.) to get a final concentration of 42.5% iodixanol. Samples were transferred into ultracentrifuge tubes, overlaid with 40 – 0% of iodixanol in TN (containing 10% sucrose, 1% Triton X-100, 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, protease inhibitors) buffer and centrifuged at 100 000 *g* for 14 hours at 4 °C. Fractions of 400 µl each were collected from top-to-bottom and further analyzed for protein and mtDNA.

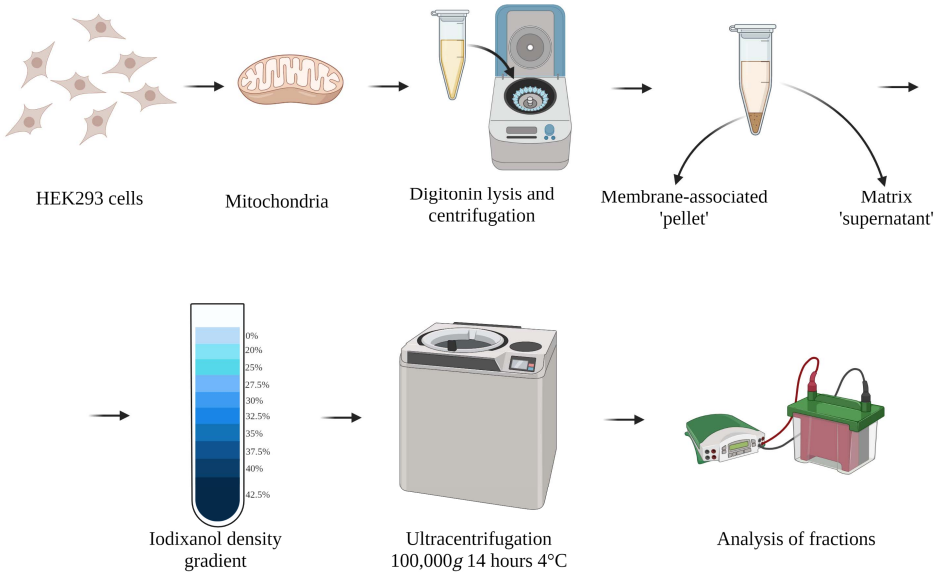

Figure 7. Floatation gradient analysis workflow. Mitochondria were isolated from HEK293 cells and sub-fractioned into membrane-associated insoluble fraction (pellet) and soluble matrix fraction (supernatant) using mild detergent digitonin at a ratio of 1:2 (µg protein:µg digitonin). Obtained fractions were subjected to flotation/fractionation on an iodixanol density gradient (42,5% – 0%) and centrifuged at 100 000 *g* for 14 hours at 4 °C. Upon ultracentrifugation fractions (10) were carefully collected from top-to-bottom and subsequently analyzed.

3.3. Immunofluorescence

U2OS cells grown on 22 mm coverslips were transfected with either 25 nM of TWNK siRNA SMARTpool or TFAM siRNA SMARTpool as described above. Cells were fixed 3 days later with 3.3% formaldehyde in complete medium for 25 minutes, washed three times with PBS and permeabilized with 0.5% Triton X-100 in 10% FBS/PBS for 15 minutes. After rinsing with PBS primary antibodies (anti-TFAM from Abcam, anti-FACL4 from Santa Cruz, anti-TOMM20 from Sigma, anti-DNA from Abcam) were added at 1:500 dilution in 10% FBS/PBS overnight at 4 °C. Coverslips were washed thrice with PBS and incubated with Alexa-Fluor conjugated secondary antibodies (anti-mouse and anti-rabbit AlexaFluor 488 conjugates, and anti-rabbit and anti-mouse AlexaFluor 568 conjugates from ThermoFisher Scientific) at 1:1000 dilution in 10% FBS/PBS for 1 hour. Coverslips with fluorescently labelled cells were immersed in ProLong™ Gold Antifade Mountant with DAPI and imaged using Zeiss LSM710 confocal microscope with ZEN software.

3.4. Copy number analysis

For mtDNA copy number determination total cellular DNA was extracted using a classical phenol:cholroform method with modifications (Barker et al., 1998). Cells were harvested, washed with PBS and resuspended in DNA lysis buffer supplemented with 200 μg of proteinase K. After incubation for 30 minutes at 37 °C 2 volumes of phenol:chloroform:isoamyl alcohol 25:24:1 pH 6.8 (ACROS Organics™) was added, vigorously mixed and centrifuged at 5000 g for 5 minutes. The aqueous phase was transferred into new tubes and extraction repeated. DNA was precipitated by the addition of 2.5 volumes of 96% ethanol at –20 °C, centrifuged at 14 000 *g* for 30 minutes, washed once with 70% ethanol and reconstituted in 20 mM HEPES, pH 7.4. Quantitative PCR was carried out using 5X HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne OÜ) and a total of 50 ng of DNA. MtDNA copy number was normalized to nuclear DNA by amplifying human *MT-CYB* fragment (*MT-CYB* forward: 5'-GCCTG-CCTGATCCTCCAAAT-3 and reverse: 5'-AAGGTAGCGGATGATTCAGCC-3') as compared to a fragment of a single copy nuclear gene *APP* (*APP* forward: 5'-TTTTTGTGTGCTCTCCCAGGTCT-3', *APP* reverse: 5'-TGGTCACTGGTT-GGTTGGC-3'). Calculation of fold change was carried out using the -ΔΔCt algorithm.

For dot blot analysis of mtDNA samples were diluted with 2X SSC, boiled for 15 minutes at 95°C, snap-cooled and transferred onto nylon membrane (GE Healthcare Amersham Hybond^{TM}-N^{+}) using Bio-Rad Bio-Dot® apparatus. Upon UV crosslinking with 120 mJ/cm^2 membrane was hybridized at 65°C using radio-actively labelled mitochondrial probe (human MT-CYB gene amplification forward 5'-TGAAACTTCGGCTCACTCCT-3' and reverse 5'-GTTGTTTGA-TCCCGTTTCGT-3' primers) for 3 hours. Membranes were exposed to phosphor screen and imaged using Typhoon Phosphorimager with ImageQuant TL soft-ware.

3.5. Protein analysis

Protein samples were analyzed using SDS-PAGE and subsequent blotting. Samples were boiled at 95 °C for 5 minutes and loaded onto 8%–15% poly-acrylamide gels, resolved in denaturing SDS buffer (Bio-Rad Mini-PROTEAN® Tetra Cell system) and transferred onto GE Healthcare Amersham^{TM} Protran^{TM} 0,45 μm nitrocellulose membrane. Membranes blocked for 1 hour with 5% skim milk in TBST and incubated with primary antibodies in 3% BSA in 1X TBST followed by incubation with secondary antibodies. Primary antibodies with respective dilutions used are listed in the table 2.

Table 2. List of antibodies used.

Antibody	Species	Supplier	Catalog number	Dilution factor
TFAM	Mouse	Abcam	ab119684	1:5000
Twinkle	Mouse	Kind gift of Dr. Anu Suomalainen-Wartiovaara	–	1:1000
POLG	Goat	Santa Cruz Biotech.	sc-5931	1:500
VDAC1	Mouse	Abcam	ab14734	1:1000
SSBP	Rabbit	Sigma	HPA002866	1:3000
HSP60	Mouse	Santa Cruz Biotech.	sc-376240	1:1000
ATAD3	Rabbit	Kind gift of Dr. Ian Holt	–	1:10 000
ATAD3A	Rat	HMGU	FLJ4D5	1:1000
Mitofilin	Rabbit	Abcam	ab137057	1:5000
COX2	Rabbit	ProteinTech	55070-1-AP	1:5000
Mitofusin 1	Mouse	Santa Cruz Biotech.	sc-166644	1:1000
Mitofusin 2	Mouse	Santa Cruz Biotech.	sc-100560	1:1000
FACL4	Mouse	Santa Cruz Biotech.	sc-271800	1:1000
POLRMT	Rabbit	Abcam	ab32988	1:1000
IRE1α	Rabbit	Cell Signaling	3294	1:1000
Calnexin	Rabbit	Cell Signaling	2679	1:1000
ATF6	Rabbit	Cell Signaling	65880T	1:1000
DNA	Mouse	Abcam	ab27156	1:1000
TOMM20	Rabbit	Sigma	A75430	1:1000
TOMM40	Rabbit	Santa Cruz Biotech.	sc-11414	1:1000
B-actin	Mouse	Cell Signaling	3700	1:2000
A-tubulin	Mouse	Abcam	ab52866	1:2000
COX4	Rabbit	Abcam	ab16056	1:1000
ATP5A	Mouse	Abcam	ab14748	1:1000
PERK	Rabbit	Cell Signaling	5683	1:1000

Signals were detected and quantified using LI-COR Odyssey® Fc Imaging System with Image Studio software.

4. RESULTS AND DISCUSSION

4.1. The importance of mitochondrial topoisomerases in mitochondrial DNA maintenance (Paper II)

It has been repeatedly shown that mtDNA occurs in different topological forms the in cells, e.g., open circles, catenanes and supercoils, but the proteins responsible for the regulation of the formation of these structures requires more elucidation. As the replication and transcription of the circular mtDNA molecule occurs bidirectionally it causes DNA supercoiling. This topological stress is resolved by catalytically active enzymes – topoisomerases, which by and large release tensions and decatenate newly replicated mtDNA.

The specific localization of (mitochondrial) DNA topoisomerases was studied here by applying density gradient centrifugation of sub-fractioned mitochondria. Firstly, the best characterized topoisomerase in mitochondria, Top1mt, was mainly found in the membrane-associated high-density fractions together with, e.g., mitochondrial RNA polymerase (POLRMT) (Figure 8). This is well in accordance with previously reported data showing that Top1mt has a significant role in the regulation of transcription (Dalla Rosa et al., 2017). However, a portion of Top1mt co-localized with mitochondrial replicating nucleoids in low-density fraction together with mtDNA and replicative helicase TWNK, thus it is also regulating the mtDNA replication process.

The second well-known type I topoisomerase with described mitochondrial function is Top3α. It has been proposed that Top3α is important for the separation of newly synthesized mtDNA molecules destined for segregation via mitochondrial fission (Nicholls et al., 2018). Reduced expression of mitochondrial isoform of Top3α resulted in a formation of enlarged nucleoids with altered intramitochondrial distribution and also an increase in high-molecular-weight mtDNA structures (catenanes) (Nicholls et al., 2018). This indicates an impaired separation of mtDNA molecules upon replication, which is a prerequisite event for the segregation. Here, similar to Top1mt, Top3α was detected in the membrane associated fractions of submitochondrial preparations and specifically in the replicating nucleoid fraction (Figure 8), which corroborates the previous findings. Hence, as mtDNA replication events occur at ER-mitochondria contact sites, which are necessary for the subsequent distribution of daughter nucleoids within mitochondrial network, the co-localization of Top3α with mtDNA replication factors here indicates its role in mtDNA maintenance.

Mitochondrial localization has also been described for two type II topoisomerases – Top2α and Top2β (Zhang et al., 2014), but the localization studies have been somewhat conflicting possibly due to dual localization and tissue specificity of their expression (Paper II, figure 1B), and various studies have failed to detect either Top2α or Top2β in mitochondrial preparations (Nicholls et al., 2018; Zhang et al., 2014). Also, the mitochondrial targeting sequence (MTS) for both Top2α and Top2β remains to be elucidated. Here, both Top2α and Top2β were

detected in sub-mitochondrial preparations of HEK293 cells (Figure 8). Both Top2α and Top2β localized to the membrane associated replicating nucleoid fraction, indicating their association with mtDNA metabolism. However, the knockdown of Top2β, but not Top2α caused an accumulation of supercoiled mtDNA intermediates and led to a significant decrease in mtDNA copy number (Paper II, figure 2). These results thus demonstrate that Top2β is essential for mtDNA maintenance most likely in a tissue specific manner.

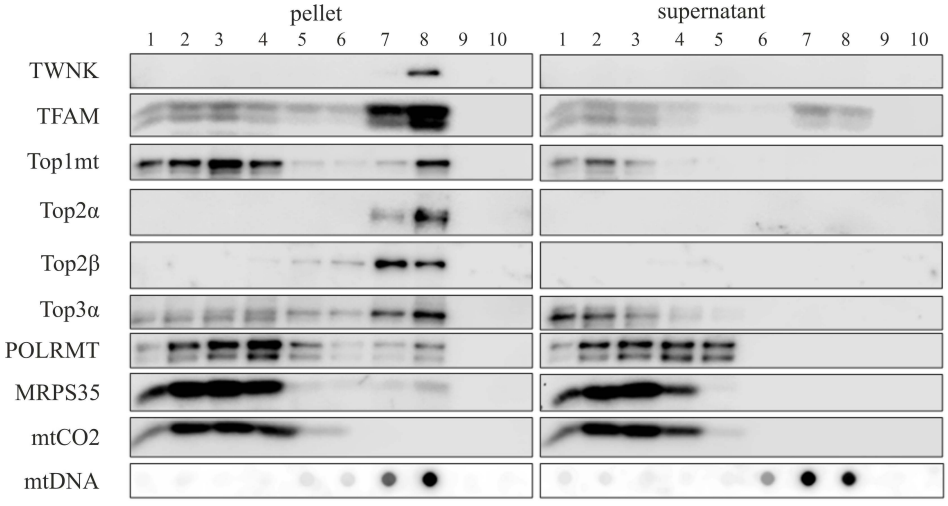

Figure 8. Localization of mitochondrial topoisomerases on floatation/fractionation gradients. While mitochondrial topoisomerase Top1mt is found both in the replicating nucleoid fraction (fraction 8 in the pellet) and in the high-density fractions together with RNA polymerase (fractions 1–4 in the pellet), the topoisomerases Top2α, Top2β and Top3α are predominantly found in the replicating nucleoid fraction (fraction 8 in the pellet) together with mitochondrial DNA. The Western blot panels presented here are cropped versions of the original exposures.

4.2. An interplay between mitochondrial DNA and RNA pools (Paper III)

Correct mitochondrial functioning requires a well-orchestrated regulation of mitochondrial RNA synthesis, processing, and correct translation of mtDNA-encoded respiratory chain proteins. Recent studies have shed more light into the role of dynamic tRNA modifications in mitochondria and described the presence of specific demethylases with polysomes and their role in mitochondrial RNA metabolism (Haag et al., 2016; Liu et al., 2016). The co-localization of specific non-heme iron (i.e., ferric iron) and α-ketoglutarate-dependent dioxygenase family enzyme AlkBh1 with mitochondrial ribosomes and RNA granules was further studied here. As previously demonstrated mtRNA granules are discrete hubs of newly synthesized RNA and are reportedly adjacent to mitochondrial nucleoids

(Hensen et al., 2019; Jourdain et al., 2013; Minczuk et al., 2011). Moreover, it was very recently demonstrated that the replicative helicase TWNK not only colocalizes with mtRNA granules, but it is important for their formation, and together with mtSSB are necessary for mtRNA metabolism (Hensen et al., 2019). Here it was found that AlkBh1 is largely concentrated in high density fractions of specific top-to-bottom gradients of submitochondrial preparations together with mtSSB and mtRNA granules proteins, such as translation elongation factor of mitochondria (TUFM) and the mitochondrial large ribosomal subunit assembling protein FASTKD2 (Figure 9). Both large (MRPL48) and small (MRPS35) ribosomal subunit proteins showed overlapping with AlkBh1 (Figure 9). Finally, mitochondrial localization of AlkBh1 was confirmed by immunofluorescence in various cell lines and an overlap analysis on high-resolution images showed its' strong colocalization with FASTKD2, but not with mtDNA (Paper III, figure 1A–C; 2).

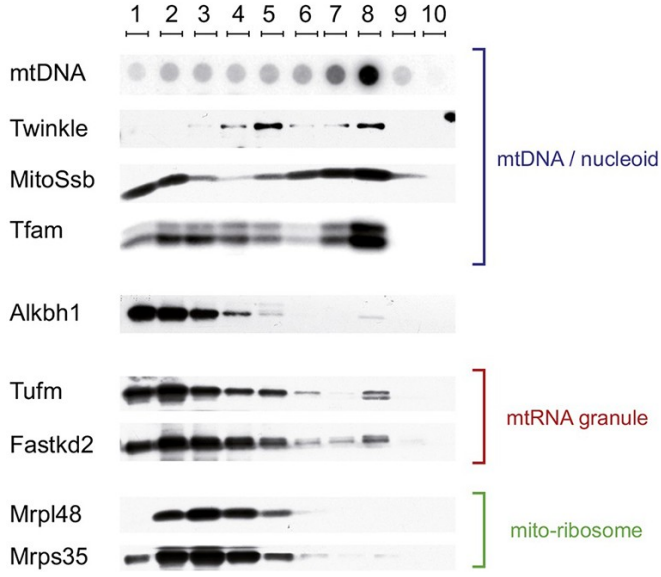

Figure 9. Isolated mitochondria were subjected to digitonin lysis, sub-fractioned into membrane insoluble pellet and soluble matrix fractions and separated on a floatation/ fractionation density gradient. Presented here are the analyzed digitonin insoluble fractions, which contains the mitochondrial membrane associated components. MtDNA co-migrates with TWNK, mtSSB and TFAM in a single fraction high up the gradient (fraction 8), Western blot panels presented here are cropped versions of the original exposures.

Taken together, these results here demonstrate the essential interconnectedness of mtDNA and mtRNA metabolism, as the disruption of either mitochondrial nucleoid integrity or mitochondrial network organization ultimately leads to altered gene expression and mitochondrial unfolded protein response (UPR^{mt}) (Paper III, figure 4).

4.3. Maintenance of mitochondrial nucleoids – the dual role of mitochondrial transcription factor A (TFAM) (Paper I)

Nuclear-encoded DNA-binding protein TFAM binds to the DNA minor groove without sequence specificity. TFAM has multiple functions in regulating mtDNA, one of which is the wrapping of the DNA into DNA-protein complexes termed mitochondrial nucleoids. Different populations of nucleoids exist, which are largely differentiated by their composition closely linked to their function. TFAM plays also an important role in the segregation of mtDNA molecules, as nucleoids are considered segregation units of mtDNA.

To investigate the possible impact of mtDNA packaging factor TFAM on mitochondrial nucleoids (populations) a 3-day TFAM-specific siRNA mediated knockdown in HEK293 cells was performed, followed by an isolation of crude mitochondria (contains, inter alia, ER membranes) and sub-fractioning into membrane-associated (insoluble) and matrix (soluble) fractions using mild detergent digitonin (See M&M Figure 7).

A short, 3-day TFAM knockdown caused significant decrease in mtDNA relative copy number (~50%) (Figure 10C). As the purpose was, foremost, the investigation of nucleoid maintenance without completely abolishing mtDNA replication we had to ensure that the mtDNA was not completely depleted. Further analysis of nucleoid proteins showed that there was an upregulation of replicative helicase TWNK in TFAM knockdown mitochondria both in membrane associated fraction and in intact mitochondria (Figure 10A). A component of mitochondrial minimal replisome, mtSSB, and mtDNA encoded complex IV enzyme COX2 did not exhibit clear alterations (Figure 10A; Paper I, Figure 2). A dot blot analysis to determine mtDNA distribution and a relative quantity in sub-fractioned mitochondria showed that in TFAM knockdown the mtDNA is more in the membrane associated fraction, i.e., only ~20% of mtDNA was soluble and 80% in the membrane fraction (in comparison the respective distribution in control mitochondria was 30% in soluble and 70% in the membrane fraction) (Figure 10D). Interestingly, a set of MAM proteins (MFN2, FACL4 and VDAC1) displayed a clear increase in TFAM knockdown mitochondria, indicative of a greater proportion of MAM with replicative nucleoids (Figure 10A).

4.3.1. ER-mitochondria membrane contacts are altered in TFAM knockdown cells

The presence of ER membranes in close vicinity of membrane associated replicating nucleoids has been previously demonstrated to be important for early mitochondrial division events and subsequent mtDNA segregation. Our initial finding that MAM proteins increase in the membrane-associated fractions upon gene-silencing of TFAM was further examined by the fractionation of crude mitochondria into MAM and ER. Isolated MAM fractions from TFAM knockdown cells showed a significant increase in MFN1/2, VDAC1 and FACL4 levels

consistent with the submitochondrial fractionation results (Figure 10B; Paper I, Figure S2). The level of TWNK was also slightly increased in isolated MAM from the knockdown cells (Figure 10B), indicative of stronger association of TWNK-containing nucleoids with mitochondrial inner membrane. Taken together, TFAM knockdown resulted in upregulation of replicative helicase TWNK and a greater retention of mtDNA in membrane associated fraction together with MAM proteins, although overall mtDNA content decreased significantly.

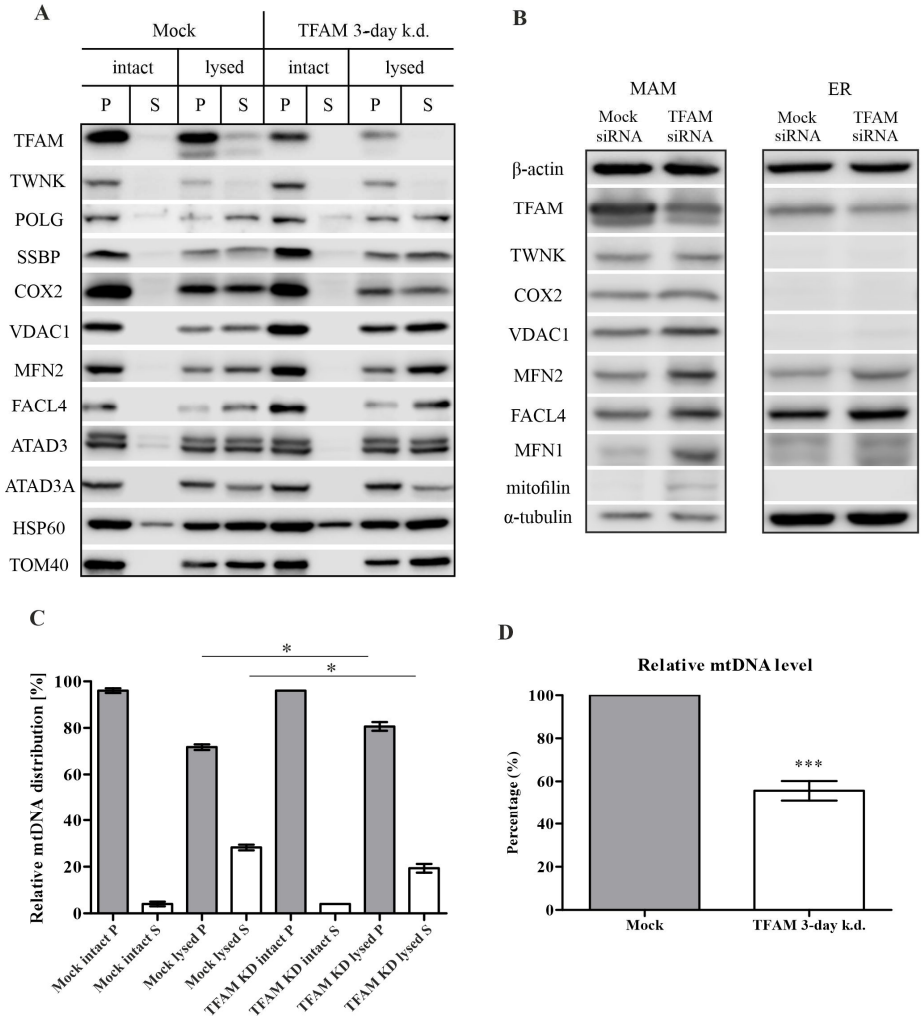

Figure 10. 3-day TFAM knockdown in HEK293 cells. (**a**) PBS-washed intact and digitonin fractioned crude mitochondria analyzed for various proteins by Western blotting. (**b**) Crude mitochondrial fractionation into MAM and ER. (**c**) TFAM knockdown caused a significant decrease in mtDNA relative copy number. For statistics, a two-tailed paired t-test comparing knockdown to the respective control set at 100 was used. Error bars indicate +/– SEM (n = 6). *** indicates a p-value ≤ 0.001. (**d**) Relative mtDNA distribution between soluble and insoluble fractions determined by dot blotting. * indicates a p-value ≤ 0.05. The Western blot panels presented here are cropped versions of the original exposure pictures.

4.3.2. TFAM knockdown does not alter the distribution of nucleoids in mitochondrial floatation gradients

Mitochondrial nucleoid populations were subsequently separated by a specific top-to-bottom density gradient centrifugation according to previously described method (Rajala et al., 2014) (See M&M Figure 7).

Firstly, the previously described replicating nucleoid fraction persists upon TFAM knockdown as mtDNA together with other replisome components occur in one discrete fraction (Figure 11). It is noteworthy, and concordant with previous digitonin fractionations here, that mtDNA is mostly present in the membrane associated fraction in TFAM knockdown, whereas the TFAM protein and mtDNA signals in the soluble matrix fractions are considerably weaker (Figure 11).

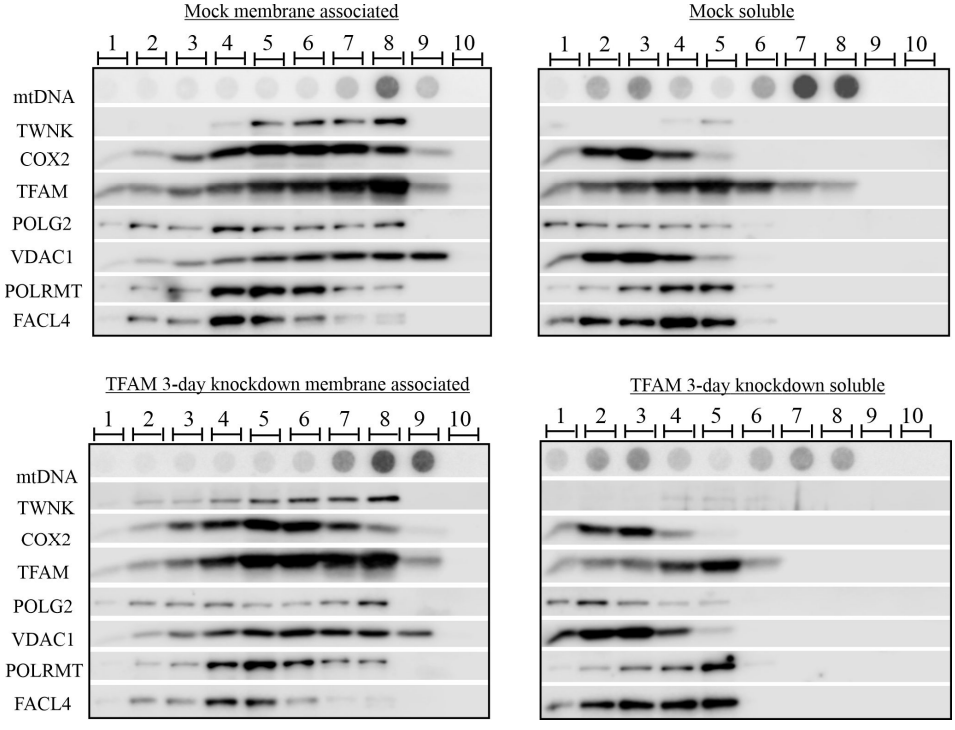

Figure 11. Separation of human mitochondrial nucleoid populations by density gradient centrifugation (floatation). The Western blot panels presented here are cropped versions of the original exposure pictures. Different density fractions are depicted with numbers 1–10.

4.3.3. TFAM knockdown causes an aggregation of nucleoids

To better understand the impact of TFAM knockdown within the dynamic mitochondrial network, a confocal microscopy in TFAM silenced U2OS cells was further applied. The staining of both mock transfected and TFAM knockdown cells with TFAM, DNA and mtSSB antibodies showed clear differences in the distribution of signals. Endogenous TFAM, mtSSB and mtDNA signals displayed

intense and enlarged fluorescence puncta compared to the control (Figure 12; Paper I, Figure 1, Figure S1), regardless of an overall decrease in TFAM and mtDNA levels (Figure 10; Paper I, Figure 1E, 2D). Interestingly, a rather similar phenotype has been previously reported in TFAM knockdown HeLa cells, where mtDNA (specifically designed mtDNA probe) and mtSSB exhibited enlarged signals indicating an enlargement of mitochondrial nucleoids (Kasashima et al., 2011). Despite of the aggregation of nucleoid components the mitochondrial thread-like network remained similar to the control cells (Paper I, Figure 1C, 1D).

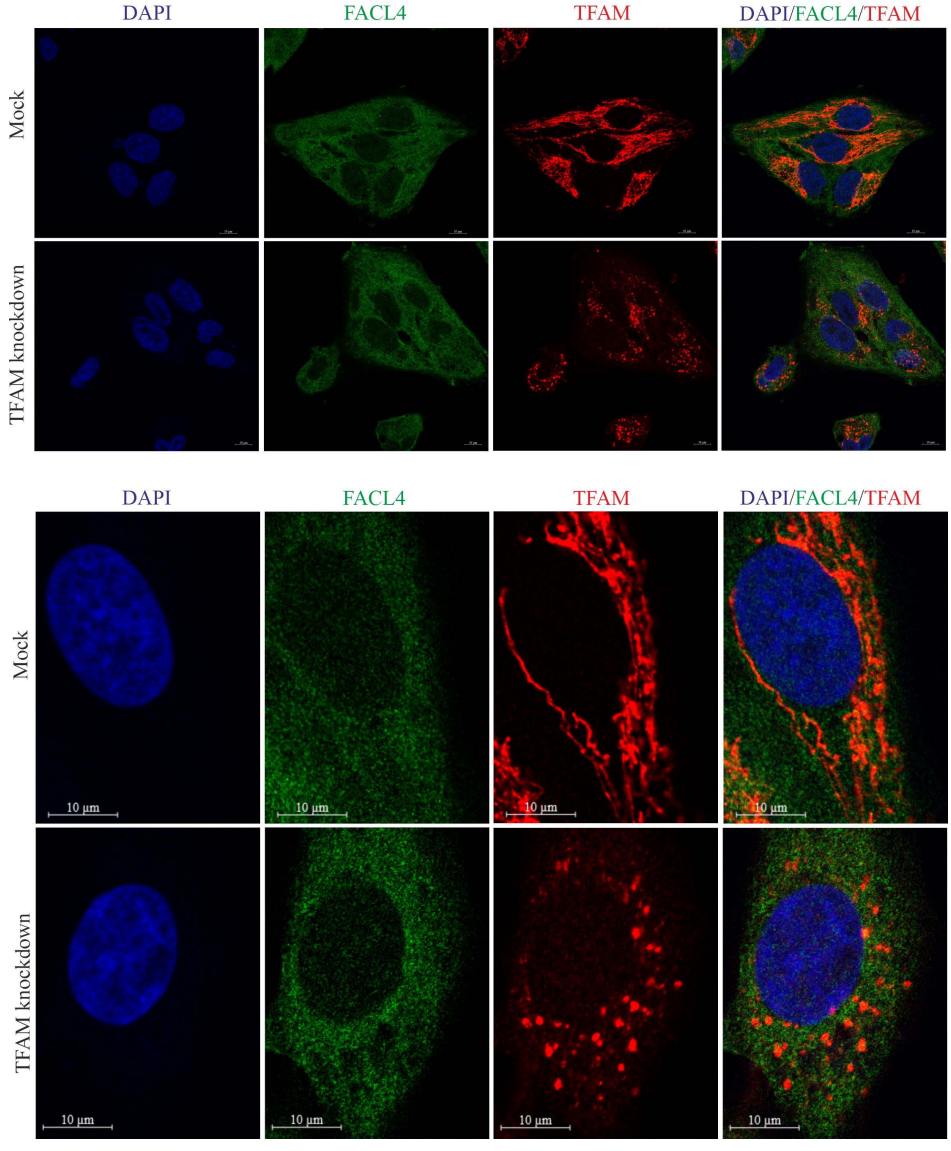

Figure 12. Confocal microscopy of U2OS cells transfected with *TFAM*-targeted and control siRNA. TFAM shows clear aggregation in knockdown U2OS cells (red). The ER-specific protein FACL4 did not exhibit clear changes (green). Knockdown of TFAM was confirmed by determining the expression level of TFAM by Western blotting.

Taken together these results indicate that mtDNA replication and segregation favorably occur at the sites of MAM contacts, corroborating the findings of Lewis et al., 2016. The reorganization of mitochondrial nucleoids via TFAM knockdown most likely triggers a compensatory response to maintain proper functioning of mitochondria (upregulation of TWNK and MAMs), which was not observed for the TWNK knockdown, i.e., TWNK knockdown at similar conditions showed weaker impact on both nucleoid and MAM proteins (Paper I, Figure 4A–4E). This is plausible since TFAM is considered as a constitutive nucleoid component, whereas TWNK dynamically associates with a small proportion of replicating nucleoids at the inner mitochondrial membrane and is also required for the maintenance of mtRNA granules.

CONCLUSIONS

The maintenance of mtDNA relies on faithful mtDNA and mtRNA metabolism, which involves for instance regulation of mtDNA topology by topoisomerases and functional gene expression system with essential role on mtRNA granules and mitochondrial ribosomes. Furthermore, mitochondrial homeostasis requires well-coordinated cellular and mitochondrial network dynamics (fusion and fission) to ensure proper segregation and allocation of nucleoids. These aspects were studied in this thesis and the following conclusions were made:

1. DNA topoisomerases Top2α and Top2β colocalized with mitochondrial replicating nucleoids, of which Top2β had a clear impact on mtDNA metabolism. Ablation of Top2β led to an accumulation of positively supercoiled mtDNA, impaired replication initiation and a decline in mtDNA copy number. Topoisomerase Top1mt showed only a partial co-existence with replicating nucleoids, majority of this protein was found together with mitochondrial ribosomal proteins and RNA polymerase (POLRMT), indicating its importance in mtRNA metabolism (transcription). Also, Top3α was found to interact with replicating nucleoids confirming its importance in mtDNA maintenance.

2. AlkBh1 protein, which has many enzymatic activities, particularly on RNA substrates, colocalized to mitochondrial RNA granules, which are hubs for the posttranscriptional processing of mtRNA and mitochondrial ribosome biogenesis. Ablation of AlkBh1 led to an inhibition of cell growth both in mammalian culture and in *C. elegans* and activated UPR^{mt} indicative of mitochondrial dysfunction.

3. The most stable component of mitochondrial nucleoids is TFAM, an abundant transcription factor in mitochondria that tightly compacts the entire mtDNA. The packaging of mtDNA by TFAM influences mtDNA transcription, translation, and segregation. The knockdown of TFAM caused an aggregation of mitochondrial nucleoids and a significant decrease in mtDNA copy number. Furthermore, depletion of TFAM resulted in increased levels of TWNK helicase and mtDNA in the membrane-associated fractions together with a transient enhancement of a specific MAM sub-compartment. Although a downregulation of a replicative helicase TWNK caused a reduction in mtDNA copy number and a moderate elevation of MAM protein levels in the membrane fractions, no aggregation of nucleoids was observed.

These results clearly demonstrate a tight interconnectedness of mtDNA and mtRNA metabolism, and mtDNA segregation via mitochondrial dynamics. Even though there are many unanswered questions about the molecular aspects of mtDNA maintenance and how this contributes to the development of mitochondrial and mtDNA-related diseases, this thesis provides many new details to this matter and opens new perspectives for future research.

REFERENCES

Alam, T.I., Kanki, T., Muta, T., Ukaji, K., Abe, Y., Nakayama, H., Takio, K., Hamasaki, N., Kang, D., 2003. Human mitochondrial DNA is packaged with TFAM. Nucleic Acids Res. 31, 1640–1645. https://doi.org/10.1093/nar/gkg251

Anand, R., Wai, T., Baker, M.J., Kladt, N., Schauss, A.C., Rugarli, E., Langer, T., 2014. The i-AAA protease YME1L and OMA1 cleave OPA1 to balance mitochondrial fusion and fission. J. Cell Biol. 204, 919–929. https://doi.org/10.1083/jcb.201308006

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. https://doi.org/10.1038/290457a0

Andersson, S.G., Zomorodipour, A., Andersson, J.O., Sicheritz-Pontén, T., Alsmark, U.C., Podowski, R.M., Näslund, A.K., Eriksson, A.S., Winkler, H.H., Kurland, C.G., 1998. The genome sequence of Rickettsia prowazekii and the origin of mitochondria. Nature 396, 133–140. https://doi.org/10.1038/24094

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. https://doi.org/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. https://doi.org/10.2353/ajpath.2009.090219

Area-Gomez, E., Del Carmen Lara Castillo, M., Tambini, M.D., Guardia-Laguarta, C., de Groof, A.J.C., Madra, M., Ikenouchi, J., Umeda, M., Bird, T.D., Sturley, S.L., Schon, E.A., 2012. Upregulated function of mitochondria-associated ER membranes in Alzheimer disease. EMBO J. 31, 4106–4123. https://doi.org/10.1038/emboj.2012.202

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. 110, 11863–11868. https://doi.org/10.1073/pnas.1301951110

Baranska, J., 1980. Phosphatidylserine biosynthesis in mitochondria from ehrlich ascites tumor cells. Biochim. Biophys. Acta BBA – Lipids Lipid Metab. 619, 258–266. https://doi.org/10.1016/0005-2760(80)90074-0

Barker et al., 1998. Phenol-Chloroform Isoamyl Alcohol (PCI) DNA Extraction. 1036–77.

Bereiter-Hahn, J., 1990. Behavior of Mitochondria in the Living Cell, in: International Review of Cytology. Elsevier, pp. 1–63. https://doi.org/10.1016/S0074-7696(08)61205-X

Berk, A.J., Clayton, D.A., 1974. Mechanism of mitochondrial DNA replication in mouse L-cells: Asynchronous replication of strands, segregation of circular daughter molecules, aspects of topology and turnover of an initiation sequence. J. Mol. Biol. 86, 801–824. https://doi.org/10.1016/0022-2836(74)90355-6

Bernhard, W., Rouiller, C., 1956. Close topographical relationship between mitochondria and ergastoplasm of liver cells in a definite phase of cellular activity. J. Biophys. Biochem. Cytol. 2, 73–78. https://doi.org/10.1083/jcb.2.4.73

Bogenhagen, D.F., Rousseau, D., Burke, S., 2008. The Layered Structure of Human Mitochondrial DNA Nucleoids. J. Biol. Chem. 283, 3665–3675. https://doi.org/10.1074/jbc.M708444200

Bogenhagen, D.F., Wang, Y., Shen, E.L., Kobayashi, R., 2003. Protein components of mitochondrial DNA nucleoids in higher eukaryotes. Mol. Cell. Proteomics MCP 2, 1205–1216. https://doi.org/10.1074/mcp.M300035-MCP200

Boldogh, I.R., Nowakowski, D.W., Yang, H.-C., Chung, H., Karmon, S., Royes, P., Pon, L.A., 2003. A protein complex containing Mdm10p, Mdm12p, and Mmm1p links mitochondrial membranes and DNA to the cytoskeleton-based segregation machinery. Mol. Biol. Cell 14, 4618–4627. https://doi.org/10.1091/mbc.e03-04-0225

Brown, T.A., 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. https://doi.org/10.1101/gad.1352105

Carrodeguas, J.A., Pinz, K.G., Bogenhagen, D.F., 2002. DNA Binding Properties of Human pol γB. J. Biol. Chem. 277, 50008–50014. https://doi.org/10.1074/jbc.M207030200

Cereghetti, G.M., Stangherlin, A., de Brito, O.M., Chang, C.R., Blackstone, C., Bernardi, P., Scorrano, L., 2008. Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria. Proc. Natl. Acad. Sci. 105, 15803–15808. https://doi.org/10.1073/pnas.0808249105

Chen, H., Vermulst, M., Wang, Y.E., Chomyn, A., Prolla, T.A., McCaffery, J.M., Chan, D.C., 2010. Mitochondrial Fusion Is Required for mtDNA Stability in Skeletal Muscle and Tolerance of mtDNA Mutations. Cell 141, 280–289. https://doi.org/10.1016/j.cell.2010.02.026

Chen, Y., Liu, Y., Dorn, G.W., 2011. Mitochondrial fusion is essential for organelle function and cardiac homeostasis. Circ. Res. 109, 1327–1331. https://doi.org/10.1161/CIRCRESAHA.111.258723

Cieri, D., Vicario, M., Giacomello, M., Vallese, F., Filadi, R., Wagner, T., Pozzan, T., Pizzo, P., Scorrano, L., Brini, M., Calì, T., 2018. SPLICS: a split green fluorescent protein-based contact site sensor for narrow and wide heterotypic organelle juxtaposition. Cell Death Differ. 25, 1131–1145. https://doi.org/10.1038/s41418-017-0033-z

Cipolat, S., de Brito, O.M., Dal Zilio, B., Scorrano, L., 2004. OPA1 requires mitofusin 1 to promote mitochondrial fusion. Proc. Natl. Acad. Sci. 101, 15927–15932. https://doi.org/10.1073/pnas.0407043101

Cortassa, S., Aon, M.A., Marbán, E., Winslow, R.L., O'Rourke, B., 2003. An integrated model of cardiac mitochondrial energy metabolism and calcium dynamics. Biophys. J. 84, 2734–2755. https://doi.org/10.1016/S0006-3495(03)75079-6

Cosson, P., Marchetti, A., Ravazzola, M., Orci, L., 2012. Mitofusin-2 independent juxtaposition of endoplasmic reticulum and mitochondria: an ultrastructural study. PloS One 7, e46293. https://doi.org/10.1371/journal.pone.0046293

Cribbs, J.T., Strack, S., 2007. Reversible phosphorylation of Drp1 by cyclic AMP-dependent protein kinase and calcineurin regulates mitochondrial fission and cell death. EMBO Rep. 8, 939–944. https://doi.org/10.1038/sj.embor.7401062

Csordás, G., Renken, C., Várnai, P., Walter, L., Weaver, D., Buttle, K.F., Balla, T., Mannella, C.A., Hajnóczky, G., 2006. Structural and functional features and significance of the physical linkage between ER and mitochondria. J. Cell Biol. 174, 915–921. https://doi.org/10.1083/jcb.200604016

Dalla Rosa, I., Zhang, H., Khiati, S., Wu, X., Pommier, Y., 2017. Transcription profiling suggests that mitochondrial topoisomerase IB acts as a topological barrier and regulator of mitochondrial DNA transcription. J. Biol. Chem. 292, 20162–20172. https://doi.org/10.1074/jbc.M117.815241

de Brito, O.M., Scorrano, L., 2008. Mitofusin 2 tethers endoplasmic reticulum to mitochondria. Nature 456, 605–610. https://doi.org/10.1038/nature07534

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. https://doi.org/10.1093/hmg/ddr559

Delettre, C., Lenaers, G., Griffoin, J.M., Gigarel, N., Lorenzo, C., Belenguer, P., Pelloquin, L., Grosgeorge, J., Turc-Carel, C., Perret, E., Astarie-Dequeker, C., Lasquellec, L., Arnaud, B., Ducommun, B., Kaplan, J., Hamel, C.P., 2000. Nuclear gene OPA1, encoding a mitochondrial dynamin-related protein, is mutated in dominant optic atrophy. Nat. Genet. 26, 207–210. https://doi.org/10.1038/79936

Desai, R., Frazier, A.E., Durigon, R., Patel, H., Jones, A.W., Dalla Rosa, I., Lake, N.J., Compton, A.G., Mountford, H.S., Tucker, E.J., Mitchell, A.L.R., Jackson, D., Sesay, A., Di Re, M., van den Heuvel, L.P., Burke, D., Francis, D., Lunke, S., McGillivray, G., Mandelstam, S., Mochel, F., Keren, B., Jardel, C., Turner, A.M., Ian Andrews, P., Smeitink, J., Spelbrink, J.N., Heales, S.J., Kohda, M., Ohtake, A., Murayama, K., Okazaki, Y., Lombès, A., Holt, I.J., Thorburn, D.R., Spinazzola, A., 2017. ATAD3 gene cluster deletions cause cerebellar dysfunction associated with altered mitochondrial DNA and cholesterol metabolism. Brain 140, 1595–1610. https://doi.org/10.1093/brain/awx094

Detmer, S.A., Chan, D.C., 2007. Complementation between mouse Mfn1 and Mfn2 protects mitochondrial fusion defects caused by CMT2A disease mutations. J. Cell Biol. 176, 405–414. https://doi.org/10.1083/jcb.200611080

Duxin, J.P., Dao, B., Martinsson, P., Rajala, N., Guittat, L., Campbell, J.L., Spelbrink, J.N., Stewart, S.A., 2009. Human Dna2 is a nuclear and mitochondrial DNA maintenance protein. Mol. Cell. Biol. 29, 4274–4282. https://doi.org/10.1128/MCB.01834-08

Ekstrand, M.I., 2004. Mitochondrial transcription factor A regulates mtDNA copy number in mammals. Hum. Mol. Genet. 13, 935–944. https://doi.org/10.1093/hmg/ddh109

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. https://doi.org/10.1101/gr.108696.110

Filadi, R., Greotti, E., Turacchio, G., Luini, A., Pozzan, T., Pizzo, P., 2015. Mitofusin 2 ablation increases endoplasmic reticulum–mitochondria coupling. Proc. Natl. Acad. Sci. 112, E2174–E2181. https://doi.org/10.1073/pnas.1504880112

Filadi, R., Pendin, D., Pizzo, P., 2018. Mitofusin 2: from functions to disease. Cell Death Dis. 9, 330. https://doi.org/10.1038/s41419-017-0023-6

Frazier, A.E., Thorburn, D.R., Compton, A.G., 2019. Mitochondrial energy generation disorders: genes, mechanisms, and clues to pathology. J. Biol. Chem. 294, 5386–5395. https://doi.org/10.1074/jbc.R117.809194

Fridlender, B., Fry, M., Bolden, A., Weissbach, A., 1972. A new synthetic RNA-dependent DNA polymerase from human tissue culture cells (HeLa-fibroblast-synthetic oligonucleotides-template-purified enzymes). Proc. Natl. Acad. Sci. U. S. A. 69, 452–455. https://doi.org/10.1073/pnas.69.2.452

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 334, 358–362. https://doi.org/10.1126/science.1207385

Fusté, J.M., Wanrooij, S., Jemt, E., Granycome, C.E., Cluett, T.J., Shi, Y., Atanassova, N., Holt, I.J., Gustafsson, C.M., Falkenberg, M., 2010. Mitochondrial RNA polymerase is needed for activation of the origin of light-strand DNA replication. Mol. Cell 37, 67–78. https://doi.org/10.1016/j.molcel.2009.12.021

Gaines, G., Attardi, G., 1984. Highly efficient RNA-synthesizing system that uses isolated human mitochondria: new initiation events and in vivo-like processing patterns. Mol. Cell. Biol. 4, 1605–1617. https://doi.org/10.1128/MCB.4.8.1605

Garrido, N., Griparic, L., Jokitalo, E., Wartiovaara, J., van der Bliek, A.M., Spelbrink, J.N., 2003. Composition and dynamics of human mitochondrial nucleoids. Mol. Biol. Cell 14, 1583–1596. https://doi.org/10.1091/mbc.e02-07-0399

Gensler, S., 2001. Mechanism of mammalian mitochondrial DNA replication: import of mitochondrial transcription factor A into isolated mitochondria stimulates 7S DNA synthesis. Nucleic Acids Res. 29, 3657–3663. https://doi.org/10.1093/nar/29.17.3657

Gerhold, J.M., Cansiz-Arda, Ş., Lõhmus, M., Engberg, O., Reyes, A., van Rennes, H., Sanz, A., Holt, I.J., Cooper, H.M., Spelbrink, J.N., 2015. Human Mitochondrial DNA-Protein Complexes Attach to a Cholesterol-Rich Membrane Structure. Sci. Rep. 5, 15292. https://doi.org/10.1038/srep15292

Gilquin, B., Taillebourg, E., Cherradi, N., Hubstenberger, A., Gay, O., Merle, N., Assard, N., Fauvarque, M.-O., Tomohiro, S., Kuge, O., Baudier, J., 2010. The AAA+ ATPase ATAD3A Controls Mitochondrial Dynamics at the Interface of the Inner and Outer Membranes. Mol. Cell. Biol. 30, 1984–1996. https://doi.org/10.1128/MCB.00007-10

Gincel, D., Zaid, H., Shoshan-Barmatz, V., 2001. Calcium binding and translocation by the voltage-dependent anion channel: a possible regulatory mechanism in mitochondrial function. Biochem. J. 358, 147–155. https://doi.org/10.1042/0264-6021:3580147

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. https://doi.org/10.1038/348651a0

Gray, M.W., 1989. The evolutionary origins of organelles. Trends Genet. TIG 5, 294–299. https://doi.org/10.1016/0168-9525(89)90111-x

Gray, M.W., Burger, G., Lang, B.F., 2001. The origin and early evolution of mitochondria. Genome Biol. 2, reviews1018.1. https://doi.org/10.1186/gb-2001-2-6-reviews1018

Grünewald, A., Rygiel, K.A., Hepplewhite, P.D., Morris, C.M., Picard, M., Turnbull, D.M., 2016. Mitochondrial DNA Depletion in Respiratory Chain-Deficient Parkinson Disease Neurons. Ann. Neurol. 79, 366–378. https://doi.org/10.1002/ana.24571

Haag, S., Sloan, K.E., Ranjan, N., Warda, A.S., Kretschmer, J., Blessing, C., Hübner, B., Seikowski, J., Dennerlein, S., Rehling, P., Rodnina, M.V., Höbartner, C., Bohnsack, M.T., 2016. NSUN 3 and ABH 1 modify the wobble position of mt-t RNA ^{Met} to expand codon recognition in mitochondrial translation. EMBO J. 35, 2104–2119. https://doi.org/10.15252/embj.201694885

Hanekamp, T., Thorsness, M.K., Rebbapragada, I., Fisher, E.M., Seebart, C., Darland, M.R., Coxbill, J.A., Updike, D.L., Thorsness, P.E., 2002. Maintenance of mitochondrial morphology is linked to maintenance of the mitochondrial genome in Saccharomyces cerevisiae. Genetics 162, 1147–1156.

He, J., Cooper, H.M., Reyes, A., Di Re, M., Sembongi, H., Litwin, T.R., Gao, J., Neuman, K.C., Fearnley, I.M., Spinazzola, A., Walker, J.E., Holt, I.J., 2012. Mitochondrial nucleoid interacting proteins support mitochondrial protein synthesis. Nucleic Acids Res. 40, 6109–6121. https://doi.org/10.1093/nar/gks266

He, J., Mao, C.-C., Reyes, A., Sembongi, H., Di Re, M., Granycome, C., Clippingdale, A.B., Fearnley, I.M., Harbour, M., Robinson, A.J., Reichelt, S., Spelbrink, J.N., Walker, J.E., Holt, I.J., 2007. The AAA+ protein ATAD3 has displacement loop binding properties and is involved in mitochondrial nucleoid organization. J. Cell Biol. 176, 141–146. https://doi.org/10.1083/jcb.200609158

Hensen, F., Cansiz, S., Gerhold, J.M., Spelbrink, J.N., 2014. To be or not to be a nucleoid protein: A comparison of mass-spectrometry based approaches in the identification of potential mtDNA-nucleoid associated proteins. Biochimie 100, 219–226. https://doi.org/10.1016/j.biochi.2013.09.017

Hensen, F., Potter, A., van Esveld, S.L., Tarrés-Solé, A., Chakraborty, A., Solà, M., Spelbrink, J.N., 2019. Mitochondrial RNA granules are critically dependent on mtDNA replication factors Twinkle and mtSSB. Nucleic Acids Res. 47, 3680–3698. https://doi.org/10.1093/nar/gkz047

Herrera-Cruz, M.S., Simmen, T., 2017. Cancer: Untethering Mitochondria from the Endoplasmic Reticulum? Front. Oncol. 7, 105. https://doi.org/10.3389/fonc.2017.00105

Hobbs, A.E.A., Srinivasan, M., McCaffery, J.M., Jensen, R.E., 2001. Mmm1p, a Mitochondrial Outer Membrane Protein, Is Connected to Mitochondrial DNA (Mtdna) Nucleoids and Required for Mtdna Stability. J. Cell Biol. 152, 401–410. https://doi.org/10.1083/jcb.152.2.401

Hoke, G.D., Pavco, P.A., Ledwith, B.J., Van Tuyle, G.C., 1990. Structural and functional studies of the rat mitochondrial single strand DNA binding protein P16. Arch. Biochem. Biophys. 282, 116–124. https://doi.org/10.1016/0003-9861(90)90094-f

Holt, I.J., Harding, A.E., Morgan-Hughes, J.A., 1988. Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. Nature 331, 717–719. https://doi.org/10.1038/331717a0

Holt, I.J., He, J., Mao, C.-C., Boyd-Kirkup, J.D., Martinsson, P., Sembongi, H., Reyes, A., Spelbrink, J.N., 2007. Mammalian mitochondrial nucleoids: organizing an independently minded genome. Mitochondrion 7, 311–321. https://doi.org/10.1016/j.mito.2007.06.004

Honrath, B., Metz, I., Bendridi, N., Rieusset, J., Culmsee, C., Dolga, A.M., 2017. Glucose-regulated protein 75 determines ER–mitochondrial coupling and sensitivity to oxidative stress in neuronal cells. Cell Death Discov. 3, 17076. https://doi.org/10.1038/cddiscovery.2017.76

Hutchinson, J., 1879. On Ophthalmoplegia Externa, or Symmetrical Immobility (partial) of the Eyes, with Ptosis. Medico-Chir. Trans. 62, 307–329. https://doi.org/10.1177/095952877906200120

Ishihara, N., 2004. Mitofusin 1 and 2 play distinct roles in mitochondrial fusion reactions via GTPase activity. J. Cell Sci. 117, 6535–6546. https://doi.org/10.1242/jcs.01565

Issop, L., Fan, J., Lee, S., Rone, M.B., Basu, K., Mui, J., Papadopoulos, V., 2015. Mitochondria-Associated Membrane Formation in Hormone-Stimulated Leydig Cell Steroidogenesis: Role of ATAD3. Endocrinology 156, 334–345. https://doi.org/10.1210/en.2014-1503

Joost, K., Rodenburg, R.J., Piirsoo, A., van den Heuvel, L., Zordania, R., Põder, H., Talvik, I., Kilk, K., Soomets, U., Ounap, K., 2012. A Diagnostic Algorithm for Mitochondrial Disorders in Estonian Children. Mol. Syndromol. 3, 113–119. https://doi.org/10.1159/000341375

Jourdain, A.A., Koppen, M., Wydro, M., Rodley, C.D., Lightowlers, R.N., Chrzanowska-Lightowlers, Z.M., Martinou, J.-C., 2013. GRSF1 regulates RNA processing in mitochondrial RNA granules. Cell Metab. 17, 399–410. https://doi.org/10.1016/j.cmet.2013.02.005

Kasamatsu, H., Robberson, D.L., Vinograd, J., 1971. A Novel Closed-Circular Mitochondrial DNA with Properties of a Replicating Intermediate. Proc. Natl. Acad. Sci. 68, 2252–2257. https://doi.org/10.1073/pnas.68.9.2252

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, 210–220. https://doi.org/10.1016/j.yexcr.2010.10.008

Kaufman, B.A., Durisic, N., Mativetsky, J.M., Costantino, S., Hancock, M.A., Grutter, P., Shoubridge, E.A., 2007. The Mitochondrial Transcription Factor TFAM Coordinates the Assembly of Multiple DNA Molecules into Nucleoid-like Structures. Mol. Biol. Cell 18, 3225–3236. https://doi.org/10.1091/mbc.e07-05-0404

Kim, J., Moody, J.P., Edgerly, C.K., Bordiuk, O.L., Cormier, K., Smith, K., Beal, M.F., Ferrante, R.J., 2010. Mitochondrial loss, dysfunction and altered dynamics in Huntington's disease. Hum. Mol. Genet. 19, 3919–3935. https://doi.org/10.1093/hmg/ddq306

Korhonen, J.A., Gaspari, M., Falkenberg, M., 2003. TWINKLE Has 5' –> 3' DNA helicase activity and is specifically stimulated by mitochondrial single-stranded DNA-binding protein. J. Biol. Chem. 278, 48627–48632. https://doi.org/10.1074/jbc.M306981200

Korhonen, J.A., Pham, X.H., Pellegrini, M., Falkenberg, M., 2004. Reconstitution of a minimal mtDNA replisome in vitro. EMBO J. 23, 2423–2429. https://doi.org/10.1038/sj.emboj.7600257

Kornmann, B., Currie, E., Collins, S.R., Schuldiner, M., Nunnari, J., Weissman, J.S., Walter, P., 2009. An ER-Mitochondria Tethering Complex Revealed by a Synthetic Biology Screen. Science 325, 477–481. https://doi.org/10.1126/science.1175088

Kukat, C., Davies, K.M., Wurm, C.A., Spåhr, H., Bonekamp, N.A., Kühl, I., Joos, F., Polosa, P.L., Park, C.B., Posse, V., Falkenberg, M., Jakobs, S., Kühlbrandt, W., Larsson, N.-G., 2015. Cross-strand binding of TFAM to a single mtDNA molecule forms the mitochondrial nucleoid. Proc. Natl. Acad. Sci. 112, 11288–11293. https://doi.org/10.1073/pnas.1512131112

Kukat, C., Wurm, C.A., Spahr, 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. 108, 13534–13539. https://doi.org/10.1073/pnas.1109263108

Lamantea, E., Tiranti, V., Bordoni, A., Toscano, A., Bono, F., Servidei, S., Papadimitriou, A., Spelbrink, H., Silvestri, L., Casari, G., Comi, G.P., Zeviani, M., 2002. Mutations of mitochondrial DNA polymerase gammaA are a frequent cause of autosomal dominant or recessive progressive external ophthalmoplegia. Ann. Neurol. 52, 211–219. https://doi.org/10.1002/ana.10278

Larsson, N.-G., Wang, J., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., Barsh, G.S., Clayton, D.A., 1998. Mitochondrial transcription factor A is necessary for mtDNA maintance and embryogenesis in mice. Nat. Genet. 18, 231–236. https://doi.org/10.1038/ng0398-231

Lee, S., Min, K.-T., 2018. The Interface Between ER and Mitochondria: Molecular Compositions and Functions. Mol. Cells 41, 1000–1007. https://doi.org/10.14348/molcells.2018.0438

Legros, F., Malka, F., Frachon, P., Lombès, A., Rojo, M., 2004. Organization and dynamics of human mitochondrial DNA. J. Cell Sci. 117, 2653–2662. https://doi.org/10.1242/jcs.01134

Lestienne, P., Ponsot, G., 1988. Kearns-Sayre syndrome with muscle mitochondrial DNA deletion. Lancet Lond. Engl. 1, 885. https://doi.org/10.1016/s0140-6736(88)91632-7

Lewis, S.C., Uchiyama, L.F., Nunnari, J., 2016. ER-mitochondria contacts couple mtDNA synthesis with mitochondrial division in human cells. Science 353, aaf5549–aaf5549. https://doi.org/10.1126/science.aaf5549

Liu, F., Clark, W., Luo, G., Wang, Xiaoyun, Fu, Y., Wei, J., Wang, Xiao, Hao, Z., Dai, Q., Zheng, G., Ma, H., Han, D., Evans, M., Klungland, A., Pan, T., He, C., 2016. ALKBH1-Mediated tRNA Demethylation Regulates Translation. Cell 167, 816-828.e16. https://doi.org/10.1016/j.cell.2016.09.038

Liu, J., Rone, M.B., Papadopoulos, V., 2006. Protein-Protein Interactions Mediate Mitochondrial Cholesterol Transport and Steroid Biosynthesis. J. Biol. Chem. 281, 38879–38893. https://doi.org/10.1074/jbc.M608820200

Longley, M.J., Nguyen, D., Kunkel, T.A., Copeland, W.C., 2001. The Fidelity of Human DNA Polymerase γ with and without Exonucleolytic Proofreading and the p55 Accessory Subunit. J. Biol. Chem. 276, 38555–38562. https://doi.org/10.1074/jbc.M105230200

Lu, B., Yadav, S., Shah, P.G., Liu, T., Tian, B., Pukszta, S., Villaluna, N., Kutejová, E., Newlon, C.S., Santos, J.H., Suzuki, C.K., 2007. Roles for the human ATP-dependent Lon protease in mitochondrial DNA maintenance. J. Biol. Chem. 282, 17363–17374. https://doi.org/10.1074/jbc.M611540200

Macao, B., Uhler, J.P., Siibak, T., Zhu, X., Shi, Y., Sheng, W., Olsson, M., Stewart, J.B., Gustafsson, C.M., Falkenberg, M., 2015. The exonuclease activity of DNA polymerase γ is required for ligation during mitochondrial DNA replication. Nat. Commun. 6, 7303. https://doi.org/10.1038/ncomms8303

Maniura-Weber, K., Goffart, S., Garstka, H.L., Montoya, J., Wiesner, R.J., 2004. Transient overexpression of mitochondrial transcription factor A (TFAM) is sufficient to stimulate mitochondrial DNA transcription, but not sufficient to increase mtDNA copy number in cultured cells. Nucleic Acids Res. 32, 6015–6027. https://doi.org/10.1093/nar/gkh921

Manor, U., Bartholomew, S., Golani, G., Christenson, E., Kozlov, M., Higgs, H., Spudich, J., Lippincott-Schwartz, J., 2015. A mitochondria-anchored isoform of the actin-nucleating spire protein regulates mitochondrial division. eLife 4, e08828. https://doi.org/10.7554/eLife.08828

Margulis, L., 1981. Symbiosis in cell evolution: Life and its environment on the early earth.

Margulis, L., 1970. Origin of eukaryotic cells: Evidence and research implications for a theory of the origin and evolution of microbial, plant and animal cells on the precambrian Earth. Yale University Press.

Martens, P.A., Clayton, D.A., 1979. Mechanism of mitochondrial DNA replication in mouse L-cells: localization and sequence of the light-strand origin of replication. J. Mol. Biol. 135, 327–351. https://doi.org/10.1016/0022-2836(79)90440-6

McCormack, J.G., Denton, R.M., 1984. Role of Ca2+ ions in the regulation of intramitochondrial metabolism in rat heart. Evidence from studies with isolated mitochondria that adrenaline activates the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes by increasing the intramitochondrial concentration of Ca2+. Biochem. J. 218, 235–247. https://doi.org/10.1042/bj2180235

Meeusen, S., Nunnari, J., 2003. Evidence for a two membrane–spanning autonomous mitochondrial DNA replisome. J. Cell Biol. 163, 503–510. https://doi.org/10.1083/jcb.200304040

Mignotte, B., Marsault, J., Barat-Gueride, M., 1988. Effects of the Xenopus laevis mitochondrial single-stranded DNA-binding protein on the activity of DNA polymerase gamma. Eur. J. Biochem. 174, 479–484. https://doi.org/10.1111/j.1432-1033.1988.tb14123.x

Minczuk, M., He, J., Duch, A.M., Ettema, T.J., Chlebowski, A., Dzionek, K., Nijtmans, L.G.J., Huynen, M.A., Holt, I.J., 2011. TEFM (c17orf42) is necessary for transcription of human mtDNA. Nucleic Acids Res. 39, 4284–4299. https://doi.org/10.1093/nar/gkq1224

Murley, A., Lackner, L.L., Osman, C., West, M., Voeltz, G.K., Walter, P., Nunnari, J., 2013. ER-associated mitochondrial division links the distribution of mitochondria and mitochondrial DNA in yeast. eLife 2, e00422. https://doi.org/10.7554/eLife.00422

Naia, L., Ferreira, I.L., Ferreiro, E., Rego, A.C., 2017. Mitochondrial Ca2+ handling in Huntington's and Alzheimer's diseases – Role of ER-mitochondria crosstalk. Biochem. Biophys. Res. Commun. 483, 1069–1077. https://doi.org/10.1016/j.bbrc.2016.07.122

Naon, D., Zaninello, M., Giacomello, M., Varanita, T., Grespi, F., Lakshminaranayan, S., Serafini, A., Semenzato, M., Herkenne, S., Hernández-Alvarez, M.I., Zorzano, A., De Stefani, D., Dorn, G.W., Scorrano, L., 2016. Critical reappraisal confirms that Mitofusin 2 is an endoplasmic reticulum–mitochondria tether. Proc. Natl. Acad. Sci. 113, 11249–11254. https://doi.org/10.1073/pnas.1606786113

Nass, M.M., 1969. Mitochondrial DNA. II. Structure and physicochemical properties of isolated DNA. J. Mol. Biol. 42, 529–545. https://doi.org/10.1016/0022-2836(69)90241-1

Nicholls, T.J., Gustafsson, C.M., 2018. Separating and Segregating the Human Mitochondrial Genome. Trends Biochem. Sci. 43, 869–881. https://doi.org/10.1016/j.tibs.2018.08.007

Nicholls, T.J., Nadalutti, C.A., Motori, E., Sommerville, E.W., Gorman, G.S., Basu, S., Hoberg, E., Turnbull, D.M., Chinnery, P.F., Larsson, N.-G., Larsson, E., Falkenberg, M., Taylor, R.W., Griffith, J.D., Gustafsson, C.M., 2018. Topoisomerase 3α Is Required for Decatenation and Segregation of Human mtDNA. Mol. Cell 69, 9–23.e6. https://doi.org/10.1016/j.molcel.2017.11.033

Oka, S., Leon, J., Sakumi, K., Ide, T., Kang, D., LaFerla, F.M., Nakabeppu, Y., 2016. Human mitochondrial transcriptional factor A breaks the mitochondria-mediated vicious cycle in Alzheimer's disease. Sci. Rep. 6, 37889. https://doi.org/10.1038/srep37889

Olichon, A., Emorine, L.J., Descoins, E., Pelloquin, L., Brichese, L., Gas, N., Guillou, E., Delettre, C., Valette, A., Hamel, C.P., Ducommun, B., Lenaers, G., Belenguer, P., 2002. The human dynamin-related protein OPA1 is anchored to the mitochondrial inner membrane facing the inter-membrane space. FEBS Lett. 523, 171–176. https://doi.org/10.1016/s0014-5793(02)02985-x

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. https://doi.org/10.1083/jcb.201007152

Oxenoid, K., Dong, Y., Cao, C., Cui, T., Sancak, Y., Markhard, A.L., Grabarek, Z., Kong, L., Liu, Z., Ouyang, B., Cong, Y., Mootha, V.K., Chou, J.J., 2016. Architecture of the mitochondrial calcium uniporter. Nature 533, 269–273. https://doi.org/10.1038/nature17656

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. https://doi.org/10.1038/embor.2011.54

Papanicolaou, K.N., Kikuchi, R., Ngoh, G.A., Coughlan, K.A., Dominguez, I., Stanley, W.C., Walsh, K., 2012. Mitofusins 1 and 2 are essential for postnatal metabolic remodeling in heart. Circ. Res. 111, 1012–1026. https://doi.org/10.1161/CIRCRESAHA.112.274142

Patergnani, S., Suski, J.M., Agnoletto, C., Bononi, A., Bonora, M., De Marchi, E., Giorgi, C., Marchi, S., Missiroli, S., Poletti, F., Rimessi, A., Duszynski, J., Wieckowski, M.R., Pinton, P., 2011. Calcium signaling around Mitochondria Associated Membranes (MAMs). Cell Commun. Signal. CCS 9, 19. https://doi.org/10.1186/1478-811X-9-19

Pellegrini, M., Asin-Cayuela, J., Erdjument-Bromage, H., Tempst, P., Larsson, N.-G., Gustafsson, C.M., 2009. MTERF2 is a nucleoid component in mammalian mitochondria. Biochim. Biophys. Acta 1787, 296–302. https://doi.org/10.1016/j.bbabio.2009.01.018

Pera, M., Larrea, D., Guardia-Laguarta, C., Montesinos, J., Velasco, K.R., Agrawal, R.R., Xu, Y., Chan, R.B., Di Paolo, G., Mehler, M.F., Perumal, G.S., Macaluso, F.P., Freyberg, Z.Z., Acin-Perez, R., Enriquez, J.A., Schon, E.A., Area-Gomez, E., 2017. Increased localization of APP-C99 in mitochondria-associated ER membranes causes mitochondrial dysfunction in Alzheimer disease. EMBO J. 36, 3356–3371. https://doi.org/10.15252/embj.201796797

Peralta, S., Goffart, S., Williams, S.L., Diaz, F., Garcia, S., Nissanka, N., Area-Gomez, E., Pohjoismäki, J., Moraes, C.T., 2018. ATAD3 controls mitochondrial cristae structure in mouse muscle, influencing mtDNA replication and cholesterol levels. J. Cell Sci. 131, jcs217075. https://doi.org/10.1242/jcs.217075

Pohjoismäki, J.L.O., Goffart, S., 2011. Of circles, forks and humanity: Topological organisation and replication of mammalian mitochondrial DNA. BioEssays 33, 290–299. https://doi.org/10.1002/bies.201000137

Pohjoismäki, J.L.O., Wanrooij, S., Hyvärinen, A.K., Goffart, S., Holt, I.J., Spelbrink, J.N., Jacobs, H.T., 2006. Alterations to the expression level of mitochondrial transcription factor A, TFAM, modify the mode of mitochondrial DNA replication in cultured human cells. Nucleic Acids Res. 34, 5815–5828. https://doi.org/10.1093/nar/gkl703

Polyak, E., Zhang, Z., Falk, M.J., 2012. Molecular profiling of mitochondrial dysfunction in Caenorhabditis elegans. Methods Mol. Biol. Clifton NJ 837, 241–255. https://doi.org/10.1007/978-1-61779-504-6_17

Radloff, R., Bauer, W., Vinograd, J., 1967. A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Proc. Natl. Acad. Sci. 57, 1514–1521. https://doi.org/10.1073/pnas.57.5.1514

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. https://doi.org/10.1093/nar/gkt988

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. https://doi.org/10.1093/nar/gkr052

Reynier, P., May-Panloup, P., Chrétien, M.F., Morgan, C.J., Jean, M., Savagner, F., Barrière, P., Malthièry, Y., 2001. Mitochondrial DNA content affects the fertilizability of human oocytes. Mol. Hum. Reprod. 7, 425–429. https://doi.org/10.1093/molehr/7.5.425

Rice, A.C., Keeney, P.M., Algarzae, N.K., Ladd, A.C., Thomas, R.R., Bennett Jr., J.P., 2014. Mitochondrial DNA Copy Numbers in Pyramidal Neurons are Decreased and Mitochondrial Biogenesis Transcriptome Signaling is Disrupted in Alzheimer's Disease Hippocampi. J. Alzheimers Dis. 40, 319–330. https://doi.org/10.3233/JAD-131715

Rizzuto, R., 1998. Close Contacts with the Endoplasmic Reticulum as Determinants of Mitochondrial Ca2+ Responses. Science 280, 1763–1766. https://doi.org/10.1126/science.280.5370.1763

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. https://doi.org/10.1073/pnas.69.3.737

Ruhanen, H., Borrie, S., Szabadkai, G., Tyynismaa, H., Jones, A.W.E., Kang, D., Taanman, J.-W., Yasukawa, T., 2010. Mitochondrial single-stranded DNA binding protein is required for maintenance of mitochondrial DNA and 7S DNA but is not required for mitochondrial nucleoid organisation. Biochim. Biophys. Acta BBA – Mol. Cell Res. 1803, 931–939. https://doi.org/10.1016/j.bbamcr.2010.04.008

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. https://doi.org/10.1016/S0021-9258(18)47012-3

Rutkowski, D.T., Kaufman, R.J., 2004. A trip to the ER: coping with stress. Trends Cell Biol. 14, 20–28. https://doi.org/10.1016/j.tcb.2003.11.001

Santel, A., Fuller, M.T., 2001. Control of mitochondrial morphology by a human mitofusin. J. Cell Sci. 114, 867–874.

Sasaki, T., Sato, Y., Higashiyama, T., Sasaki, N., 2017. Live imaging reveals the dynamics and regulation of mitochondrial nucleoids during the cell cycle in Fucci2-HeLa cells.

Satoh, M., Hamamoto, T., Seo, N., Kagawa, Y., Endo, H., 2003. Differential sub-localization of the dynamin-related protein OPA1 isoforms in mitochondria. Biochem. Biophys. Res. Commun. 300, 482–493. https://doi.org/10.1016/s0006-291x(02)02874-7

Schaefer, A.M., McFarland, R., Blakely, E.L., He, L., Whittaker, R.G., Taylor, R.W., Chinnery, P.F., Turnbull, D.M., 2008. Prevalence of mitochondrial DNA disease in adults. Ann. Neurol. 63, 35–39. https://doi.org/10.1002/ana.21217

Schein, S.J., Colombini, M., Finkelstein, A., 1976. Reconstitution in planar lipid bilayers of a voltage-dependent anion-selective channel obtained from paramecium mitochondria. J. Membr. Biol. 30, 99–120.

Sharma, N., Arora, S., Saurav, S., Motiani, R.K., 2020. Pathophysiological significance of calcium signaling at Mitochondria-Associated Endoplasmic Reticulum Membranes (MAMs). Curr. Opin. Physiol. 17, 234–242. https://doi.org/10.1016/j.cophys.2020.08.012

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. https://doi.org/10.1016/0092-8674(90)90059-n

Silva Ramos, E., Motori, E., Brüser, C., Kühl, I., Yeroslaviz, A., Ruzzenente, B., Kauppila, J.H.K., Busch, J.D., Hultenby, K., Habermann, B.H., Jakobs, S., Larsson, N.-G., Mourier, A., 2019. Mitochondrial fusion is required for regulation of mitochondrial DNA replication. PLOS Genet. 15, e1008085. https://doi.org/10.1371/journal.pgen.1008085

Simmen, T., Aslan, J.E., Blagoveshchenskaya, A.D., Thomas, L., Wan, L., Xiang, Y., Feliciangeli, S.F., Hung, C.-H., Crump, C.M., Thomas, G., 2005. PACS-2 controls endoplasmic reticulum–mitochondria communication and Bid-mediated apoptosis. EMBO J. 24, 717–729. https://doi.org/10.1038/sj.emboj.7600559

Smirnova, E., Shurland, D.L., Ryazantsev, S.N., van der Bliek, A.M., 1998. A human dynamin-related protein controls the distribution of mitochondria. J. Cell Biol. 143, 351–358. https://doi.org/10.1083/jcb.143.2.351

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, 749–755. https://doi.org/10.1083/jcb.200704110

Sood, A., Jeyaraju, D.V., Prudent, J., Caron, A., Lemieux, P., McBride, H.M., Laplante, M., Tóth, K., Pellegrini, L., 2014. A Mitofusin-2-dependent inactivating cleavage of Opa1 links changes in mitochondria cristae and ER contacts in the postprandial liver. Proc. Natl. Acad. Sci. U. S. A. 111, 16017–16022. https://doi.org/10.1073/pnas.1408061111

Spelbrink, J.N., 2009. Functional organization of mammalian mitochondrial DNA in nucleoids: History, recent developments, and future challenges. IUBMB Life n/a-n/a. https://doi.org/10.1002/iub.282

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. https://doi.org/10.1038/90058

Steuerwald, N., Barritt, J.A., Adler, R., Malter, H., Schimmel, T., Cohen, J., Brenner, C.A., 2000. Quantification of mtDNA in single oocytes, polar bodies and subcellular components by real-time rapid cycle fluorescence monitored PCR. Zygote Camb. Engl. 8, 209–215. https://doi.org/10.1017/s0967199400001003

Stoica, R., De Vos, K.J., Paillusson, S., Mueller, S., Sancho, R.M., Lau, K.-F., Vizcay-Barrena, G., Lin, W.-L., Xu, Y.-F., Lewis, J., Dickson, D.W., Petrucelli, L., Mitchell, J.C., Shaw, C.E., Miller, C.C.J., 2014. ER–mitochondria associations are regulated by the VAPB–PTPIP51 interaction and are disrupted by ALS/FTD-associated TDP-43. Nat. Commun. 5, 3996. https://doi.org/10.1038/ncomms4996

Stroud, D.A., Oeljeklaus, S., Wiese, S., Bohnert, M., Lewandrowski, U., Sickmann, A., Guiard, B., van der Laan, M., Warscheid, B., Wiedemann, N., 2011. Composition and Topology of the Endoplasmic Reticulum–Mitochondria Encounter Structure. J. Mol. Biol. 413, 743–750. https://doi.org/10.1016/j.jmb.2011.09.012

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. https://doi.org/10.1083/jcb.200608073

Tan, J., Wagner, M., Stenton, S.L., Strom, T.M., Wortmann, S.B., Prokisch, H., Meitinger, T., Oexle, K., Klopstock, T., 2020. Corrigendum to "Lifetime risk of autosomal recessive mitochondrial disorders calculated from genetic databases" [EBioMedicine 54 (2020) 102730]. EBioMedicine 61, 103072. https://doi.org/10.1016/j.ebiom.2020.103072

Tan, W., Colombini, M., 2007. VDAC closure increases calcium ion flux. Biochim. Biophys. Acta BBA – Biomembr. 1768, 2510–2515. https://doi.org/10.1016/j.bbamem.2007.06.002

Thal, D.R., Ronisz, A., Tousseyn, T., Rijal Upadhaya, A., Balakrishnan, K., Vandenberghe, R., Vandenbulcke, M., von Arnim, C.A.F., Otto, M., Beach, T.G., Lilja, J., Heurling, K., Chakrabarty, A., Ismail, A., Buckley, C., Smith, A.P.L., Kumar, S., Farrar, G., Walter, J., 2019. Different aspects of Alzheimer's disease-related amyloid β-peptide pathology and their relationship to amyloid positron emission tomography imaging and dementia. Acta Neuropathol. Commun. 7, 178. https://doi.org/10.1186/s40478-019-0837-9

Tiivoja, E., Õunap, K., Muru, K., Kool, P., Reinson, K., Rähn, K., 2020. Pärilike ainevahetushaiguste diagnoosimise kokkuvõte ajavahemikul 1990–2017 TÜ Kliinikumi ühendlabori kliinilise geneetika keskuses. Eesti Arst 99, 537–545.

Tiranti, V., Rocchi, M., DiDonato, S., Zeviani, M., 1993. Cloning of human and rat cDNAs encoding the mitochondrial single-stranded DNA-binding protein (SSB). Gene 126, 219–225. https://doi.org/10.1016/0378-1119(93)90370-I

Tubbs, E., Rieusset, J., 2016. Study of Endoplasmic Reticulum and Mitochondria Interactions by In Situ Proximity Ligation Assay in Fixed Cells. J. Vis. Exp. JoVE. https://doi.org/10.3791/54899

Valm, A.M., Cohen, S., Legant, W.R., Melunis, J., Hershberg, U., Wait, E., Cohen, A.R., Davidson, M.W., Betzig, E., Lippincott-Schwartz, J., 2017. Applying systems-level spectral imaging and analysis to reveal the organelle interactome. Nature 546, 162–167. https://doi.org/10.1038/nature22369

Vance, J.E., 1990a. Phospholipid synthesis in a membrane fraction associated with mitochondria. J. Biol. Chem. 265, 7248–7256.

Vance, J.E., 1990b. Phospholipid synthesis in a membrane fraction associated with mitochondria. J. Biol. Chem. 265, 7248–7256.

Vance, J.E., Vance, D.E., 1986. Specific pools of phospholipids are used for lipoprotein secretion by cultured rat hepatocytes. J. Biol. Chem. 261, 4486–4491.
Vergano, S.S., Rao, M., McCormack, S., Ostrovsky, J., Clarke, C., Preston, J., Bennett, M.J., Yudkoff, M., Xiao, R., Falk, M.J., 2014. In vivo metabolic flux profiling with stable isotopes discriminates sites and quantifies effects of mitochondrial dysfunction in C. elegans. Mol. Genet. Metab. 111, 331–341. https://doi.org/10.1016/j.ymgme.2013.12.011
Vila, M., Ramonet, D., Perier, C., 2008. Mitochondrial alterations in Parkinson's disease: new clues. J. Neurochem. 107, 317–328. https://doi.org/10.1111/j.1471-4159.2008.05604.x
Voelker, D.R., 1989. Phosphatidylserine translocation to the mitochondrion is an ATP-dependent process in permeabilized animal cells. Proc. Natl. Acad. Sci. U. S. A. 86, 9921–9925. https://doi.org/10.1073/pnas.86.24.9921
von Graefe, 1856. Verhandlungen ärztlicher Gesellschaften. Berl. Klin. Wochenschr. 5.
Wallace, D.C., 1993. Mitochondrial diseases: genotype versus phenotype. Trends Genet. TIG 9, 128–133. https://doi.org/10.1016/0168-9525(93)90207-x
Wallace, D.C., 1986. Mitotic segregation of mitochondrial DNAs in human cell hybrids and expression of chloramphenicol resistance. Somat. Cell Mol. Genet. 12, 41–49. https://doi.org/10.1007/BF01560726
Wallace, D.C., Singh, G., Lott, M.T., Hodge, J.A., Schurr, T.G., Lezza, A.M., Elsas, L.J., Nikoskelainen, E.K., 1988. Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. Science 242, 1427–1430. https://doi.org/10.1126/science.3201231
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. https://doi.org/10.1074/jbc.M604501200
Wanrooij, S., Falkenberg, M., 2010. The human mitochondrial replication fork in health and disease. Biochim. Biophys. Acta BBA – Bioenerg. 1797, 1378–1388. https://doi.org/10.1016/j.bbabio.2010.04.015
Wanrooij, S., Fusté, J.M., Farge, G., Shi, Y., Gustafsson, C.M., Falkenberg, M., 2008. Human mitochondrial RNA polymerase primes lagging-strand DNA synthesis in vitro. Proc. Natl. Acad. Sci. U. S. A. 105, 11122–11127. https://doi.org/10.1073/pnas.0805399105
Xie, X., Venit, T., Drou, N., Percipalle, P., 2018. In Mitochondria β-Actin Regulates mtDNA Transcription and Is Required for Mitochondrial Quality Control. iScience 3, 226–237. https://doi.org/10.1016/j.isci.2018.04.021
Zeviani, M., Moraes, C.T., DiMauro, S., Nakase, H., Bonilla, E., Schon, E.A., Rowland, L.P., 1988. Deletions of mitochondrial DNA in Kearns-Sayre syndrome. Neurology 38, 1339–1346. https://doi.org/10.1212/wnl.38.9.1339
Zhang, H., Pommier, Y., 2008. Mitochondrial Topoisomerase I Sites in the Regulatory D-Loop Region of Mitochondrial DNA. Biochemistry 47, 11196–11203. https://doi.org/10.1021/bi800774b
Zhang, H., Zhang, Y.-W., Yasukawa, T., Dalla Rosa, I., Khiati, S., Pommier, Y., 2014. Increased negative supercoiling of mtDNA in TOP1mt knockout mice and presence of topoisomerases IIα and IIβ in vertebrate mitochondria. Nucleic Acids Res. 42, 7259–7267. https://doi.org/10.1093/nar/gku384
Zhou, R., Yazdi, A.S., Menu, P., Tschopp, J., 2011. A role for mitochondria in NLRP3 inflammasome activation. Nature 469, 221–225. https://doi.org/10.1038/nature09663

Zorzano, A., Pich, S., 2006. What is the biological significance of the two mitofusin proteins present in the outer mitochondrial membrane of mammalian cells? IUBMB Life Int. Union Biochem. Mol. Biol. Life 58, 441–443. https://doi.org/10.1080/15216540600644838

Züchner, S., Mersiyanova, I.V., Muglia, M., Bissar-Tadmouri, N., Rochelle, J., Dadali, E.L., Zappia, M., Nelis, E., Patitucci, A., Senderek, J., Parman, Y., Evgrafov, O., Jonghe, P.D., Takahashi, Y., Tsuji, S., Pericak-Vance, M.A., Quattrone, A., Battaloglu, E., Polyakov, A.V., Timmerman, V., Schröder, J.M., Vance, J.M., Battologlu, E., 2004. Mutations in the mitochondrial GTPase mitofusin 2 cause Charcot-Marie-Tooth neuropathy type 2A. Nat. Genet. 36, 449–451. https://doi.org/10.1038/ng1341

SUMMARY IN ESTONIAN

Inimese mitokondriaalsete nukleoidide dünaamika mitokondriaalses võrgustikus

Mitokondrid on eukarüootse raku organellid, mis on muuhulgas spetsialiseerunud ATP tootmisele. ATP kui raku energiavaluuta on vajalik mitmete biokeemiliste ning füsioloogiliste protsesside tööshoidmiseks.

Peale tuumagenoomi esineb peaaegu kõigil eukarüootidel väike ning kompaktne mitokondriaalne genoom (mtDNA), mis on organiseeritud DNA-valk kompleksidesse ehk nukleoididesse. Replikatsiooniprotsessis osalevad nukleoidid on muuhulgas assotsieerunud mitokondrite sisemembraaniga, mis on oluline mtDNA säilitamise ning segregatsiooni aspektist (Rajala et al., 2014). Nukleoididega interakteeruvad valgud koordineerivad erineval moel mtDNA ja mtRNA metabolismi ning mitokondrite dünaamikat.

Mitokondrid signaliseerivad teiste organellidega, sh endoplasmaatilise retiikulumiga (ER). Neid funktsionaalseid signaliseerimiskeskuseid, kus ER ja mitokonder füüsiliselt interakteeruvad, nimetatakse MAM-ideks (ingl *mitochondria associated ER-membranes*). Vastavad kontaktid kohandavad dünaamiliselt raku erinevaid metaboolseid vajadusi ning vahendavad mitmeid signaale. Viimastel aastatel on esile kerkinud nende kontaktpunktide oluline panus näiteks vähi ja neurodegeneratiivsete haiguste puhul (Stoica et al., 2014; Thal et al., 2019; Vila et al., 2008). Lisaks sellele on MAM kontaktid olulised mtDNA segregatsiooniks, kuna on leitud, et replitseeruvad nukleoidid asuvad MAM kontaktide vahetus läheduses. Samas pole täpselt teada, kuidas nukleoidi staatus võiks mõjutada MAM struktuuride terviklikkust ja funktsiooni. Käesoleva doktoritöö käigus uuriti mitokondriaalsete nukleoidide kooslust, mtDNA säilitamist ning nukleoidide ja MAM kontaktide omavahelist koosmõju.

Antud doktoritöös mitokondriaalsete nukleoidide populatsioonide valgulise koosluse uurimiseks mtDNA säilitamise kontekstis kasutati spetsiifilist biokeemilist fraktsioneerimist ning nn flotatsiooni. Katsete üheks eesmärgiks oli tuvastada mtDNA topoisomeraaside võimalik lokalisatsioon mitokondriaalsete nukleoidide populatsioonides. Esmalt selgus, et topoisomeraasid Top2α ning Top2β lokaliseeruvad membraaniga seotud replitseeruvates nukleoidides, mida iseloomustab muuhulgas mtDNA ning helikaas TWNK esinemine. Antud tulemust kinnitas ka immuunofluorestsents mikroskoopia. Samas, varasemalt hästikirjeldatud mitokondriaalne topoisomeraas Top1mt lokaliseerus vaid osaliselt replitseeruvate nukleoidide fraktsioonis. Enamus Top1mt esines koos ribosomaalsete valkudega ning mitokondriaalse RNA polümeraasiga, mis kinnitab selle valgu olulisust eelkõige transkriptsioonis.

Edasised katsed näitasid, et ainult Top2β mõjutab selgelt mtDNA säilitamist. Top2β valgu *knockdown* põhjustas positiivselt superkeerdunud (ingl *supercoiled*) mtDNA topoloogiliste vormide osakaalu suurenemist ning mtDNA koopiaarvu olulist langust. Lisaks, potentne topoisomeraaside inhibiitor, fluorokinoloon

antibiootikumide klassi kuuluv tsiprofloksatsiin pärssis Top2β aktiivsust *in vitro* ja *in vivo* ning põhjustas tõsist 7S DNA langust, seeläbi takistades oluliselt replikatsiooni initsiatsiooni.

Doktoritöö raames uuriti ka spetsiaalse nukleiinhappe dioksügenaasi AlkBh1 täpset lokalisatsiooni ja funktsioone, kuna eelnevalt selgus, et AlkBh1 asub mitokondrites ning selle üheks interaktsioonipartneriks on just mitokondriaalsete nukleoidide struktuurne valk ATAD3 (publitseerimata andmed). Mitokondrite fraktsioneerimiskatsed näitasid, et AlkBh1 lokaliseerub spetsiifiliselt mitokondriaalse RNA graanulites (FASTKD2, TUFM) koos mitokondri ribosoomidega (MRPL48, MRPS35), viidates AlkBh1 rollile transkriptsioonis ja/või translatsioonis. Antud tulemusi kinnitasid ka immuunofluorestsents ning kõrge resolutsiooniga 3D-SIM mikroskoopilised analüüsid. Edasi uuriti AlkBh1 vähenemise mõju ka mudelorganismis *Caenorhabditis elegans* ning selgus, et valgu kadu põhjustas ebakorrektselt volutunud valkude kuhjumist mitokondris (ingl *mitochondrial unfolded protein response*), viidates ebanormaalsele translatsioonile.

Viimaseks, nukleoidide terviklikkuse uurimiseks kasutati mtDNA pakkimisvalgu ja transkriptsioonifaktori TFAM transientset allareguleerimist. Käesolevas töös leiti, et TFAM valgu taseme langus põhjustas olulist mtDNA koopiaarvu vähenemist ning nukeoidide agregeerumist. Digitoniiniga fraktsioneeritud mitokondrite analüüsimisel selgus, et replikatiivne helikaas TWNK tase suurenes oluliselt membraaniga seotud nukleoidide fraktsioonis koos MAM markervalkude FACL4, MFN2, VDAC1-ga. Muuhulgas suurenes ka mtDNA osakaal antud fraktsioonis viidates intensiivistunud mtDNA replikatsioonile, et kompenseerida üldist mtDNA langust. Edasine MAM väljapuhastamine TFAM *knockdown* rakkudest näitas olulist MAM markervalkude tasemete suurenemist koos TWNK helikaasiga toetades eelnevat hüpoteesi, et nukleoidide terviklikkuse häirumisel toimub kompensatoorne replikatsioon sisemembraaniga assotsieerunud nukleoidides. Seejuures tugevneb antud piirkonnas mitokondri ning ER vaheline interakteerumine, mis on vajalik mtDNA segregatsiooniks ja seeläbi ka mtDNA korrektseks säilitamiseks.

Tulemustest selgus ka see, et ajalises skaalas oli tegu pöörduva reaktsiooniga, kuna pikaaegsel TFAM *knockdown* rakkude kasvatamisel (5–6 päeva) olid MAM markervalkude tasemed võrreldavad kontrollrakkude valkude tasemetega.

Kokkuvõttes aitavad antud doktoritöö tulemused paremini mõista mtDNA ja mtRNA omavahelisi seoseid mitokondri genoomi säilitamise kontekstis. Seejuures on väga oluline roll ka mitokondriaalsel dünaamikal, st liitumisel ja jagunemisel tagamaks nukleoidide korrektse jaotumise tütarmitokondritesse. Mitokondriaalne dünaamika on omakorda tihedalt seotud ER-mitokonder interaktsioonide moodustumisega kohtades, kus lokaliseeruvad TWNK-sisaldavad replikatiivsed nukleoidid. Kuigi mitmed olulised mtDNA säilitamise ning segregatsiooni molekulaarsed mehhanismid vajavad edasist uurimist, muuhulgas ka mitokondriaalsete haiguste kontekstis, on käesoleva töö tulemused selle oluliseks panuseks.

ACKNOWLEDGEMENTS

Current PhD thesis was carried out at the University of Tartu, Institute of Technology in the field of organelle biochemistry.

First, I wish to thank my main supervisor **Joachim Matthias Gerhold** for a long-term cooperation and a possibility to conduct my research at the Biochemistry of Organelles Research Group. His dedication and relentless determination were overwhelming and helped me to accomplish this research. His scholarly advice, meticulous scrutiny and inspiration deserve my profuse thanks. My sincere thanks also go to Yuliya Basikhina and Julia Borovikova, who constantly injected others, including me, with loads of positivity and motivation.

The completion of my research could not have been possible without the assistance from Maie Loorits and Mikk Välbe. Their contribution is deeply appreciated and gratefully acknowledged.

I am also very thankful to my co-supervisor **Hannes Kollist** from the Plant Signal Research Group for providing us the lab space and introducing me with a new research field (plant biology).

I owe a deep sense of gratitude to Prof. **Allen Kaasik** (and his lab), whose comments and general feedback to my thesis were duly appreciated.

I am beyond grateful to my awesome colleagues at the University of Eastern Finland, who have given me an opportunity to proceed conducting mitochondrial research in their lab. I wish to emphasize the contribution of **Steffi Goffart** and **Jaakko L Pohjoismäki**, who have provided me with so much help regarding the publications and the thesis.

Also, my sincere thanks to Anu Hangas, Georgios Fragkoulis, Petra Tikkanen, Riikka Tapanainen and Chiara Rutanen, for keeping up the great spirit in the lab and accepting me open-heartedly.

Special thanks to all my family members for their enormous support, encouragement, and inspiration throughout my research studies.

Finally, I would like to express my deep appreciation to all my friends for their endless encouragement and understanding that helped me to keep my perspective on my research and develop my ideas. It is my privilege to thank Ats Joorits, who enthusiastically offered new perspectives and fascinating scientific approaches. His unflagging support always made me hit the ground running.

PUBLICATIONS

CURRICULUM VITAE

Name: Koit Aasumets
Date of birth: 8th of March 1993, Lääne-Virumaa, Rakvere
Citizenship: Estonia
Contact: Yliopistokatu 7, Natura 247, 80130 Joensuu, Finland
koit.aasumets@uef.fi

Education:

2017–…	Doctoral studies, Engineering and Technology, University of Tartu
2015–2017	Master's studies, Gene Technology, University of Tartu
2012–2015	Bachelor's studies, Gene Technology, University of Tartu
2009–2012	Rakvere Reaalgümnaasium, Natural Sciences
2000–2009	Muuga Basic School

Professional employment:

2020–…	Project researcher, University of Eastern Finland, Department of Environmental and Biological Sciences
2020–2021	Junior research fellow in Plant biochemistry, University of Tartu, Institute of Technology
2014–2016	Laboratory assistant, University of Tartu, Institute of Biomedicine and Translational Medicine

List of publications:

Aasumets, K., Basikhina, Y., Pohjoismäki, J. L., Goffart, S., Gerhold, J. (2021). TFAM knockdown-triggered mtDNA-nucleoid aggregation and a decrease in mtDNA copy number induce the reorganization of nucleoid populations and mitochondria-associated ER-membrane contacts. Biochemistry and Biophysics Reports *28:* 1–8.

Hangas, A., **Aasumets, K.**, Kekäläinen, N. J., Paloheinä, M., Pohjoismäki, J. L., Gerhold, J. M., Goffart, S. (2018). Ciprofloxacin impairs mitochondrial DNA replication initiation through inhibition of Topoisomerase 2. Nucleic Acids Research *46*: 9625–9636.

Wagner, A., Hofmeister, O., Rolland, S. G., Maiser, A., **Aasumets, K.**, Schmitt, S., Schorpp, K., Feuchtinger, A., Hadian, K., Schneider, S., Zischka, H., Leonhardt, H., Conradt, B., Gerhold, J. M., Wolf, A. (2019). Mitochondrial Alkbh1 localizes to mtRNA granules and its knockdown induces the mitochondrial UPR in humans and *C. elegans*. Journal of Cell Science *132*: 1–12.

Ianevski, A., Kulesskiy, E., Krpina, K., Lou, G., Aman, Y., Bugai, A., **Aasumets, K.**, Akimov, Y., Bulanova, D., Gildemann, K., Arutyunyan, A.F., Susova, O. Y., Zhuze, A. L., Ji, P., Wang, W., Holien, T., Bugge, M., Zusinaite, E., Oksenych, V., Lysvand, H., Gerhold, J. M., Bjørås, M., Johansen, P., Waage, A., Heckman, C. A., Fang, E. F., Kainov, D. E. (2020). Chemical, Physical and Biological Triggers of Evolutionary Conserved Bcl-xL-Mediated Apoptosis. Cancers *12*: 1–21.

ELULOOKIRJELDUS

Nimi: Koit Aasumets
Sünniaeg: 8. märts 1993, Lääne-Virumaa, Rakvere
Kodakondsus: Eesti
Kontakt: Yliopistokatu 7, Natura 247, 80130 Joensuu, Soome
koit.aasumets@uef.fi

Hariduskäik:

2017–…	Doktoriõpe, tehnika ja tehnoloogia, Tartu Ülikool
2015–2017	Magistriõpe, geenitehnoloogia, Tartu Ülikool
2012–2015	Bakalaureuseõpe, geenitehnoloogia, Tartu Ülikool
2009–2012	Rakvere Reaalgümnaasium, reaal-loodus
2000–2009	Muuga Põhikool

Erialane teenistuskäik:

2020–…	Projektiteadur, Ida-Soome Ülikool, keskkonna- ja bioteaduste osakond
2020–2021	Taimebiokeemia nooremteadur, Tartu Ülikool, tehnoloogiainstituut
2014–2016	Laboriassistent, Tartu Ülikool, bio- ja siirdemeditsiini instituut

List of publications:

Aasumets, K., Basikhina, Y., Pohjoismäki, J. L., Goffart, S., Gerhold, J. (2021). TFAM knockdown-triggered mtDNA-nucleoid aggregation and a decrease in mtDNA copy number induce the reorganization of nucleoid populations and mitochondria-associated ER-membrane contacts. Biochemistry and Biophysics Reports *28:* 1–8.

Hangas, A., **Aasumets, K.**, Kekäläinen, N. J., Paloheinä, M., Pohjoismäki, J. L., Gerhold, J. M., Goffart, S. (2018). Ciprofloxacin impairs mitochondrial DNA replication initiation through inhibition of Topoisomerase 2. Nucleic Acids Research *46*: 9625–9636.

Wagner, A., Hofmeister, O., Rolland, S. G., Maiser, A., **Aasumets, K.**, Schmitt, S., Schorpp, K., Feuchtinger, A., Hadian, K., Schneider, S., Zischka, H., Leonhardt, H., Conradt, B., Gerhold, J. M., Wolf, A. (2019). Mitochondrial Alkbh1 localizes to mtRNA granules and its knockdown induces the mitochondrial UPR in humans and *C. elegans*. Journal of Cell Science *132*: 1–12.

Ianevski, A., Kulesskiy, E., Krpina, K., Lou, G., Aman, Y., Bugai, A., **Aasumets, K.**, Akimov, Y., Bulanova, D., Gildemann, K., Arutyunyan, A.F., Susova, O. Y., Zhuze, A. L., Ji, P., Wang, W., Holien, T., Bugge, M., Zusinaite, E., Oksenych, V., Lysvand, H., Gerhold, J. M., Bjørås, M., Johansen, P., Waage, A., Heckman, C. A., Fang, E. F., Kainov, D. E. (2020). Chemical, Physical and Biological Triggers of Evolutionary Conserved Bcl-xL-Mediated Apoptosis. Cancers *12*: 1–21.

DISSERTATIONES TECHNOLOGIAE UNIVERSITATIS TARTUENSIS

1. **Imre Mäger**. Characterization of cell-penetrating peptides: Assessment of cellular internalization kinetics, mechanisms and bioactivity. Tartu 2011, 132 p.
2. **Taavi Lehto**. Delivery of nucleic acids by cell-penetrating peptides: application in modulation of gene expression. Tartu 2011, 155 p.
3. **Hannes Luidalepp**. Studies on the antibiotic susceptibility of *Escherichia coli*. Tartu 2012, 111 p.
4. **Vahur Zadin**. Modelling the 3D-microbattery. Tartu 2012, 149 p.
5. **Janno Torop**. Carbide-derived carbon-based electromechanical actuators. Tartu 2012, 113 p.
6. **Julia Suhorutšenko**. Cell-penetrating peptides: cytotoxicity, immunogenicity and application for tumor targeting. Tartu 2012, 139 p.
7. **Viktoryia Shyp**. G nucleotide regulation of translational GTPases and the stringent response factor RelA. Tartu 2012, 105 p.
8. **Mardo Kõivomägi**. Studies on the substrate specificity and multisite phosphorylation mechanisms of cyclin-dependent kinase Cdk1 in *Saccharomyces cerevisiae*. Tartu, 2013, 157 p.
9. **Liis Karo-Astover**. Studies on the Semliki Forest virus replicase protein nsP1. Tartu, 2013, 113 p.
10. **Piret Arukuusk**. NickFects–novel cell-penetrating peptides. Design and uptake mechanism. Tartu, 2013, 124 p.
11. **Piret Villo**. Synthesis of acetogenin analogues. Asymmetric transfer hydrogenation coupled with dynamic kinetic resolution of α-amido-β-keto esters. Tartu, 2013, 151 p.
12. **Villu Kasari**. Bacterial toxin-antitoxin systems: transcriptional cross-activation and characterization of a novel *mqsRA* system. Tartu, 2013, 108 p.
13. **Margus Varjak**. Functional analysis of viral and host components of alphavirus replicase complexes. Tartu, 2013, 151 p.
14. **Liane Viru**. Development and analysis of novel alphavirus-based multifunctional gene therapy and expression systems. Tartu, 2013, 113 p.
15. **Kent Langel**. Cell-penetrating peptide mechanism studies: from peptides to cargo delivery. Tartu, 2014, 115 p.
16. **Rauno Temmer**. Electrochemistry and novel applications of chemically synthesized conductive polymer electrodes. Tartu, 2014, 206 p.
17. **Indrek Must**. Ionic and capacitive electroactive laminates with carbonaceous electrodes as sensors and energy harvesters. Tartu, 2014, 133 p.
18. **Veiko Voolaid**. Aquatic environment: primary reservoir, link, or sink of antibiotic resistance? Tartu, 2014, 79 p.
19. **Kristiina Laanemets**. The role of SLAC1 anion channel and its upstream regulators in stomatal opening and closure of *Arabidopsis thaliana.* Tartu, 2015, 115 p.

20. **Kalle Pärn**. Studies on inducible alphavirus-based antitumour strategy mediated by site-specific delivery with activatable cell-penetrating peptides. Tartu, 2015, 139 p.
21. **Anastasia Selyutina**. When biologist meets chemist: a search for HIV-1 inhibitors. Tartu, 2015, 172 p.
22. **Sirle Saul**. Towards understanding the neurovirulence of Semliki Forest virus. Tartu, 2015, 136 p.
23. **Marit Orav**. Study of the initial amplification of the human papillomavirus genome. Tartu, 2015, 132 p.
24. **Tormi Reinson**. Studies on the Genome Replication of Human Papillomaviruses. Tartu, 2016, 110 p.
25. **Mart Ustav Jr**. Molecular Studies of HPV-18 Genome Segregation and Stable Replication. Tartu, 2016, 152 p.
26. **Margit Mutso**. Different Approaches to Counteracting Hepatitis C Virus and Chikungunya Virus Infections. Tartu, 2016, 184 p.
27. **Jelizaveta Geimanen**. Study of the Papillomavirus Genome Replication and Segregation. Tartu, 2016, 168 p.
28. **Mart Toots**. Novel Means to Target Human Papillomavirus Infection. Tartu, 2016, 173 p.
29. **Kadi-Liis Veiman**. Development of cell-penetrating peptides for gene delivery: from transfection in cell cultures to induction of gene expression *in vivo*. Tartu, 2016, 136 p.
30. **Ly Pärnaste**. How, why, what and where: Mechanisms behind CPP/cargo nanocomplexes. Tartu, 2016, 147 p.
31. **Age Utt**. Role of alphavirus replicase in viral RNA synthesis, virus-induced cytotoxicity and recognition of viral infections in host cells. Tartu, 2016, 183 p.
32. **Veiko Vunder**. Modeling and characterization of back-relaxation of ionic electroactive polymer actuators. Tartu, 2016, 154 p.
33. **Piia Kivipõld**. Studies on the Role of Papillomavirus E2 Proteins in Virus DNA Replication. Tartu, 2016, 118 p.
34. **Liina Jakobson**. The roles of abscisic acid, CO_2, and the cuticle in the regulation of plant transpiration. Tartu, 2017, 162 p.
35. **Helen Isok-Paas**. Viral-host interactions in the life cycle of human papillomaviruses. Tartu, 2017, 158 p.
36. **Hanna Hõrak**. Identification of key regulators of stomatal CO_2 signalling via O_3-sensitivity. Tartu, 2017, 260 p.
37. **Jekaterina Jevtuševskaja**. Application of isothermal amplification methods for detection of *Chlamydia trachomatis* directly from biological samples. Tartu, 2017, 96 p.
38. **Ülar Allas**. Ribosome-targeting antibiotics and mechanisms of antibiotic resistance. Tartu, 2017, 152 p.
39. **Anton Paier**. Ribosome Degradation in Living Bacteria. Tartu, 2017, 108 p.
40. **Vallo Varik**. Stringent Response in Bacterial Growth and Survival. Tartu, 2017, 101 p.

41. **Pavel Kudrin**. In search for the inhibitors of *Escherichia coli* stringent response factor RelA. Tartu, 2017, 138 p.
42. **Liisi Henno**. Study of the human papillomavirus genome replication and oligomer generation. Tartu, 2017, 144 p.
43. **Katrin Krõlov**. Nucleic acid amplification from crude clinical samples exemplified by *Chlamydia trachomatis* detection in urine. Tartu, 2018, 118 p.
44. **Eve Sankovski**. Studies on papillomavirus transcription and regulatory protein E2. Tartu, 2018, 113 p.
45. **Morteza Daneshmand**. Realistic 3D Virtual Fitting Room. Tartu, 2018, 233 p.
46. **Fatemeh Noroozi**. Multimodal Emotion Recognition Based Human-Robot Interaction Enhancement. Tartu, 2018, 113 p.
47. **Krista Freimann**. Design of peptide-based vector for nucleic acid delivery in vivo. Tartu, 2018, 103 p.
48. **Rainis Venta**. Studies on signal processing by multisite phosphorylation pathways of the *S. cerevisiae* cyclin-dependent kinase inhibitor Sic1. Tartu, 2018, 155 p.
49. **Inga Põldsalu**. Soft actuators with ink-jet printed electrodes. Tartu, 2018, 85 p.
50. **Kadri Künnapuu**. Modification of the cell-penetrating peptide PepFect14 for targeted tumor gene delivery and reduced toxicity. Tartu, 2018, 114 p.
51. **Toomas Mets**. RNA fragmentation by MazF and MqsR toxins of *Escherichia coli*. Tartu, 2019, 119 p.
52. **Kadri Tõldsepp**. The role of mitogen-activated protein kinases MPK4 and MPK12 in CO_2-induced stomatal movements. Tartu, 2019, 259 p.
53. **Pirko Jalakas**. Unravelling signalling pathways contributing to stomatal conductance and responsiveness. Tartu, 2019, 120 p.
54. **S. Sunjai Nakshatharan**. Electromechanical modelling and control of ionic electroactive polymer actuators. Tartu, 2019, 165 p.
55. **Eva-Maria Tombak**. Molecular studies of the initial amplification of the oncogenic human papillomavirus and closely related nonhuman primate papillomavirus genomes. Tartu, 2019, 150 p.
56. **Meeri Visnapuu**. Design and physico-chemical characterization of metal-containing nanoparticles for antimicrobial coatings. Tartu, 2019, 138 p.
57. **Jelena Beljantseva**. Small fine-tuners of the bacterial stringent response – a glimpse into the working principles of Small Alarmone Synthetases. Tartu, 2020, 104 p.
58. **Egon Urgard**. Potential therapeutic approaches for modulation of inflammatory response pathways. Tartu, 2020, 120 p.
59. **Sofia Raquel Alves Oliveira**. HPLC analysis of bacterial alarmone nucleotide (p)ppGpp and its toxic analogue ppApp. Tartu, 2020, 122 p.
60. **Mihkel Örd**. Ordering the phosphorylation of cyclin-dependent kinase Cdk1 substrates in the cell cycle. Tartu, 2021, 228 p.

61. **Fred Elhi.** Biocompatible ionic electromechanically active polymer actuator based on biopolymers and non-toxic ionic liquids. Tartu, 2021, 140 p.
62. **Liisi Talas.** Reconstructing paleo-diversity, dynamics and response of eukaryotes to environmental change over the Late-Glacial and Holocene period in lake Lielais Svētiņu using sedaDNA. Tartu, 2021, 118 p.
63. **Livia Matt.** Novel isosorbide-based polymers. Tartu, 2021, 118 p.