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INVESTIGATING THE ROLE OF TATDN3
IN
MITOCHONDRIA

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Mitochondria are the powerhouses of cells, and they have their own genome. Hundreds to thousands of copies of mammalian mitochondrial DNA (mtDNA) are present in each cell and encode subunits of oxidative phosphorylation (OXPHOS) complexes, complex I, III, IV, and V for oxidative energy production. In mammalian cells, the total mtDNA copy number is under distinct cellular control and responds to changes in mitochondrial metabolism. The conservation and integrity of this genome are vital because high levels of alterations such as insertions, point mutations, and deletions, or depletion (low copy number) decrease mitochondrial function. This can result in insufficiency of adenosine triphosphate (ATP) production or other cellular metabolic components which are produced in mitochondria and might cause multisystemic heterogeneous disorders such as MELAS (mitochondrial encephalopathy-lactic acidosis and stroke-like episodes), MERRF syndrome (myoclonic epilepsy-ragged red fibers) and LHON (Leber's hereditary optic neuropathy). Mitochondrial dysfunction is also connected to type 2 diabetes mellitus, cancer, neurodegenerative disorders, and aging progression. To maintenance of mtDNA, for instance mtDNA replication, transcription, degradation, and repair mechanisms are always regulated, otherwise the cellular respiration system will be disrupted. In this *pro gradu* research project, I aimed to investigate the role of the mitochondrial protein TATDN3 in the maintenance of the mitochondrial genome in mammalian cells. This uncharacterized protein has been predicted to be a deoxyribonuclease (DNase) localized in mitochondria. As mitochondrial DNA endonuclease TATDN3 could play a role as decatenase separating freshly replicated mtDNA molecules, but it could also be involved in mtDNA repair, creating strand breaks required for repair, polymerization or recombination. Potentially TATDN3 could also be selectively degrading mtDNA molecules, a process that is required e.g. upon mtDNA damage and during oocyte fertilization. The nuclease activity of TATDN3 should alter the topological form of mtDNA, but overexpression of wild-type and mutant TATDN3 or knock-down in mammalian cultured cells did not influence the topological shape of mtDNA isomers. Equally no effect on mitochondrial damage and mtDNA copy number was observed during PCR analysis. Immunoprecipitation screening has shown no known factor of the replication machinery to interact with TATDN3. Also, mitochondrial transcript levels were analyzed by Northern blot, but only a small nonsignificant effect was examined. Transient knockdown of TATDN3 by siRNA revealed that deficiency of TATDN3 reduces cell growth compared to control cells.

ABBREVIATIONS

2DNAGE	two-dimensional neutral/neutral agarose gel electrophoresis
ATP	adenosine triphosphate
D-loop	displacement loop
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
EtBr	ethidium bromide
ETC	electron transport chain
FBS	fetal bovine serum
GE	gel electrophoresis
HEK293	Human embryonic kidney 293 cell line
hTATDN3	human TATDN3
IP	Immunoprecipitation
MELAS	mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes
MERRF	myoclonic epilepsy with ragged red fibers
mRNA	messenger RNA
mtDNA	mitochondrial DNA
mtSSB	single-stranded DNA-binding protein, mitochondrial
Mut	mutant
NCR	non coding region
NTC	non template control
O_H	heavy strand replication origin
O_L	light strand replication origin
OXPHOS	oxidative phosphorylation
PBS	phosphate buffer saline
POLRMT	mitochondrial RNA polymerase
RC	respiratory chain
RITOLS	ribonucleotide incorporation throughout the lagging-stand
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT	room temperature
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

siRNA	small interfering RNA
TAE	mixture of tris base, acetic acid and EDTA
TATDN3	TatD DNase Domain Containing 3
TBE	mixture of tris/Borate/EDTA
TFAM	mitochondrial transcription factor A
TFB1M	mitochondrial transcription factor B1
Top 2β	topoisomerase II beta
Top 3α	topoisomerase 3 alpha
tRNA	transfer RNA
TWNK	mitochondrial DNA helicase TWINKLE
WT	wild- type

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1 INTRODUCTION

Mitochondria are fabulous and enigmatic structures in cells. Despite a long evolutionary history mitochondria were originally thought the powerhouse of cells. Importantly, recent various studies showed that these organelles are harboring a diversity of cellular functions and are connected to pathobiologies regarding different biological aspects including cell signaling, apoptosis, metabolism, aging, and cancer (Wallace 2012). In the field of molecular biology, the discovery of the mitochondrial genome and protein synthesis in mitochondria as well as the import of nuclear-encoded proteins carried into mitochondria opened up unique research field in bioscience. The studies of mitochondrial biogenesis expanded a novel chapter of gene expression in eukaryotic organisms.

Each mammalian cell has two DNA containing organelles- nucleus and mitochondria. In each cell hundreds to thousands of copies of mitochondrial DNA (mtDNA) are present. Mitochondria contain the main machinery of ATP (adenosine triphosphate) production, the mitochondrial genome-encoded polypeptides are the subunits of enzyme complexes of oxidative phosphorylation (OXPHOS). The synthesis of ATP in mitochondria requires OXPHOS complex I (NADH-ubiquinone oxidoreductase), complex II (succinate dehydrogenase), complex III (cytochrome reductase), complex IV (cytochrome oxidase) and complex V (ATP synthase). Mitochondrial encoded subunits are part of complex I, III, IV, and V.

mtDNA is maintained by nuclear-encoded factors, but very little is known yet about the molecular mechanism of mtDNA maintenance. Troubles of mtDNA maintenance can lead to deletions or depletion of mtDNA molecules and ultimately mitochondrial dysfunction and thus cause many rare diseases directly- MELAS (Mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes) (Henry *et al.* 2017), MERRF (myoclonic epilepsy with ragged red fibers) (Zhou *et al.* 1997). Disturbances of mtDNA maintenance might also indirectly influence many other diseases likely mitochondrial diabetes (Sivitz *et al.*2010), Parkinson's disease (Swerdlow 2012), aging (Shadel 2008), and cancer (Wallace 2010).

The study of protein interaction in mitochondria is an important way to identify the mechanisms of mitochondrial replication, transcription, repair, and maintenance. This *pro gradu* research is focused on the analysis of the mitochondrial protein TATDN3 to find out the role of this protein in mitochondrial metabolism. TATDN3 is a newly identified protein and yet uncharacterized. In vertebrates, the only studied protein of the TATD protein family is

TATDN1, a nuclearly localized metal-dependent DNase essential for chromosomal segregation and cell cycle progression (Hui *et al.* 2012). TATDN3 might be part of the mtDNA maintenance machinery since it is localized in mitochondria and likely is a nuclease. Especial emphasis is given on the role of TATDN3 in the regulation of mtDNA copy number, topological isomers, mitochondrial damage repair, mitochondrial DNA degradation, mode of transcription, and maintenance of cells growth. Techniques employed are overexpression, knock-down, and expression of a non-functional mutation of TATDN3 in mammalian cultured cells.

2 LITERATURE REVIEW

2.1 Mitochondria

Mitochondria are organelles found in most cells of living organisms in large numbers. In 1890, Richard Altmann, first noticed that mitochondria are ubiquitous in eukaryotic cells and called them "bioblasts", a cytoplasmic structure resembling bacteria. A few years later in 1898, Carl Benda introduced the term "mitochondria" which came from the Greek words "mitos" (thread) and "chondros" (granule) and referred to the structure of these organelles (Ernster & Schatz 1981). Mitochondria form a complex network in living cells (Figure 1) (Pohjoismäki 2008, 13).

In eukaryotic cells, mitochondria are double membrane-bounded organelles (Figure 2). They are semi-independent and pleomorphic organelles (Logan 2006). The outer membrane is merely permeable to small molecules (*M*r _5,000) and ions through transmembrane channels which are formed by proteins called porins. The inner membrane is impermeable to ions, including protons (H^+) and most small molecules; it contains the respiratory electron carriers (complexes I-IV) and the F_1F_0-ATP synthase (complex V) (Nelson & Cox 2013). The infoldings of inner membrane of mitochondria generate cristae and provide an increased internal surface area to facilitate the chemical reactions in the mitochondrial compartment (Ernster & Schatz 1981).

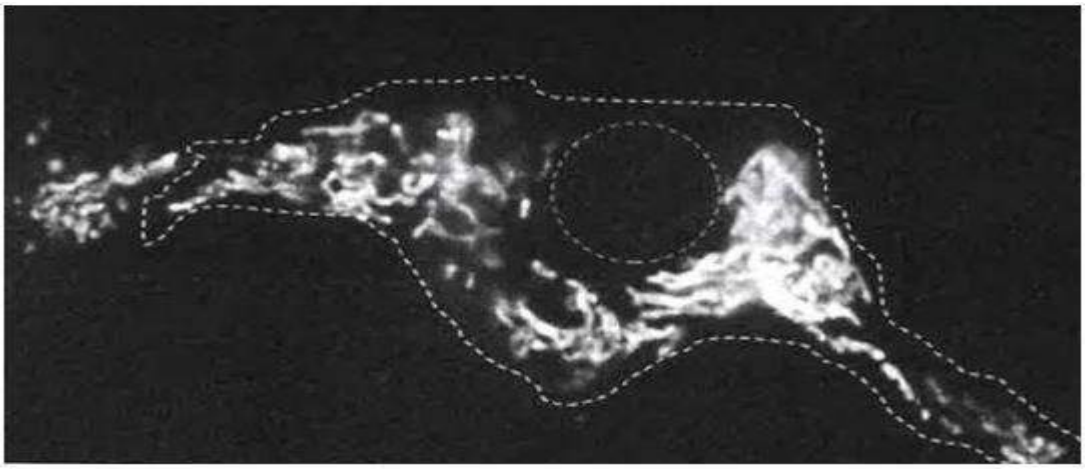


Figure 1. Mitochondrial network outside of nucleus (dashed circular outlined) (obtained from Pohjoismäki 2008, 13)

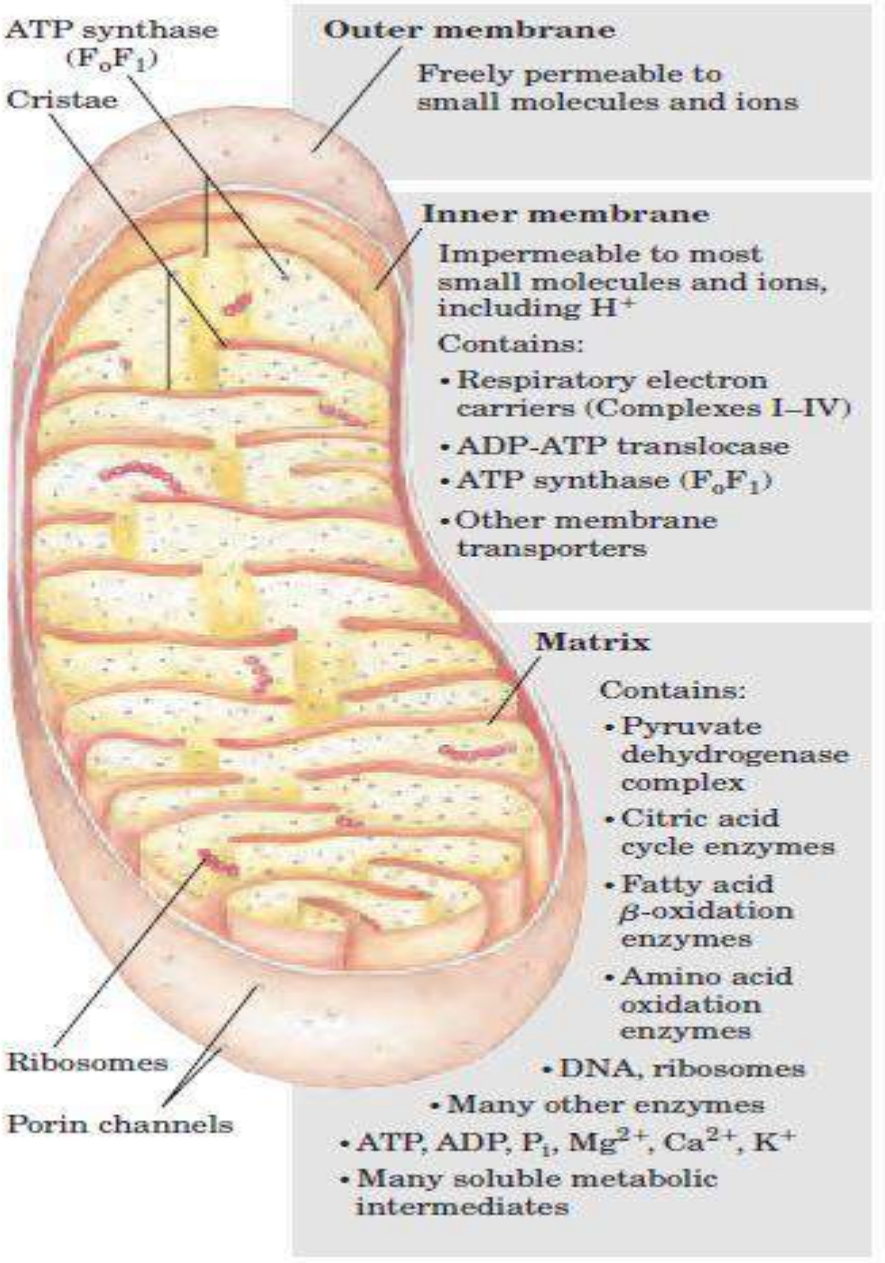


Figure 2. Biochemical anatomy of a mitochondrion (obtained from Nelson & Cox 2013).

In 1948, Eugene Kennedy and Albert Lehninger discovered oxidative phosphorylation (OXPHOS) in eukaryotic organisms and proved that this metabolic pathway occurs in mitochondria for energy transduction (Nelson & Cox 2013). In aerobic organisms, OXPHOS facilitates ATP production by cellular respiration and oxidative degradation of carbohydrates, fats, and amino acids. ATP synthesis in mitochondria was explained Peter Mitchell's hypothesis which was introduced in 1961. He proposed that during OXPHOS a transmembrane proton gradient is produced and used for energy production in mitochondria from biological oxidation reactions (Nelson & Cox 2013).

The mitochondrial matrix surrounded by the inner membrane contains mitochondrial DNA and ribosomes. It also contains all enzymes of fuel oxidation pathways including the pyruvate dehydrogenase complex, amino acid oxidation, citric acid cycle, and fatty acid oxidation, except glycolysis, which takes place in the cytosol. The biochemical reactions in the matrix produce -NADH that is used by inner membrane-embedded respiration complexes to generate ATP (Figure 3) (Nelson & Cox 2013).

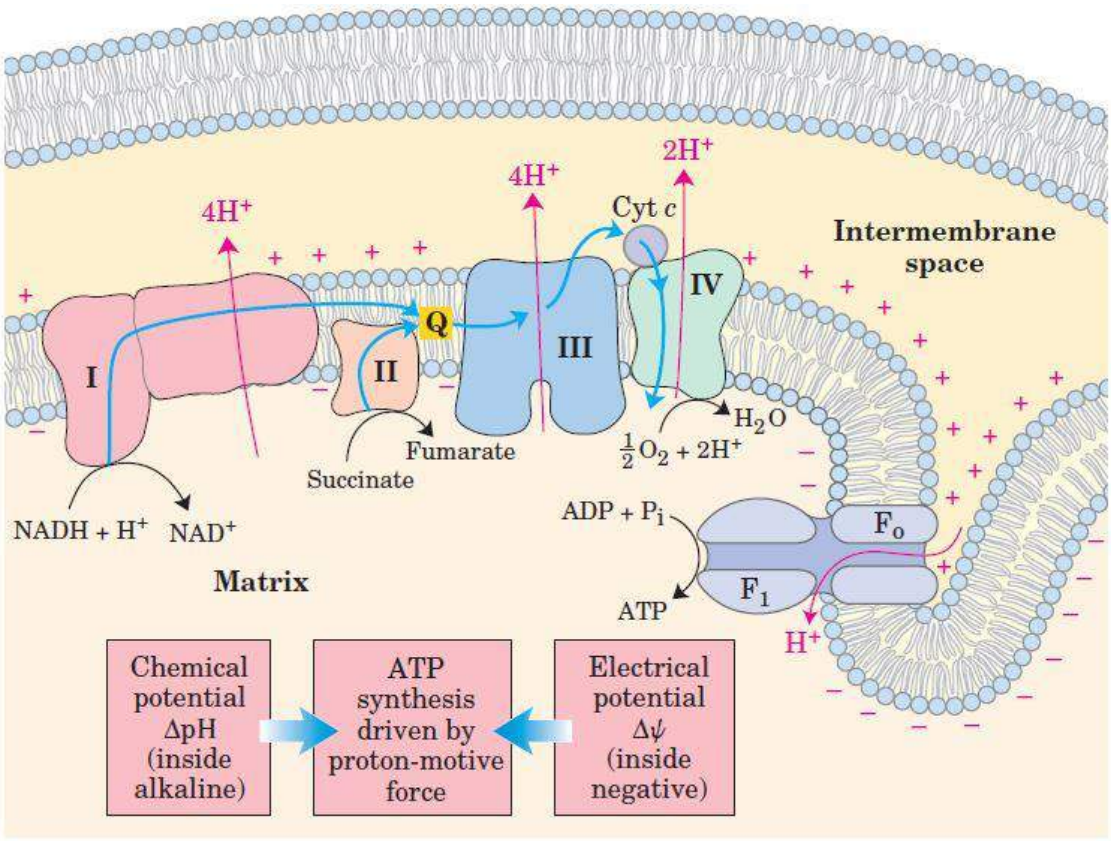


Figure 3. Electron transport chain (ETC) and ATP synthesis in the mitochondrial inner membrane. Electrons are carried by NADH into the electron transport chain (ETC) via complex I (NADH-ubiquinone oxidoreductase), that donates them to the ubiquinone pool (UbQ). The complex II (Succinate dehydrogenase), that is also part of the TCA cycle, transports electrons via FAD-coenzyme from succinate to UbQ. In ETC, electrons are carried through electron transporters and finally used to reduce O_2 to H_2O. Through complexes CI, CIII and CIV protons are pumped into the intermembrane space. Thus the electrochemical gradient is higher in intermembrane space and lower in mitochondrial matrix. The protons are released through F_1F_0-ATP synthase (CV), and their energy used to produce ATP (Figure obtained from Nelson & Cox 2013).

Along with ATP synthesis for energy conversion, mitochondria also play a vital role in iron metabolism (Horowitz & Greenamyre 2011) and calcium homeostasis (Paupe & Prudent

2018) via biosynthesis of various compounds such as steroids and heme group in living cells. In many cases, apoptosis or programmed cell death is maintained by mitochondria and they are the fundamental players for maintaining tissue homeostasis, cellular development and immunological responses (Wang & Youle 2016).

2.2 Mitochondrial genome and function

Mitochondria contain their own mitochondrial genome which requires replication, transcription, repair maintenance, and degradation. The mitochondrial genome is located within the mitochondrial matrix (Nelson & Cox 2013).

The tight regulation of morphology of mitochondria is crucial for the mitochondrial function and mtDNA stability in living cells. Mitochondrial morphology is regulated by fission and fusion of mitochondrial membrane (Bleazard *et al.* 1999*).* Fission and fusion both are opposite forces that should be in a balanced state (Okamoto & Shaw 2005). Any kind of disturbance of mitochondrial fission and fusion state would create heterogeneous disorder in cells and generate different physical disorders (Smirnova *et al.* 2001). Recent biological advances have suggested that mitochondrial fission is responsible for programmed cell death and mitochondrial fusion does vice versa (Chen & Chan 2005). Two genetically transmitted diseases have been identified which are caused by mutation in pro-fusion genes of mitochondria, namely- MFN2 and OPA1 (Olichon *et al.* 2006).

In mammalian mitochondria about 2000 proteins are present, but only 13 proteins are encoded in mtDNA. The remaining proteins are encoded in the nucleus and imported post-translationally from the cytosol (Koehler 2004).

The mitochondrial genome looks very similar in all animals, but plants and microorganisms have huge varieties due to various gene organization, size, and expression mode. However, the basic functions of the mitochondrial genome in all species are remarkably similar to each other (Wanrooij 2007, 17). In 1981, the human mitochondrial genome was first sequenced entirely and reported to be 16 569 base pairs of double-stranded DNA (Anderson *et al.* 1981) (Figure 4). One of the two strands is guanine-rich and therefore called heavy (H) strand, the other strand is cytosine-rich strand and called light (L) strand. There are only 37 genes encoded in the mitochondrial genome, 22 tRNAs, 2 rRNAs and 13 mRNAs encoding polypeptide subunits of the electron transport chain. The coding sequence is very compact in a sense that the genes do not contain introns and some genes are even overlapping; this is an

example of extremely economic genes organization for cellular function (Fernandez-Silva *et al.* 2003).

mtDNA contains a non-coding region that contains two origins of transcription and the origin of heavy-strand replication (O_H). In this region a third strand (7S DNA) can be integrated into the mtDNA genome, creating the so-called Displacement loop or D-loop. The function of this structure is not yet understood, but it might be connected to the start of replication at O_H site (Figure 4).

The genetical information is distributed asymmetrically in the double strands of human mtDNA; the heavy strand contain most genes, while the light strand encodes only for one protein-coding gene (ND6) and several tRNAs (Figure 4) and functionally both strands are essential in different biological aspects (Anderson *et al.* 1981).

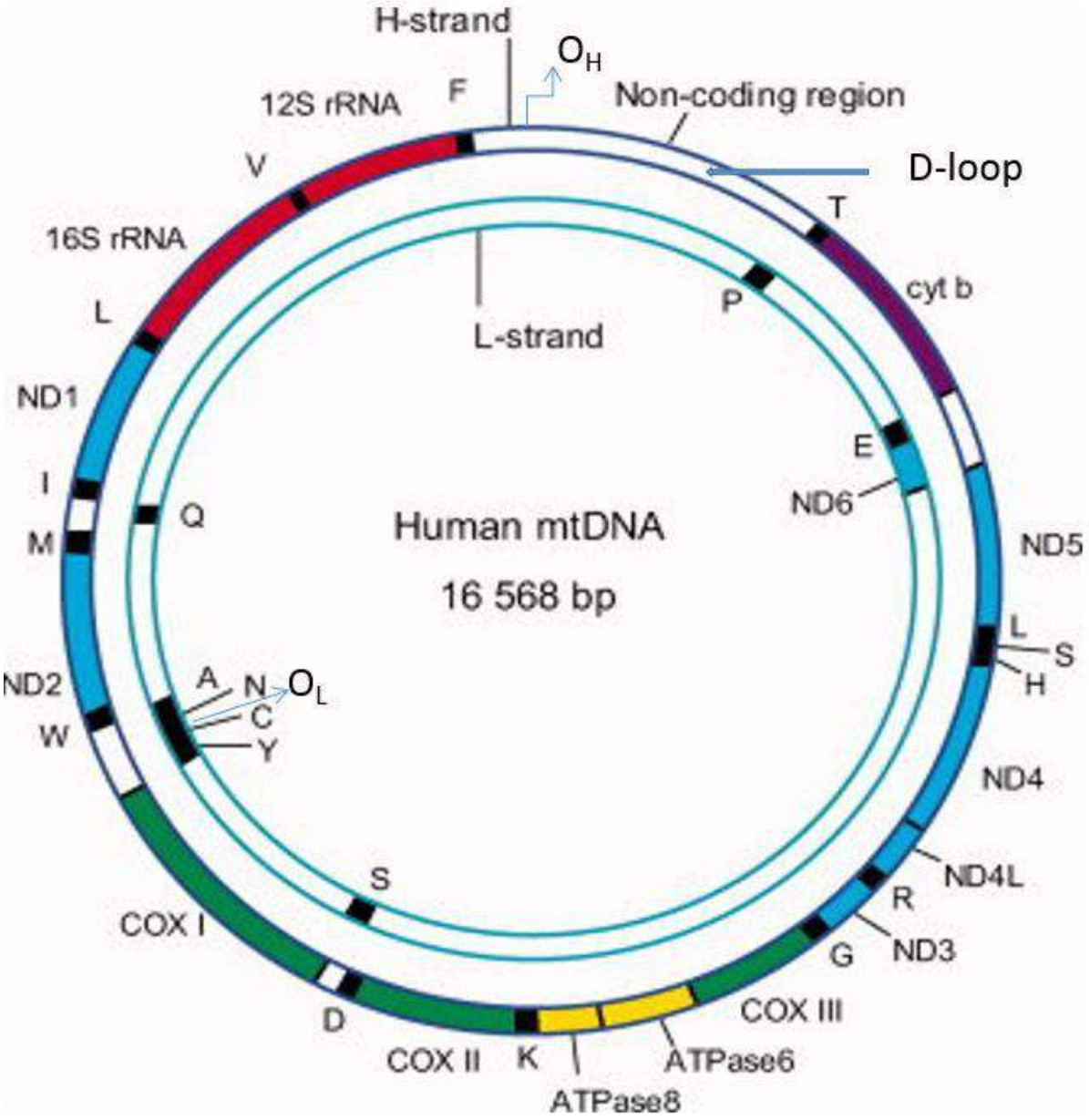


Figure 4. The human mitochondrial genome (mtDNA). The outer strand is named heavy strand (H-strand) due to a greater density of nucleotides, -especially guanine, than the inner light strand (L-strand) which is cytosine-rich. The non-coding region contains the D-loop and the heavy strand replication origin (O_H). O_L indicates the replication origin of the light strand. Genes for the NADH dehydrogenase (ND)-complex I are shown in light blue. Cytochrome b (Cytb)-complex III genes are shown in violet. Genes for subunits of the Cytochrome c oxidase (COX) - complex IV are shown in green. ATP synthase (ATPase) - complex V genes are shown in yellow. Ribosomal RNA (rRNA) genes in red. Transfer RNA (tRNA) genes in black boxes. F (phenylalanine), V (valine), L (leusine), I (isoleusine), M (methionine) , W (tryptophan), Q (glutamine), A (alanine), N (asparagine), C (cysteine), Y (tyrosine), S (serine), D (aspartic acid), K (lysine), G (glycine), R (arginine), H (histadine), H (glutamic acid), T (threonine) and P (proline). Black thin lines in between ATP8-ATP6 and ND4L-ND4 indicate two genes overlap each other (Wanrooij 2007, 18; Tynismaa 2007, 13).

The mitochondrial genome in vertebrates is double-stranded circular DNA which is covalently linked (Bogenhagen & Claytons 1974). But, in fact it differs from nuclear genome in many aspects, for instance by its unique genetic code of mRNAs that cannot be translated by the cytosolic machinery (Wanrooij 2007, 18). The mitochondrial genome is fully maternal inherited (Giles *et al.* 1980). However, during the fertilization of an egg cell about hundred mitochondria come from sperm. These sperm mitochondria are destroyed selectively during fertilization by the cytoplasmic destruction machineries of the egg (Sutovsky *et al.* 1999). The mitochondrial genome is a molecular marker of diversity in mammals and predominantly it shows a higher mutation rate compared to nuclear genome (Nabholz *et al.* 2008). However, recent research findings manifest that mtDNA is more resistant to induced point and deletion mutations than the nuclear genome (Valente *et al.* 2016). Interestingly, mutated mitochondrial DNA can co-exist with wild-type mtDNA, and it is proposed that accumulation of mutations in mitochondrial DNA can cause dysfunction of mitochondria when rising over a certain threshold level of mutation. Ultimately this results in diseases such as MELAS or MERFF (Dimauro & Davidzon 2005).

2.3 Mitochondrial transcription

Each strand of mtDNA contains a promoter for transcription initiation that is named according to the transcription direction - heavy strand promoter (HSP) and light strand promoter (LSP). The transcription that initiates from these promoters regions creates precursor RNA which encodes the genetic information for each of the strands (Montoya *et al*. 1982).

The interaction of several proteins is necessary for mitochondrial transcription. The catalytic enzyme involved in mtDNA transcription is mitochondrial RNA polymerase (POLRMT). POLRMT works with the help of the mitochondrial transcription factors A (TFAM) and mitochondrial transcription factors B1 (TFB1M) or B2 (TFB2M) (Falkenberg *et al*. 2002; Gaspari *et al.* 2004). Also, the mitochondrial transcription termination factor (MTERF) is important for the regulation of mitochondrial transcription; it is hypothesized to regulate initiation and termination simultaneously (Martin *et al.* 2005).

2.4 Mitochondrial translation

Mitochondria have their own machineries for the translation of mRNAs encoded by mtDNA. The mtDNA encodes all RNA components such as rRNA, mRNA, and tRNA in higher animals, but all necessary proteins for translation are imported. The mtDNA-encoded 12S and 16S rRNA form the mitochondrial ribosome (mitoribosome) along with nuclear-derived proteins (Jacobs & Turnbull 2005).

2.5 TatD DNase Domain Containing 3 (TATDN3)

The novel mitochondrial protein TATDN3 is a putative endonuclease found in mammalian cells. It belongs to the TATD family that comprises of DNase domain containing proteins from prokaryotes to eukaryotes (Qiu *et al.* 2005). The name-giving TATD proteins belong to the twin arginine translocation (Tat) system of Escherichia coli (E. Coli), a bacterial operon containing TATA, TATB, TATC, and TATD genes (Wexler *et al.* 2000). Except TATD, all other operon's genes help to translocate fully folded proteins through the Tat system across the cytoplasmic membrane in prokaryotes (Müller & Klösgen 2005).

However, TATD is a cytoplasmic protein not involved to folded protein export. Instead, experiments revealed that in Saccharomyces cerevisiae it is an endonuclease and exonuclease (Wexler *et al.* 2000). Other studies revealed that in Leishmania and *S. cerevisiae* TATD plays a vital role in DNA degradation of apoptotic cells (Bosedasgupta *et al.* 2008; Qiu *et al.* 2005). In protozoa species such as in *Trypanosoma brucei* it helps to accelerate the programmed cell death (Gannavaram & Debrabant 2012).

In higher organisms three proteins of the TATD family have been found, TATDN1, TATDN2, and TATDN3 (Yang *et al*. 2012). It is still unclear whether TATD family proteins work as DNase in mammalian cells (Wexler *et al.* 2000), but recently *in vitro* experiments showed that purified TATDN3 is a dimeric endonuclease. In the presence of Mg^{2+} TATDN3 relaxes and linearizes supercoiled dsDNA (Figure 5).

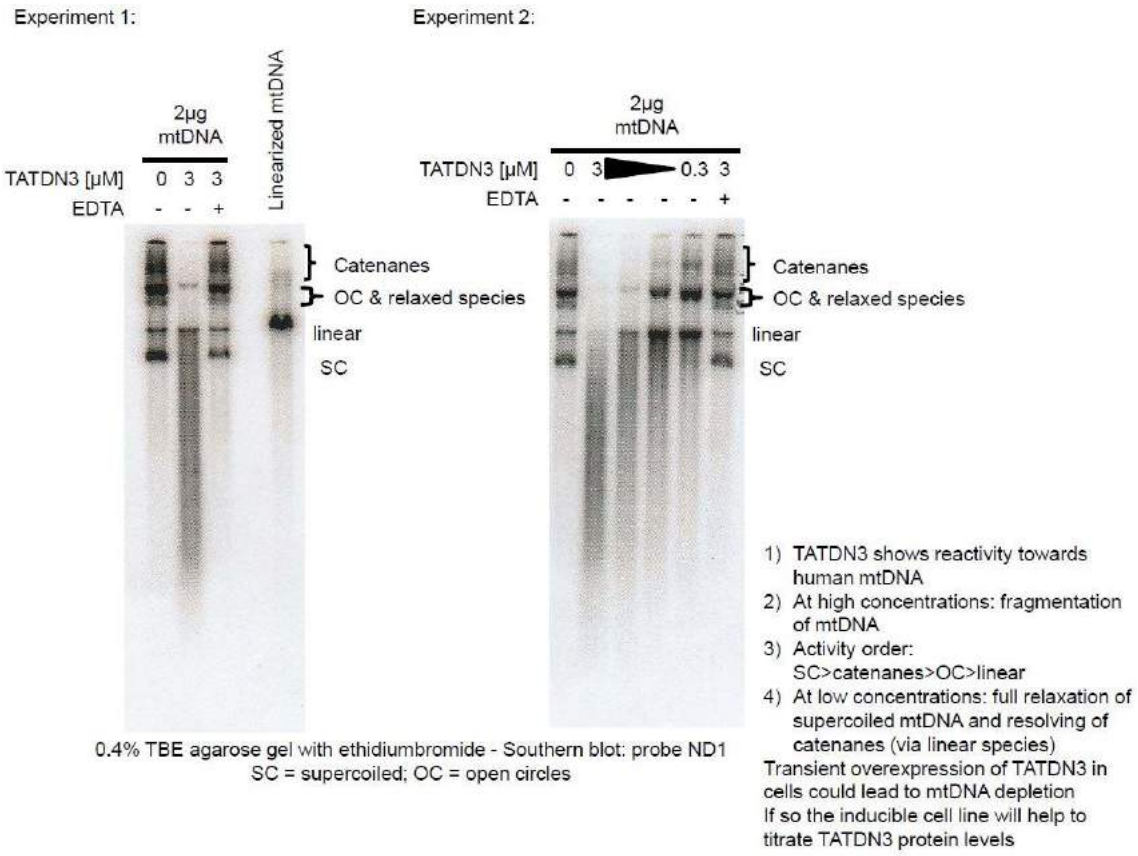


Figure 5. *In vitro* biochemical assay of purified TATDN3. A high concentration of TATDN3 with human mtDNA in test tube generates fragmented mtDNA (done by Al-Furoukh et al. unpublished data, Umeå University, Sweden)

TATDN1 in zebrafish is the only vertebrate member of the TATD protein family. *In vitro* biochemical assays with extracted zebrafish TATDN1 revealed it to be an endonuclease protein which degrades the dsDNA by double strand break catalysis. It was also shown that knockdown of zTATDN1 in zebrafish showed a negative effect on eye development. In case of zebrafish, the researchers claimed that zTATDN1 has an important role in chromosomal segregation and eye development (Yang *et al*. 2012).

3 OBJECTIVE OF THE STUDY

TATDN3 is an uncharacterized protein belonging to the family of TATD proteins and found in mammalian mitochondria.

First *in vitro* experiments with purified TATDN3 have shown that like other TAD proteins also this mitochondrial TATDN3 protein is a metal-dependent DNase that relaxes and cuts circular DNA molecules such as plasmid, Trypanosoma kinetoplast mtDNA and human mtDNA.

As a mitochondrial DNA endonuclease, TATDN3 could play a role as decatenase separating freshly replicated mtDNA molecules, but it could equally well be involved in mtDNA repair, creating strand breaks required for repair polymerization or recombination. Potentially TATDN3 could also be selectively degrading mtDNA molecules, a process that is required e.g. upon mtDNA damage and during oocyte fertilization.

My Master's thesis project aimed to identify the role of TATDN3 in human mitochondria and its effect on mitochondrial DNA metabolism using cultured human cell lines. I have investigated whether an excess of TATDN3 causes alterations in mtDNA topology or strand breaks and whether mtDNA replication or segregation are disturbed if TATDN3 is non-functional.

4 MATERIALS AND METHODS

4.1 Cell culture

The human embryonic kidney cell line (HEK293) and 293 Flp-In™ T-REx™ TATDN3 wild-type and mutant cell lines (created by Sjoerd Wanrooij's lab using the 293 Flp-In™ T-REx™ system) were cultured in DMEM-Dulbecco's Modified Eagle's Medium (BioWest Cat.-No. L0103) containing 10% FBS-fetal bovine serum (BioWest Cat.-No. S181G). The medium was pre-warmed in the 37°C water bath before using and humidified incubator with 8.5 % CO_2 was used for cell culture. To wash cells 1x PBS (10 mM phosphate p^H 7.4, 137 mM NaCl, 2.7 mM KCl) was used and pre-warmed 1x Trypsin/EDTA (Millipore Cat.-No. #2153) was used to detach the cells from the surface. If cells were completely detached and formed a cell suspension, they were centrifuged for 4 minutes at 400 g. After trypsinization cells were seeded in new cell culture 6-well plates, 35 mm or 10 cm dishes.

4.1.1 Transient transfection for overexpression of TATDN3 protein

During transient transfection TATDN3 wild-type FLAG-tagged plasmid (PC DNA5/FRT/TO) (cloned by Sjoerd Wanrooij's lab, pcDNA5/FRT/TO vector sold by Thermo Fisher as part of the 293 Flp-In™ T-REx™ system) was used to transfect HEK293 cultured cells. Transfected cells were cultured on 6-well plate and the medium was changed one hour before transfection. 4 μg of plasmid DNA was diluted in 400 μL serum-free DMEM and 8 μL of Turbofect (Thermo Fisher) was added. The solution was incubated at room temperature (RT) for 15 minutes and added to the cultured cells drop-wise. Two or three days later cells were collected to extract protein, DNA and RNA samples.

4.1.2 siRNA transient transfection to knock down TATDN3 gene

siRNA was used for transient knockdown of TATDN3 in HEK293 cultured cells. Two different siRNAs (LifeTechnologies Silencer select siRNA s43305 and s43307) were used for transfection and compared to siRNA negative control. Cultured cells were transfected with siRNA 1, siRNA 2, a combination of both siRNAs (siRNA1+siRNA2) or the siRNA negative control. 4 μL of Lipofectamine RNAiMAX reagent (Thermo Fisher) was mixed into 75 μL of

Opti-MEM medium (Gibco Cat-No.31985070). 1.25 µL siRNA (12.5 pmol) was diluted into 75 µL of the Opti-MEM medium and both solutions were combined, incubated for 5 minutes at RT and added to the 6-well plate. Two days later cells were collected for DNA, RNA and protein extraction.

4.2 Immunoprecipitation (IP)

HEK293 cells were harvested with 1x PBS and pelleted at 800 g for 5 minutes at 4°C. The cell pellet was resuspended in lysis buffer (30 mM HEPES pH 7.4, 5% glycerol, 2 mM DTT, 70 mM NaCl, 1 mM EDTA, 1.2% Triton X-100, 1x complete EDTA-free protease inhibitor cocktail, Roche) and incubated for 30 minutes on ice. 2 ml lysis buffer were used for one 10-cm plate. The lysate was sonicated for 10 minutes on ice. After sonication 20 µL lysate were collected as "Cell Lysate" sample. The remaining lysate was centrifuged at 15 000 g for 15 minutes at 4°C. After centrifugation, 20 µL of supernatant was taken as "Input" sample and the pellet fraction as "Pellet sample". 40 µL beads of anti-FLAG M2 magnetic beads (Sigma #M8823) were added into the rest of supernatant and rotated at 4°C for 30 minutes. Beads were pelleted using a magnetic stand; the supernatant was removed and kept as "Flowthrough" sample. The beads were washed 3 times with 1 ml lysis buffer and the supernatant was collected as "Wash samples 1-3". For elution, the beads were mixed with 100 µL glycine buffer (100-mM glycine, 2 M urea pH 2.9) and incubated for 5 minutes at RT. The supernatant was taken as "Elution 1" sample, after this the bead pellet was eluted with two times bead volume of 1x SDS protein loading buffer (ca. 100 µL) and the supernatant taken as "Elution 2" sample. All IP samples were collected from both TATDN3 FLAG-tagged overexpressed HEK293 cells and control HEK293 cells without TATDN3 FLAG transient transfection.

4.3 RNA extraction

For RNA extraction cells were grown on a 3 cm dish. The cultured medium was removed and 500 µL Trizol reagent (Thermo Fisher) were added immediately. The cell lysate was stored at -80°C if necessary. For RNA extraction 100 µL Chloroform were added and mixed properly by vortexing, then incubated for 3 minutes at RT. The mixture was centrifuged at 12 000 g for 15 minutes at 4°C and the water phase (ca. 300 µL) taken into a fresh tube. 250 µL isopropanol were added to the water phase and incubated for 10 minutes at RT. Then mixture was

centrifuged at 12 000 g for 10 minutes at 4°C, the liquid removed and the pellet washed with 1- ml 70% ethanol. After another centrifugation for 5 minutes at 15 000 g at 4°C the liquid was taken off and the RNA pellet was air dried. The RNA was re-dissolved in distilled water and stored at -80°C for later use.

4.3.1 Northern Blot

4.3.1.1 Gel preparation

Gel tray, chamber, lid and comb were cleaned thoroughly with Hydrogen peroxide solution. 1.2% agarose was molten in RNase free H_2O by microwaving and cooled down up to 65°C. Under a fume hood 10x MOPS (0.4 M morpholinopropanesulfonic acid, 0.1 M Na-Acetate, 10- mM Na-EDTA pH 7.2) was added to a final concentration of 1x MOPS with 14.6 ml formaldehyde in a final volume of 100 ml. The gel suspension was poured onto the gel tray and let settle.

4.3.1.2 RNA Sample preparation

2-10 µg total RNA was prepared. The concentration and purity of RNA was measured with a Nanodrop ND-1000 spectrophotometer. If the sample volume was less than 5.5 µL, then RNase-free water was added up to 5.5 µL, 16 µL ready-mixed RNA loading buffer (0.05% Bromophenol Blue and/or Xylene Cyanol in 100% formamide) was added. The samples were heated at 65°C for 15 minutes and snap-cooled on ice.

4.3.1.3 Gel running and blotting

The gel was pre-run for 15 minutes at 5 V/cm (60V) in 1x MOPS buffer with 5% formaldehyde prior to loading the samples. Samples were loaded and the gel was run at 30V for overnight. After the run, the gel was rinsed once with dH_2O and soaked in 20x SSC (3 M NaCl, 0.3 M Na-Citrate, pH 7.2) for 20 minutes with shaking. Capillary blotting was done onto Nylon membrane (Hybond-XL from Amersham) for >6 hours at RT. The membrane was soaked briefly in 6x SSC and the RNA cross-linked at 80°C for 2 hours.

4.3.1.4 Northern blot hybridization

The Northern blot membrane was pre-hybridized in Church´s buffer (0.25 M Na_2PO_4 pH 7.0, 7% SDS, 10 mM EDTA) for >1h at 65ºC and hybridized with a probe of interest (Table 1) overnight at 65ºC. After hybridization the membrane was washed once short and 3x 20 minutes in 1x SSC+ 0.1%SDS and exposed onto film or phosphorimager.

Table 1. List of all probes, source, and chromosome positions used in present study. Mitochondrial DNA encoded genes- O_H, ND5, ND6, ND5+6, ND2 and nuclear-encoded genes- 18S rRNA and 28S rRNA for doing hybridization, while 18S rRNA and 28S rRNA are the genomic DNA genes used as loading control.

Name of Probes	**Source**	**Chromosome positions**
O_H	Human	nts 35-611
ND5	Human	nts 13641-13777
ND6	Human	nts 14374-14595
ND5+6	Human	nts 13641-14374
ND2	Human	nts 4470-5511
28S rRNA	Human	nts 1259-2388 of mRNA seq
12S	Human	nts 1332-1894

4.4 Protein extraction

For protein extraction cells were grown in 10 cm dishes. The medium was removed, and the cells detached with 1x PBS. The cells were pelleted by centrifugation and the pellet lysed in 3-4 vol TOTEX buffer (20 mM HEPES pH 7.9, 400 mM NaCl, 20% glycerol, 1% NP-40, 1-mM-$MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 10 mM beta-glycerophosphate, 10 mM NaF, 5-mM DTT, 1x complete protease inhibitor cocktail). The cell lysate was incubated on ice for 10 minutes with occasional vortexing. The solution was frozen at -80°C, thawed and incubated again for 10 minutes on ice. Insoluble proteins were pelleted at 16 000 g for 3 minutes at 4ºC and the supernatant transferred to a fresh tube. This protein extract was stored at -20°C.

4.5 Determination of protein concentration

Protein concentration was measured by mixing 2 µL of the protein suspension into 1 ml Bradford reagent (Bio-Rad) and incubation on ice for 20 minutes. A standard curve was prepared using 0, 2, 4, 6, 8, 10 and 12 µL of 1 mg/ml BSA (VWR Cat-No. 0332). 200 µL Bradford mix of standards and samples were taken into a 96-well plate and absorbance measured at 595 nm.

4.5.1 Western blot

4.5.1.1 Casting of Lämmli gels

Glass plates were cleaned with ethanol and tissue paper and assembled. The gel solution (8%, 12% or 15% Acrylamide/Bis 29:1, 0.1% SDS, 375 mM Tris, pH 8.8) was mixed in a beaker. After this 0.1% APS (10% ammonium persulfate, self-made) and 0.1% TEMED (Trethylmethylethylenediamine 100%, VWR Cat-No. 443083G) were added and the gel solution poured into the gel sandwich until 2 cm below the edge of the lower glass plate. Water-saturated n-butanol was poured on top to smoothen the surface. The gel was left to settle for 30 minutes. The butanol was removed with a filter paper. The stacking gel solution (4% Acrylamide/Bis 29:1, 0.1% SDS, 125 mM Tris, pH 6.8) was prepared. 0.1% APS and 0.1% TEMED were added in the gel solution and mixed and cast to fill the sandwich completely. The gel comb was added without catching bubbles and the gel left to settle. If gel was needed days later, it was wrapped in moist tissue paper and stored at 4^{o}C.

4.5.1.2 Loading and gel running

The heat block was preheated at 95^{o}C. Protein samples were mixed with ¼ volume of 5x SDS PAGE sample buffer (250 mM Tris pH 6.8, 10% SDS, 30% glycerol, 500 mM DTT, 0.02% Bromophenol blue) and denatured for 5 minutes at 95^{o}C. After this samples were spun down briefly. The gel chamber was assembled and filled with 1x SDS buffer (10x SDS buffer: 25-mM Tris, 192 mM Glycine, 0.1% SDS) and the comb removed. The samples were loaded, and a protein size marker was loaded into one lane. The lid was closed and gel electrophoresis was run for ca. 15 minutes at 80V, then at 100-150V until the blue dye band reached the bottom

(ca. 1.5 hours total). After the gel electrophoresis, the gel chamber was disassembled and glass plates preyed apart. The gel was taken into a plastic dish, the stacking gel removed and Coomassie blue staining (Expedeon, Instant blue) or silver staining or blotting was done as described below.

4.5.1.2.1 Silver staining

For silver staining, the SDS-PAGE gel was fixed in fixing solution (30% EtOH, 10% acetic acid) overnight after gel electrophoresis. The gel was rinsed with 20% EtOH for 15 minutes and washed with dH_2O for 15 minutes. Sensitizer (300 mg thiosulfate.$5H_2O$ in 1 L H_2O) was used to sensitize the gel for 90 seconds precisely. The gel was rinsed twice for 20 seconds with dH_2O and developed for 2-5 minutes in developer solution (30g KCO_3, 15 mg Na-thiosulfate.$5H_2O$ and 700 µL formaldehyde in 1 L H_2O. The developer reaction was stopped by 2.5% acetic acid and the gel rinsed twice for 10 minutes with dH_2O.

4.5.1.3 Western Blotting

For Western blotting nitrocellulose membrane (Protran, Life technologies) and filter papers were cut to gel size and the blotting sandwich was assembled in 1x blot buffer (10x blot buffer: 25 mM Tris, 192 mM Glycine, 0.1% SDS, 20% methanol). The blotting chamber was filled with 1x blotting buffer and the gel blotted 1.2 hours at 120V at 4°C. After blotting the membrane was briefly washed with water and proteins stained for 1 minute in Ponceau S solution (0.1% in 5% acetic acid) to judge sample loading and transfer. The membrane was cut according to experimental need.

4.5.1.3.1 Western blot hybridization

The membrane was incubated for >1 hour in 5% milk powder in 1x TBST low salt (20 mM Tris pH 7.6, 150 mM NaCl, 0.1% Tween-20) with shaking to block free sites on the membrane. It was washed briefly with 1x TBST and incubated overnight with primary antibody solution in 3% BSA/1x TBST at 4°C (List of used antibodies in Table 2). The membrane was washed once briefly, then 3 times for 5 minutes in 1x TBST. It was incubated in secondary antibody

solution (Goat-anti-rabbit IgG-HRP coupled (Life Technologies #A16104) 1:15 000 in 3% BSA/1xTBST) for >1 hour, and washed 4 x 10 minutes with 1x TBST.

Table 2. List of antibodies used in Western blot hybridization at present study

Source	Name of Antibody	Dilution	Name of company	Catalog No.
Rabbit	Anti-FLAG polyclonal IgG1	1:2000	Sigma	#F7425
Rabbit	Anti-TATDN3 polyclonal IgG	1:1000	LSBio	105881
			Invitrogen	PA5-28490
Rabbit	Anti-TFAM polyclonal IgG	1:1000	Aviva biosys	ARP31400
Rabbit	Anti-mtSSB polyclonal IgG	1:1000	Sigma	HPA002866
Rabbit	Anti-PEO No.3 polyclonal IgG	1:1000	Elabscience	EAP1298
Rabbit	Anti-Top3α polyclonal IgG	1:2000	Proteintech	14525-!-AP
Rabbit	Anti-Top2β polyclonal IgG	1:2000	Genetex	GTX102640
Goat	Anti-rabbit IgG-HRP coupled	1:15 000	Life Technologies	#A16104

4.5.1.3.2 Chemiluminescent reaction

The chemiluminescent detection was done by incubating the membrane for 1-2 minutes in Luminol solution (500 µg/ml Na-Luminol, 100 mM Tris-HCl pH 8.5, 0.01% H_2O_2) with 1% Enhancer (0.11 mg/ml para-hydroxycoumaric acid in DMSO) and then exposing to X-ray film. Typical exposure times were 10 seconds to 5 minutes.

4.6 DNA extraction

For extraction of total DNA, cells were trypsinized and washed off in 1x PBS. The cells were pelleted at 12 000 g for 3 minutes and the cell pellet resuspended in 700 µL lysis buffer (10 mM Tris pH 7.4, 10 mM EDTA, 150 mM NaCl, 0.4% SDS). 20 µL of proteinase K (10 mg/ml, VWR Cat-No. #0706) was added and the samples were incubated at 37°C overnight. 50 µL of 5 M NaCl was added to aid phase separation. The solution was mixed with one volume of Phenol:Chloroform:Isoamylalcohol (25:24:1) and thoroughly mixed. The suspension was centrifuged for 15 minutes at 16 000 g and the upper water phase was recovered and transferred to a new tube with avoiding interphase. This phase separation step was repeated two or three times. The solution was once extracted with one volume of chloroform to remove phenol traces. To precipitate the DNA 50 µL 3M Na Acetate and 1 ml 100% cold EtOH were added, the solution mixed properly and incubated for 60 minutes at -20°C or 20 minutes at -80°C. The samples were centrifuged for 10 minutes at 16 000 g at 4°C and supernatants were removed. The DNA pellets were washed once with 500 µL 70% ethanol and re-centrifuged and the supernatant was again carefully removed. The washed pellets were air-dried in open tubes until no visible ethanol was remained. The DNA pellets were suspended in 100-300 µL of TE-buffer (10 mM Tris pH 8.0, 1 mM EDTA) and dissolved overnight at 4°C. Extracted total DNA was stored at -20°C.

4.6.1 Southern blot

Relative mtDNA copy number level was determined in the total DNA samples by Southern blot. 50 µL of total DNA was digested overnight at 37°C with 1 µL BamHI fast digest in 1x fast digestion buffer (Thermo Fisher). The concentration and purity of DNA in each of the digested samples was measured with a Nanodrop ND-1000 spectrophotometer. 2 µg of total DNA per sample was mixed with loading buffer and loaded onto a 0.6% or 0.4% agarose gel in 1x TBE buffer (45 mM Tris-borate pH 8.0, 1 mM EDTA) and the gel was run overnight at 30V. After electrophoresis, the gel was stained with ethidium bromide (1x TBE + 1 µg/ml EtBr) for 10-20 minutes and the loading visualized on a UV table.

For Southern blotting, the gel was washed twice for 15 minutes in depurination solution (0.25 M HCl) rinsed briefly with dH_2O. The gel was incubated twice for 20 minutes in denaturation solution (0.5 M NaOH, 1.5 M NaCl) and the DNA was transferred from the gel

onto a nylon membrane (Amersham Hybond XL, GE Healthcare) using capillary blotting. The gel was carefully lifted on plastic film on the table and the membrane was placed on the top of the gel. One piece of Whatman™ 3MM Chromatography paper was placed on top of the gel and a stack of napkins and weight were placed on the top of it for overnight blotting. After the transfer, the membrane was washed once very briefly in a 6x SSC and baked for 2 hours at 80°C.

4.6.1.1 Southern blot hybridization

The membrane was prehybridized in preheated Church's hybridization buffer (250 mM Na-phosphate, 7% SDS, pH 7.2) at 65°C for 30 minutes. Different probes were prepared in advance, for instance, mitochondrial DNA encoded O_H, ND5, ND6, ND5+6, ND2, and nuclear-encoded 18S rRNA and 28S rRNA for doing hybridization (see Table 1).

For random-primed labeling of hybridization probes, 20 ng PCR product in 45 µL TE-buffer were denatured for 5 minutes at 95°C and snap cooled on ice. The denatured probe containing solution was mixed in a random primer labeling kit reaction tube (AmershamRediprime II Labeling System, GE Healthcare) with 5 µL of [α-32P] dCTP and incubated for 20 minutes at 37°C. The labeled probe was purified by G-50 purification column (Mini Quick Spin Column, Roche) at 1600 g for 4 minutes. The purified labeled probe was denatured at 95°C for 5 minutes and added immediately into the pre-heated hybridization buffer.

The membrane was hybridized overnight at 65ºC. After hybridization the membrane was washed 1x short time (10 minutes) and 3x long time (20 minutes each) in 1x SSC+ 0.1% SDS. The membrane was wrapped in cling film and exposed to a phosphor storage screen (BAS-IP MS, GE Healthcare). Depending on the radioactive signal intensity the screen was exposed for 1-15 days and then quantified using a phosphorimager (Molecular Imager FX, Bio-Rad).

4.6.1.2 mtDNA topology identification by following Southern blot

To identify changes in the topological shape of mtDNA, the isomers were separated over a 0.4% agarose (ultrapure, Invitrogen) gel in 1x TBE (10x TBE stock solution: 1 M Tris, 0.9 M boric acid, and 0.01 M EDTA) or 1x TAE (50x TAE stock solution: 242 g Tris base in H_2O,

100 ml of 500 mM EDTA (pH 8.0), and adding 57.1 ml glacial acetic acid, and the final volume up to 1 liter) without ethidium bromide. The gel was run at 30V overnight, blotted as described above, hybridized with a mitochondrial probe (ND6 or O_H) and analyzed by a phosphor screen.

4.7 mtDNA copy number quantification by RT-PCR

The mtDNA copy number per cell was assessed using a duplex Taqman-based real time PCR that quantifies a short sequence of the mitochondrial DNA and a part of the nuclear NDUFV1 (see Table 3 for details) gene at the same time.

For this assay total DNA samples were sonicated for 30 seconds using cycle 0.5 and amplitude 90%. The DNA concentration was measured by Nanodrop and the samples diluted to 25 ng/µL. A serial dilution was prepared from one control sample for instance 200 ng/µL, 100 ng/µL, 25 ng/µL, 12.5 ng/µL, 6.25 ng/µL to create a standard curve. 50 ng DNA was used as template in a 25 µL PCR reaction containing 1x Accutaq Supermix II, 100 nM mtDNA forward and reverse primer, 300 nM nuclear forward and reverse primer and 125 nM of mitochondrial and nuclear probe (see Table 3 for details).

The PCR program was the following:

1. 3 minutes 95°C
2. 20 seconds 95°C
3. 20 seconds 58°C
4. 20 seconds 72°C

   40 cycles 2-4

The obtained template concentrations were used to calculate the relative mtDNA copy number per nuclear DNA.

4.8 mtDNA damage quantification by Long-run PCR

The integrity of mtDNA was assessed using a long-range PCR approach, where damages in the template affect the amplification of the DNA. For this 100 ng of non-sonicated total DNA were used as template in a 25 µL reaction containing 1x AccuTaq Supermix II, 500 nM 5999F and 14841R primers and 125 nM mtDNA probe (see Table 3 for details).

The PCR program used was the following:

1. 5 minutes 94°C
2. 30 seconds 94°C
3. 30 seconds 64°C
4. 7 minutes 72°C

   40 cycles 2-4

The template concentration used in this PCR was quantified by a short-range real-time PCR similar to the copy number PCR, but without the nuclear primers and probe. The relative mtDNA damage compared to controls was calculated using the CT-values obtained by long- and short-range PCR, the amplicon length and the PCR efficiency as described in Lehle *et al*. 2014.

Table 3. List of all primer sequences

Target	Primer	Sequence (5′-3′)	Remarks
mtDNA (108bp)	13456F	ACCATTGGCAGCCTAGCATT	Short forward
	13593R	TGTCAGGGAGGTAGCGATGA	Short reverse
mtDNA (6.5kb)	5999F	TCTAAGCCTCCTTATTCGAGCCGA	Long forward
	14841R	TTTCATCATGCGGAGATGTTGGATGG	Long reverse
TATDN3	F	CGTAGGCTTGGTGGACTGTC	-
	R	TTTAGTGTGACACTTCTTTGGTCT	-
18S rRNA	850 F	CCGCAGCTAGGAATAATGGA	-
	1347 R	AACTAAGAACGGCCATGCAC	-
ND2	4,470 F	GTTATACCCTTCCCGTACTA	-
	5,511 R	TATTTAACCTAAATTTCTAT	-
NDUFV1 Nuclear gene	F	ATC CAG GAT CCC ACA GAG CT	-
	R	CCT TTC CAG CAG ATG TGG GT	-
28S+ (Human rRNA)	1259F	TGG CCA TGG AAG TCG GAA TC	-
	2388R	gga acc ctt ctc cac ttc gg	-
ND1	F	AACCTAGGCCTCCTATTTATTC	-
	R	ATATGTTGTGTAGAGTTCAGGG	-
ND6	F (HS14374F)	TACCTCCATCGCTAACCCCA	-
	R (HS14595R)	GGTGTGGTCGGGTGTGTTAT	-
12S	HS827F	GGGTTGGTCAATTTCGTGCC	-
	HS1318R	TTACGTGGGTACTTGCGCTT	-
mtDNA probe	13546F	FAM-ACAAACGCCTGAGCCCTA-BHQ1	FAM
NDUFV1 probe	-	VIC-TCCATTGAGGGCAAGCAGG-MGB	HEX

4.9 Quantification of TATDN3 transcript after siRNA knockdown

The efficiency of siRNA knockdown in HEK293 cells was quantified by reverse transcription and real-time PCR

4.9.1 Reverse Transcription reaction

0.5 µg heat denatured RNA in 10 µL 1x qScriptsupermix (Quanta Bio) was incubated for 5 minutes at 25ºC, 30 minutes at 42ºC and 5 minutes at 85ºC. As a no-template control (NTC) 0.5 µg RNA was mixed with water only. All samples were diluted 1:1 with water after the reaction. One control sample was used at 2x, 1x and 0.5x concentration to obtain a standard curve.

4.9.2 Reverse Transcription-qPCR

2.5 µL Reverse Transcription reaction was used as template in 25 µL 1x SybrGreen Mastermix containing 300 nM TATDN3 forward and reverse primers (see Table 3 for details). Each sample was measured 3x and each standard twice.

The PCR program used was the following:

1. 10 minutes 95°C
2. 15 seconds 95°C
3. 30 seconds 60°C
4. 30 seconds 72°C

   40 cycles 2-4

4.10 2D Neutral - Neutral Agarose Gel Electrophoresis (2DNAGE)

4.10.1 Total nucleic acid extraction for 2D gels

Semi-confluent cells of 10 cm dishes were washed off with 1x PBS and lysed in 4 ml lysis buffer (10 mM Tris pH 7.4, 10 mM EDTA, 150 mM NaCl, 0.4% SDS). The lysate was mixed with one volume of phenol by rotation for 15 minutes at RT. The suspension was centrifuged for 5 minutes at 15 000 g and the upper phase was obtained with avoiding the middle layer. To this water phase, one volume of Chloroform was added and mixed again by rotation for 15

minutes at RT. The suspension was centrifuged for 5 minutes at 15 000 g and 4ºC and the upper phase collected. Nucleic acids were precipitated from this solution by addition of 100-mM NaCl and one volume of isopropanol. The DNA was pelleted for 10 minutes at 15 000 g and 4ºC and the supernatant removed. The pellet was washed with 1 ml of 70% EtOH and centrifuged again. The liquid was removed and the pellet air-dried and dissolved in 300 µL DNA lysis buffer. 5 µL of 20 mg/ml Proteinase-K was added and the solution was incubated at 4ºC for 30 minutes. The DNA solution was extracted with Phenol/Chloroform and precipitated with NaCl/isopropanol as before (see section 4.6). The final DNA pellet was dissolved in 300-500 µL TE buffer and the concentration determined by Nanodrop ND-1000 absorbance measurement.

4.10.2 Sample preparation and gel electrophoresis for 2Ds

For 2D gel electrophoresis 10 µg of total DNA was digested in 50 µL with HincII overnight at 37°C. The digested DNA was once extracted with one volume of phenol and the water phase mixed with 5 µL DNA loading dye (20 mM Hepes pH 7.2, 10 mM EDTA, 50% glycerol, 0.5% bromophenol blue, 0.5% Xylene cyanol) before loading onto a 0.4 % agarose (ultrapure, Invitrogen) gel in 1x TBE buffer. The gel was run at 30V for 20 h and 50V for 1 h without ethidium bromide (EtBr). After the first dimensional the gel was stained in 1x TBE + 1 µg/ml EtBr for 10-20 minutes and visualized on a UV table to cut out the DNA-containing lanes. These gel stripes were arranged horizontally on a larger gel tray and a 0.95 % agarose gel in 1x TBE with 1 µg/ml EtBr cast around them. This gel was run in 1x TBE buffer + 1- µg/ml EtBr at 105 V for 21 hours and 250V for 80 minutes. The second dimension was run at 4°C to avoid overheating of the gel chamber. When the 1n spot was ca. 2 cm from the edge, the gel was Southern blotted as described above and hybridized with mtDNA O_H probe (nts 35-611). The schematic illustration of two-dimensional gel electrophoresis is shown below (Figure 6 and Figure 7).

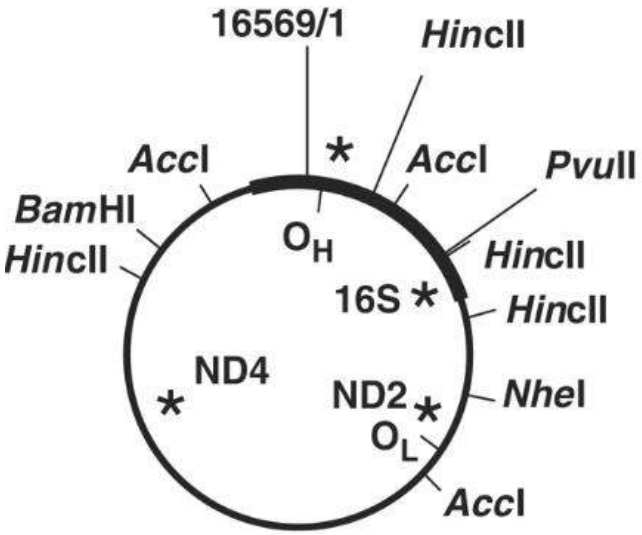


Figure 6. The typical restriction enzymes which cut mtDNA in different positions. This schematic map shows relevant restriction sites, O_H, O_L and approximate different probe locations (denoted by asterisks), and the Non-Coding Region (NCR, bold-black) (Hyvärinen *et al.* 2007).

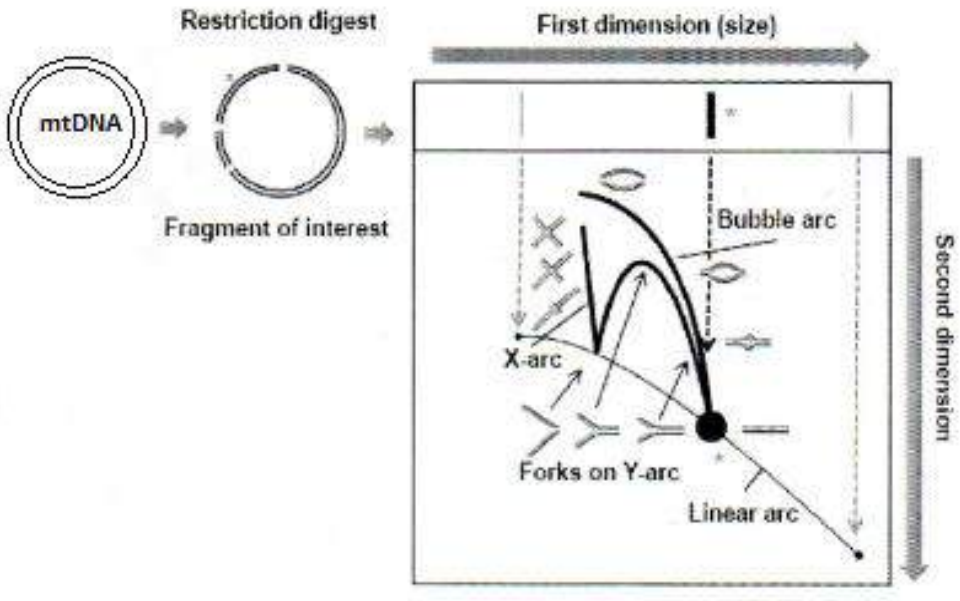


Figure 7. Schematic interpretation of 2DNAGE image (obtained from Pohjoismäki 2008, 75). The first-dimensional gel electrophoresis separates according to size of DNA, and second-dimensional gel electrophoresis separates according to size and shape after digested with a restriction enzyme of interest. Restriction enzyme cuts the mtDNA into small fragments of interest, those fragmented mtDNA are separated by size in 1st dimensional GE; In 2nd dimensional GE those separated DNA fragments of 1st dimension are placed into 90 degree angle and further separated in 1x TBE with addition of EtBr (1μg/ml) and high voltage (described in section 4.10.2). For instance, non-replicating linear fragments (3.9kb) start growing from 1n spot (big black spot) and create a linear arc, Y-shaped fragments locate on

the Y-arc, uncut bubble shaped fragments form the bubble arc, and X-shaped replicating fragments form an X-arc (Friedman & Brewer 1985; Brewer & Fangman 1987).

4.11 Determination of cellular growth rate

To determine the cellular growth rate $5x10^5$ cells per well were seeded in 6-well plates. Three wells were transfected with siRNA against TATDN3, while three served as control. Cells were counted at 22 hours, 46 hours and 68 hours time points after transfection. The cells were washed once with 1x PBS, detached by trypsinization and resuspended in 1 ml 1x PBS. Total cells were counted by Bürker's haemocytometer to determine the cellular growth rate.

5 RESULTS

5.1 TATDN3 wild-type (WT) FLAG- tagged overexpression

Overexpression of TATDN3 WT FLAG after transient transfection in Hek293 cells was visualized by Western blot analysis on a 15% SDS-PAGE gel. The expected protein size was ~30 kDa, and an intense band at this size was detected by anti-FLAG antibody (see Table 2 for details) (Figure 8).

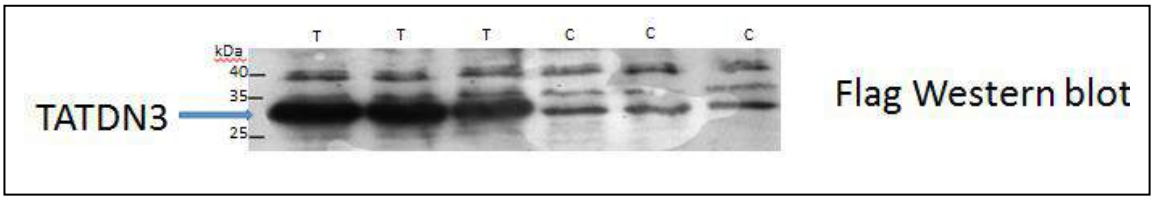


Figure 8. Transient overexpression of TATDN3 WT FLAG in HEK293 cells. T: HEK293 cells after transient transfection with pcDNA5/TO-TATDN3 WT FLAG, C: non-transfected control

5.2 Topology of mtDNA

As TATDN3 might be an endonuclease, it was assumed that TATDN3 overexpression could affect the topology of mitochondrial DNA by the creation of strand breaks or nicks. Thus different ways of altering TATDN3 levels in the cells were employed to see if this affects mtDNA topology.

First, mtDNA topology was compared in cells overexpressing TATDN3 after transient transfection with pcDNA5/TO-TATDN3 WT-FLAG and untreated control cells (Figure 9). No change of topology was observed.

(A) (B)

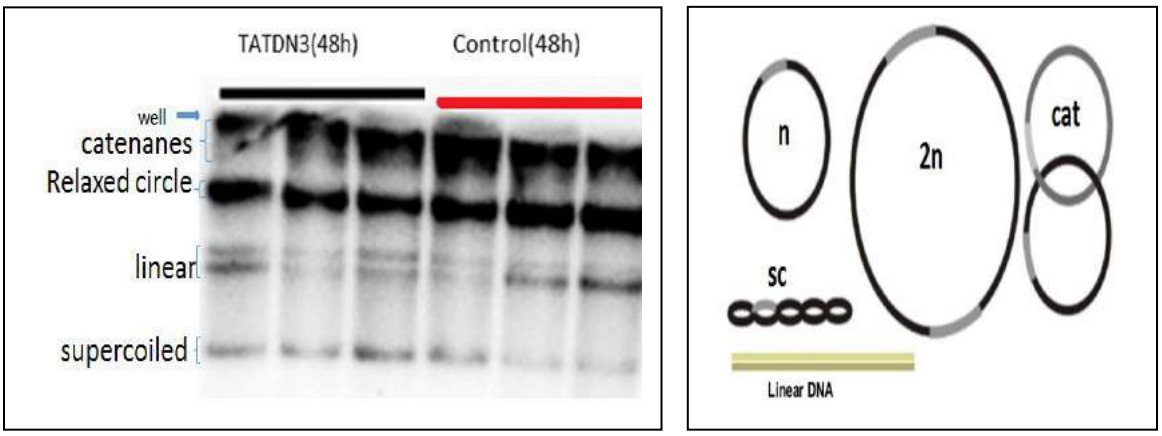


Figure 9. Topological isomers. (A) Topology of mtDNA upon TATDN3 wild-type overexpression in HEK293 cultured cell after 48 hours. (B) Typical topological isomers of mtDNA (Pohjoismäki & Goffart 2011).

5.3 mtDNA copy number

As overexpression of a mitochondrial endonuclease might affect mtDNA copy number, the levels of mtDNA were compared in HEK293 cells transiently overexpressing TATDN3-FLAG and untreated controls (Figure 10).

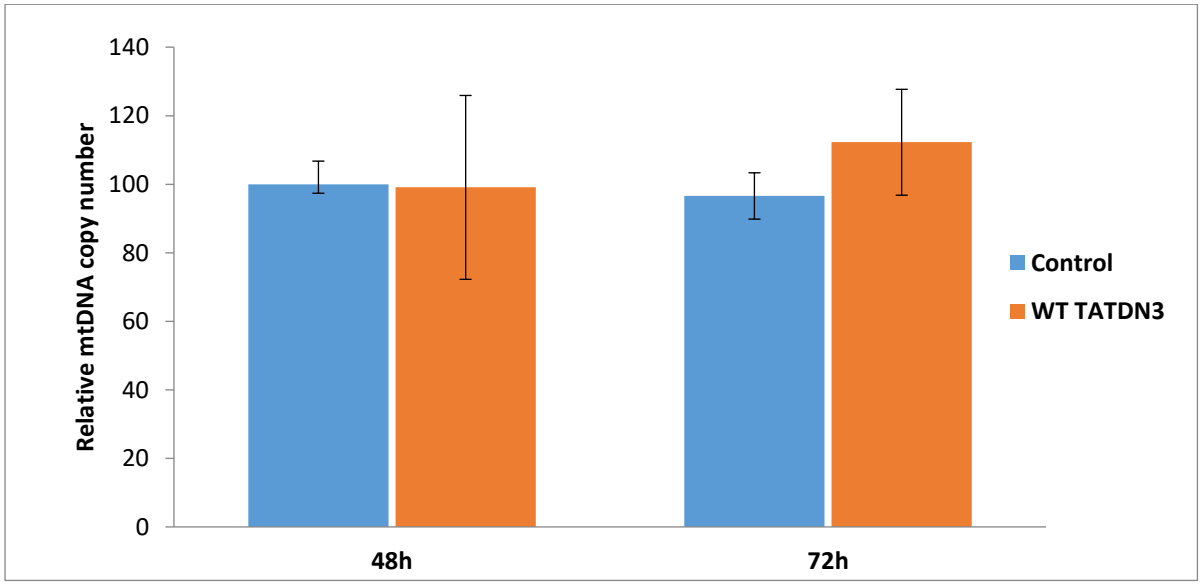


Figure 10. mtDNA copy number upon TATDN3 WT-FLAG overexpression in HEK293 cells. Control 48h Vs TATDN3 48h, *P*=0.96; Control 72h Vs TATDN3 72h *P*=0.48. No significant changes at all. (*n* = 3; two-tailed Student's t-Test; error bars represent STDEV.P).

This mtDNA copy number measurement manifested that overexpression of TATDN3 did not change mtDNA copy number compared to controls.

5.4 mtDNA damage analysis

Mitochondrial DNA damage is a substantial indicator to detect the effects of overexpressed mitochondrial nucleases in cells.

To assess whether increased levels of TATDN3 lead to mtDNA nicks or strand breaks long-range damage PCR was performed using DNA extracted from HEK293 cells transiently overexpressingTATDN3-FLAG and controls (Figure 11).

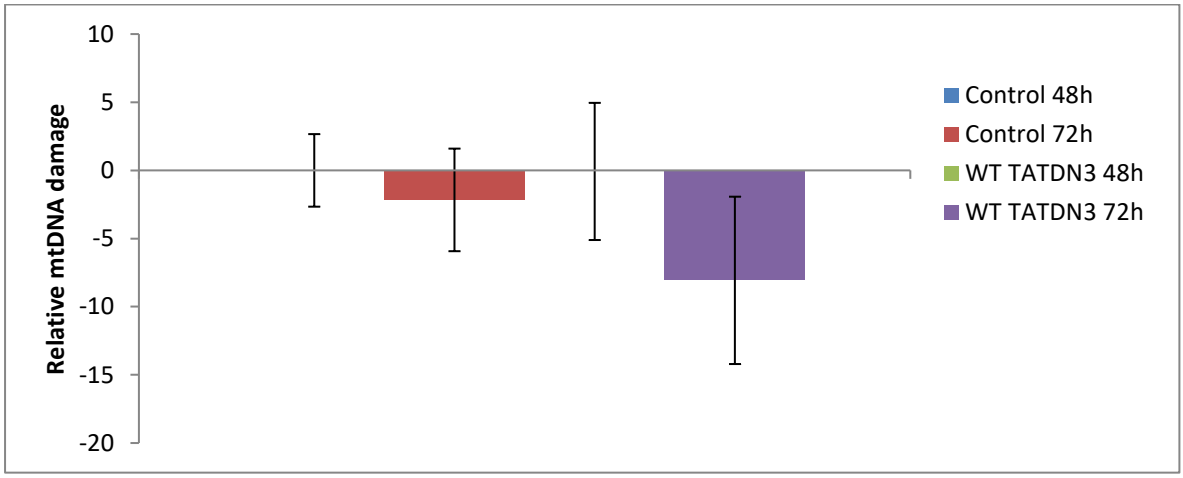


Figure 11. mtDNA damage analysis by long-range PCR upon wild-type TATDN3 FLAG-tagged overexpression in HEK293 cultured cell. No significant differences were observed in between control and wild-type TATDN3 overexpression in HEK293 cells. *P* values were insignificant. Control 48h Vs WT TATDN3 48h overexpression, *P*=0.5; Control 72h Vs WT TATDN3 72h overexpression, *P*=0.54; (*n*=3, 48h; *n*=2, 72h; two-tailed Student's t-Test; error bars represent STDEV.P).

Mitochondrial DNA damage analysis revealed that TATDN3 WT overexpression in cultured cells did not have any influence on mtDNA damage upon TATDN3 WT overexpression after 48 hours and 72 hours respectively.

5.5 Immunoprecipitation (IP) of TATDN3

Interactors of TATDN3 were investigated by IP in WT TATDN3 FLAG transiently transfected HEK293 cells (Figure 12). Anti-FLAG polyclonal IgG1 (see Table 2 for details) was used to pull down the protein of interest.

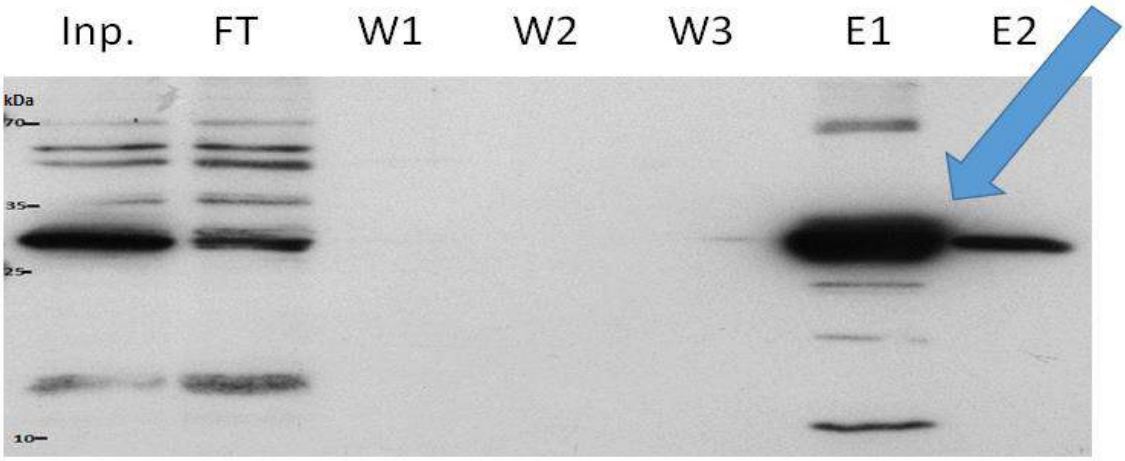


Figure 12. Immunoprecipitation of WT TATDN3 FLAG-tagged protein from HEK293 cells upon transient overexpression. The blue arrow indicates the intense band of successfully pulled-down of overexpressed TATDN3. Inp.=Input, FT=Flowthrough, W1=Wash1, W2=Wash2, W3=Wash3, E1=Elution1, E2=Elution2

The WT TATDN3 FLAG-tagged protein was successfully pulled-down by Immunoprecipitation, as it could be detected in the elution fraction by Western blot.

5.6 Identification of TATDN3 by Coomassie Blue Staining

Coomassie Brilliant blue staining was performed after gel electrophoresis to detect proteins that are pulled down in the Immunoprecipitation due to their interaction with TATDN3. Only TATDN3 itself was detected in the elution fraction (Figure 13).

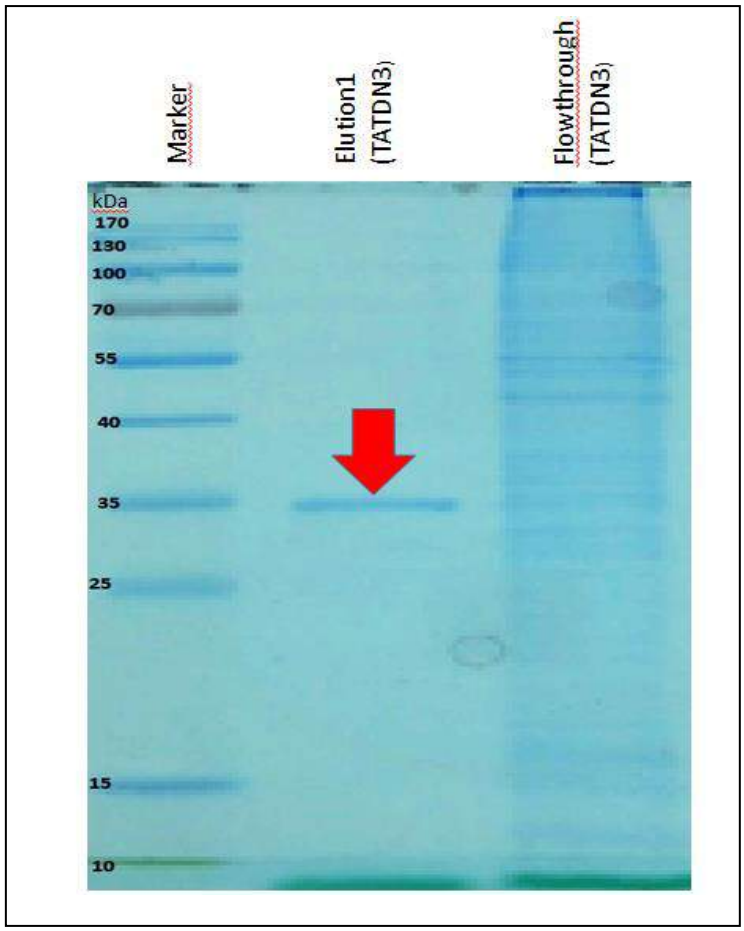


Figure 13. TATDN3 is identified by IP and Coomassie Blue staining. E_{T1}: $Elution_1$ sample from TATDN3 WT FLAG-tagged overexpression HEK293 cells. Flowthrough from TATDN3 WT FLAG-tagged overexpression HEK293 cells. Red arrow indicates that only one protein is present in the sample.

5.7 Identification of TATDN3 by Silver staining technique

To detect also low-abundant proteins in the elution fraction silver staining was performed, and the result confirmed only TATDN3 to be present in the elution (Figure 14).

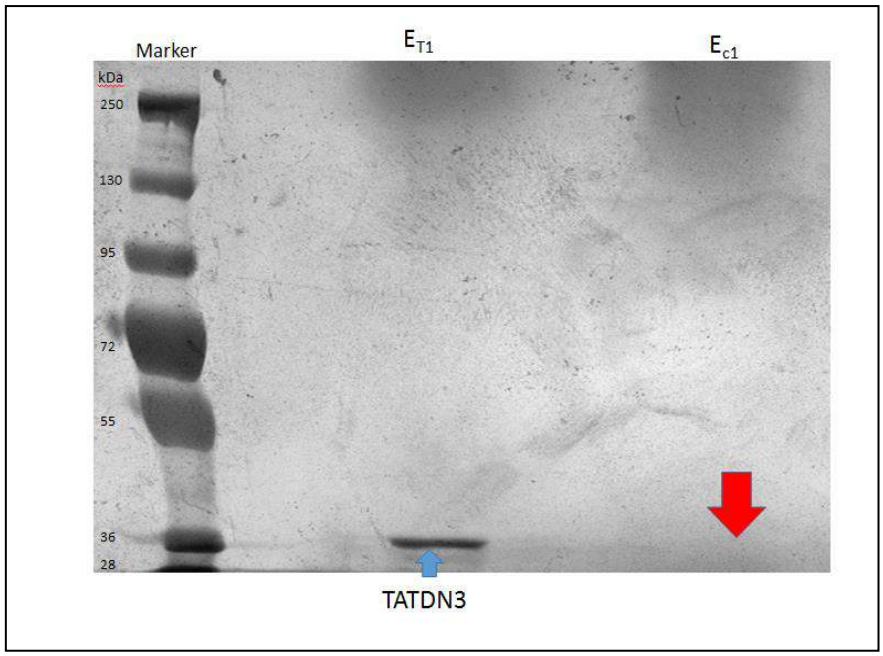


Figure 14. TATDN3 protein is pulled down by IP and clearly identified by Silver staining technique. Blue arrow indicates only TATDN3 WT-FLAG was present. Red arrow indicates that in the control sample FLAG-tagged protein is not present. E_{T1}: $Elution_{T1}$-TATDN3 WT FLAG-tagged overexpression in HEK293 cells, E_{C1}: $Elution_{C1}$-Control HEK293 cells without TATDN3 WT- FLAG transient transfection.

5.8 Identification of TATDN3 in the elution fraction by an antibody against TATDN3

To test a polyclonal antibody against TATDN3 flowthrough and elution fraction of the FLAG- tagged IP was blotted and probed with the anti-TATDN3 polyclonal IgG (see Table 2 for details) (Figure 15).

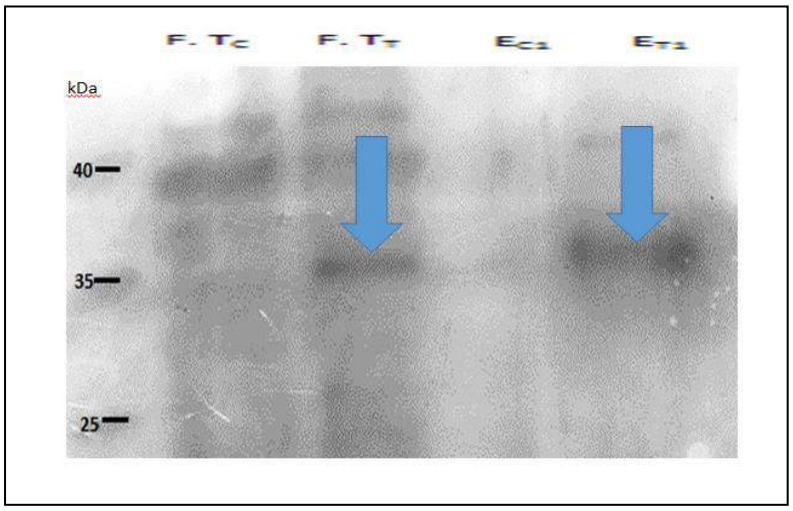


Figure 15. Western blotting to identify the TATDN3 protein by Anti-TATDN3 antibody. $F.T_C$= FlowThrough control, $F.T_T$= FlowThrough TATDN3, E_{C1}= Elution1 Control, E_{T1}= Elution1 TATDN3. Blue arrows indicated only TATDN3 was present. Stated IP samples were collected from TATDN3 WT-FLAG tagged overexpressed (transient transfection) and non-transfected (control) HEK293 cells.

Western blotting result demonstrated that the Anti-TATDN3 antibody recognized the desired TATDN3 protein, but with low efficiency. The antibody signal had also several unspecific bands that were present also in the negative control elution.

Although the above mentioned performed staining did not indicate any interactors of TATDN3 to be abundantly present in the IP elution, candidate proteins were investigated by Western blot using specific antibodies. Proteins searched for were TFAM, mtSSB, Top 2β, Top-3α and PEO (see Table 2 for details). The Western blotting results revealed that all these mitochondrial interacting proteins can be detected in the flowthrough, but are absent in both control and overexpressed TATDN3 elution sample (Figure 16).

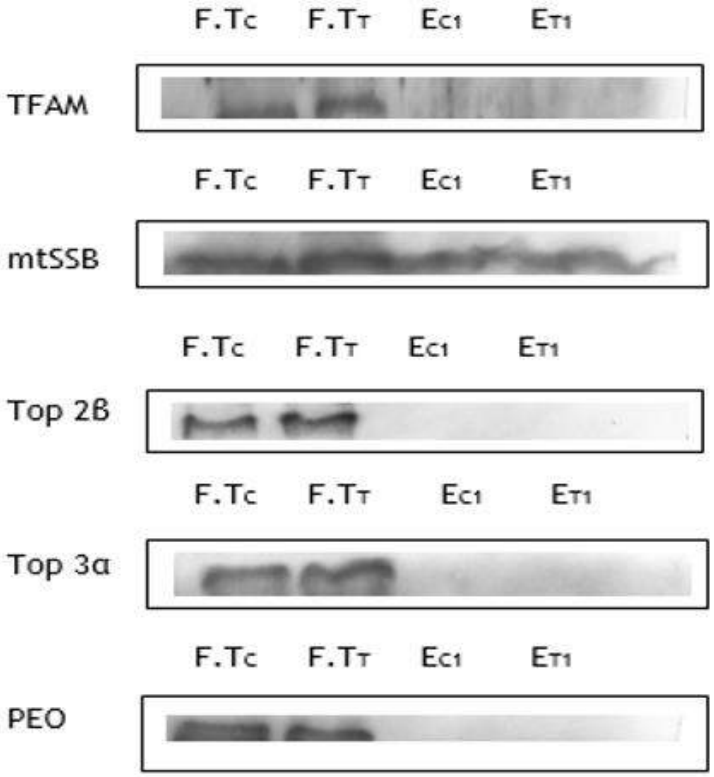


Figure 16. Mitochondrial interacting proteins were analyzed by Western blotting using IP samples. $F.T_C$=Flowthrough control, $F.T_T$=Flowthrough TATDN3, E_{C1}= Elution1 Control, E_{T1}= Elution1 TATDN3.

All above experimental results harbor that even though TATDN3 is a mitochondrial protein, it does not interact with known mtDNA maintenance proteins.

5.9 siRNA transient transfection

As overexpression of TATDN3 did not alter any features of mtDNA, I aimed to reduce TATDN3 protein levels by siRNA knockdown in HEK293 cells. Two siRNAs (siRNA1, siRNA2) and siRNA negative control agents were used to knock down TATDN3. The knockdown efficiency was quantified using Western blot (Figure 17) and mRNA quantification.

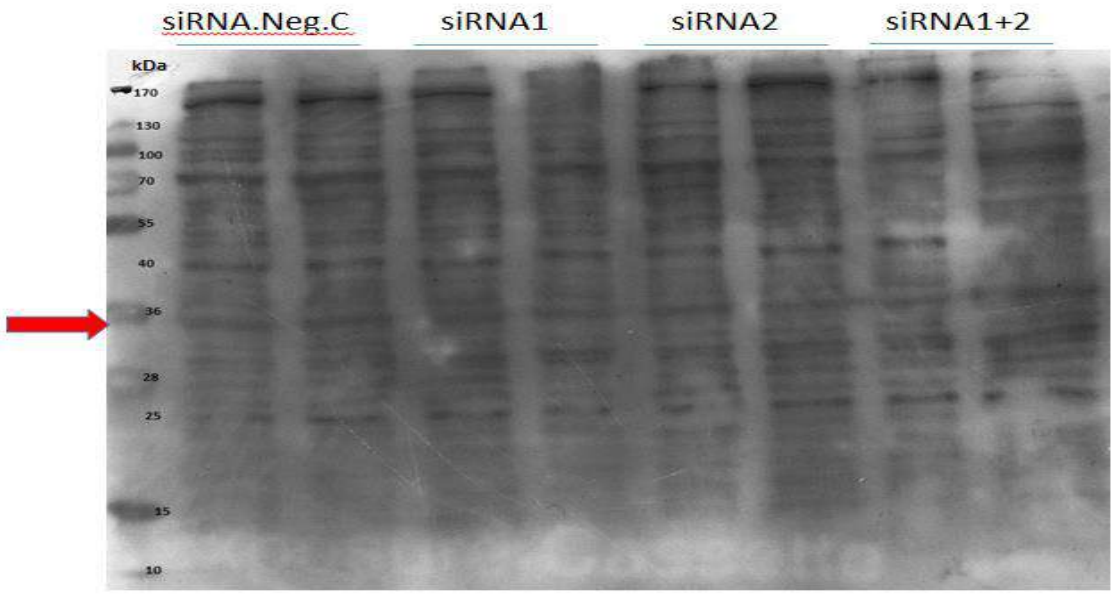


Figure 17. Western blot of HEK293 cells after knockdown of TATDN3 by siRNA transient transfection. Poorly defined non-specific protein bands were visible in the blot.

As the anti-TATDN3 antibody was too unspecific to detect endogenous TATDN3 protein, it was not possible to judge the knockdown efficiency by Western blot.

5.10 Quantification of TATDN3 mRNA levels after siRNA knockdown

As the knockdown efficiency for TATDN3 could not be judge on protein level, the mRNA levels were quantified by Reverse Transcription-qPCR (Figure 18). The results indicate that the mRNA levels were reduced by ~75% when using a combination of both siRNA1 and siRNA2 compared with siRNA negative control.

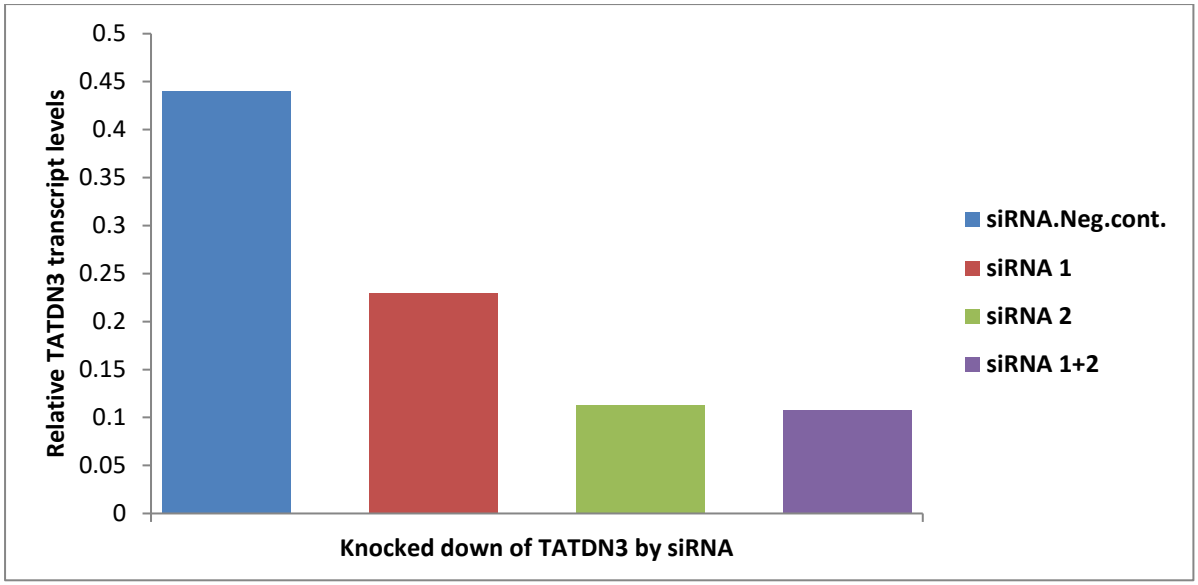


Figure 18. Quantification of TATDN3 transcription level after TATDN3 gene was knocked down by siRNA. Reverse transcription and SYBR PCR methods are used. siRNA. Neg. Cont. = siRNA negative control. (*n*=2).

As the knockdown by siRNA was successful, mtDNA topology and cellular growth rate were analyzed in TATDN3 knockdown cells.

5.11 mtDNA Topology upon TATDN3 knockdown

To investigate whether reduced levels of TATDN3 had any influence on mtDNA conformation, the topology of mtDNA in knockdown cells was compared with control-siRNA treated cells (Figure 19). The result revealed that knockdown of TATDN3 in cultured HEK293 did not have any effect on topological isomers of mitochondria.

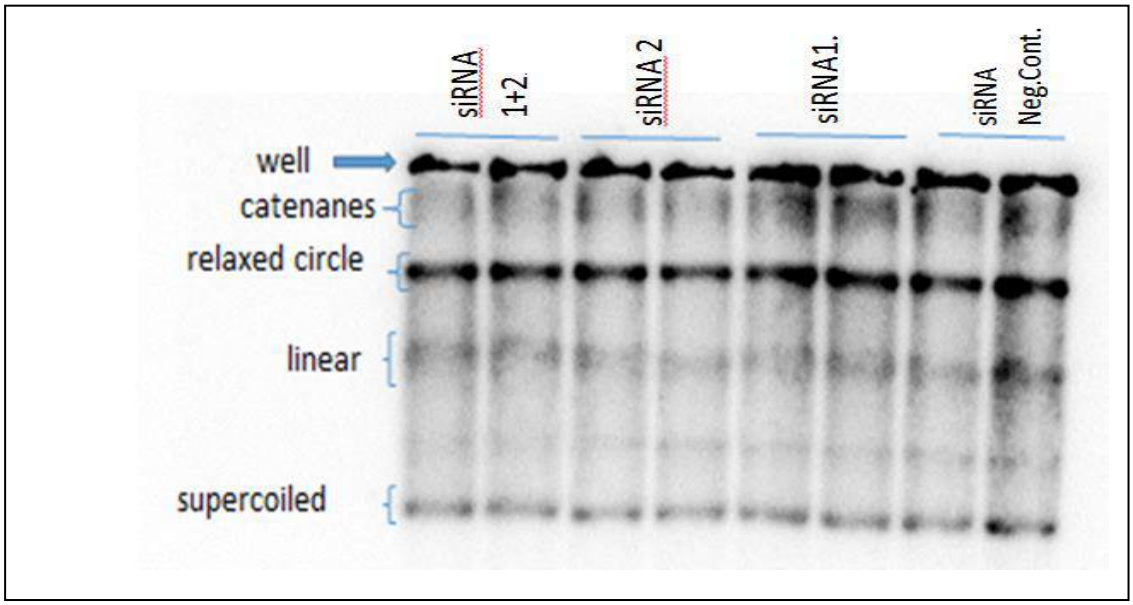


Figure 19. Topological isomers identification by Southern blotting after TATDN3 is knocked down. (*n*=2).

5.12 Mitochondrial transcription

To detect any influence of altered TATDN3 levels on mitochondrial transcription a Northern blot with RNA of control, knockdown and TATDN3 overepxressing cells was probed for ND5+6. The results revealed that ND5 and ND6 mRNA transcription levels were not changed significantly in any of the treatments compared to control (Figure 20)

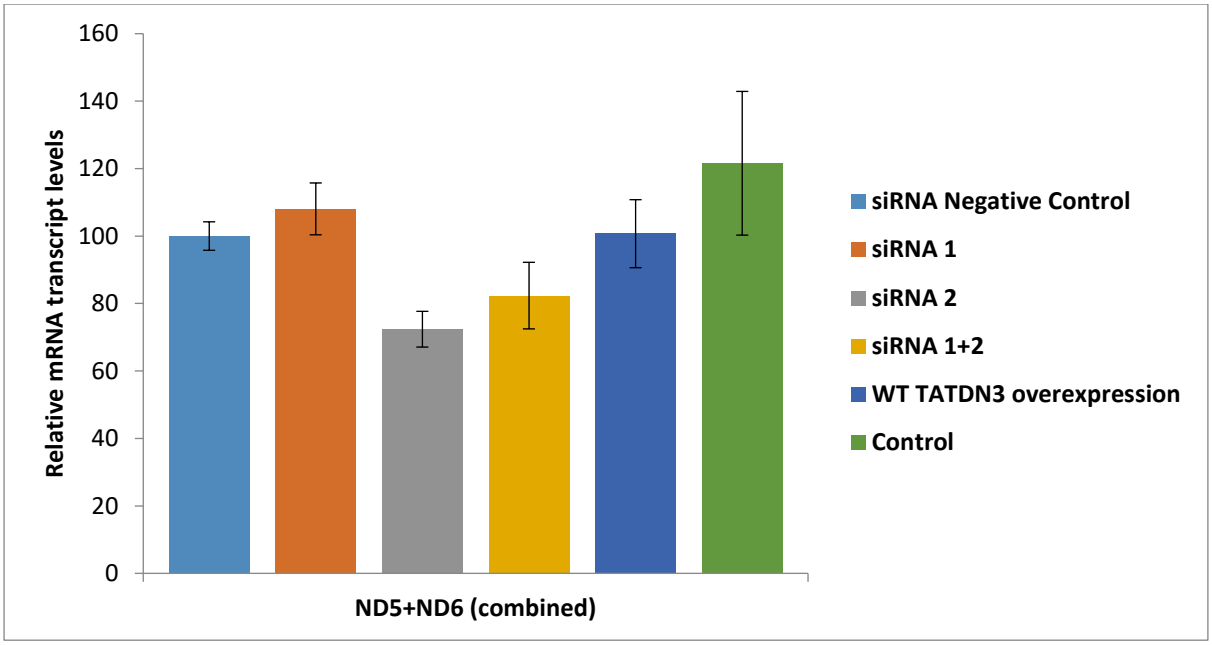


Figure 20. Mitochondrial transcription level is analyzed by Northern blotting after TATDN3 is knocked-down by siRNA and TATND3 WT overexpression in HEK293 cell line. No significant differences were observed in between knocked down TATDN3 and siRNA negative control; wild-type overexpressed and control HEK293 cells. siRNA Negative Control Vs siRNA2, P=0.059; Control Vs WT TATDN3 overexpression, P=0.49; (n = 2; two-tailed Student's t-Test; error bars represent STDEV.P).

5.13 Cellular proliferation rate

A growth curve experiment was done to observe the effect of TATDN3 knockdown of on cell proliferation rate in HEK293 cells. The analysis (see Table 4 for total amount of cells) revealed that TATDN3 knockdown slowed down the cellular proliferation rate compared to control (Figure 21).

Table 4. Cells counting by Bürker's Haemocytometer

Sample name	Seeded amount	Day 1 (22h)	Day 2 (46h)	Day 3 (68h)
TATDN3 knocked down by siRNA1+2	$5x10^5$ cells	103 cells in 5 large squares =103 cells in 0.5 µL =206 cells in 1 µL $=2.06x10^5$ cells in 1 ml $=3.09x10^5$ cells in 1.5 ml (cells were diluted in 1.5 ml PBS)	$1.24x10^6$ cells(counted as Day1)	$4.0x10^6$ cells (counted as Day1)

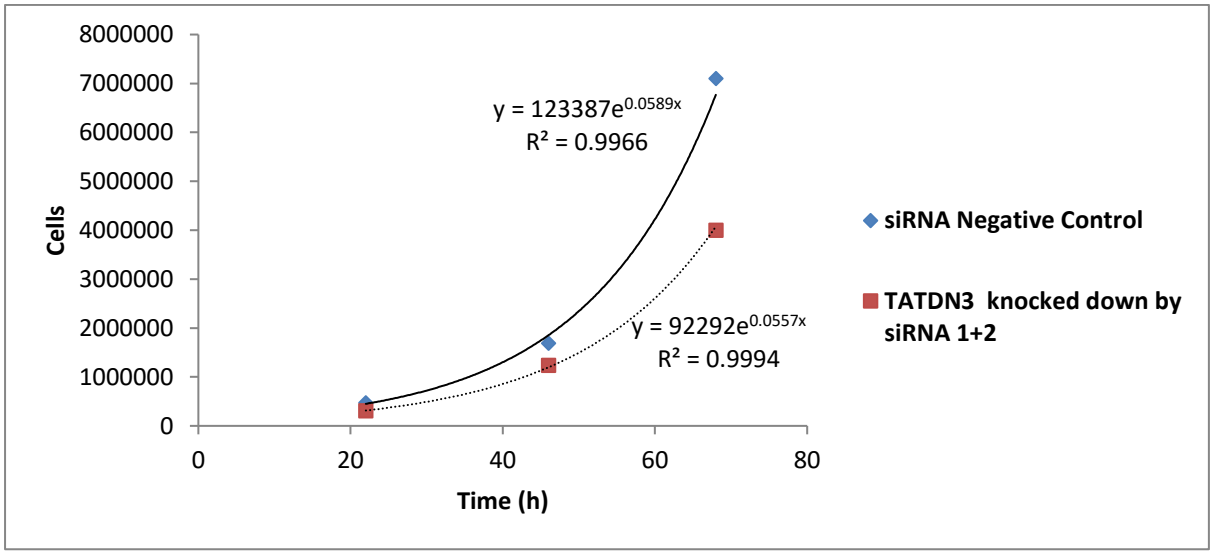


Figure 21. Cellular growth curve after TATDN3 knock-down in HEK293 cell line at 22h, 46h, and 68h time points. TATDN3 gene was knocked-down by the combination of siRNA1+2. (*n*=1).

5.14 TATDN3 overexpression in an inducible stable cell line

During all above-mentioned experiments, TATDN3 was overexpressed and knocked-down by transient transfection. As the transfection rate is variable, making experiments difficult to

reproduce, I switched to stable inducible wild-type and catalytically mutant Flp-In™ T-REx™ 293 cells. This is a stable cell line system allowing the induction of TATDN3 transgene expression by addition of doxycycline to the culture medium. In the following experiments expression of FLAG-tagged TATDN3 wild-type and D218A mutant protein was induced by 10 ng/ml doxycycline. Initially overexpression was checked by Western blotting (Figure 22). While TATDN3-FLAG was detectable after doxycycline addition, no FLAG-tagged protein was detected in the non-induced cells, indicating that the system is not leaky.

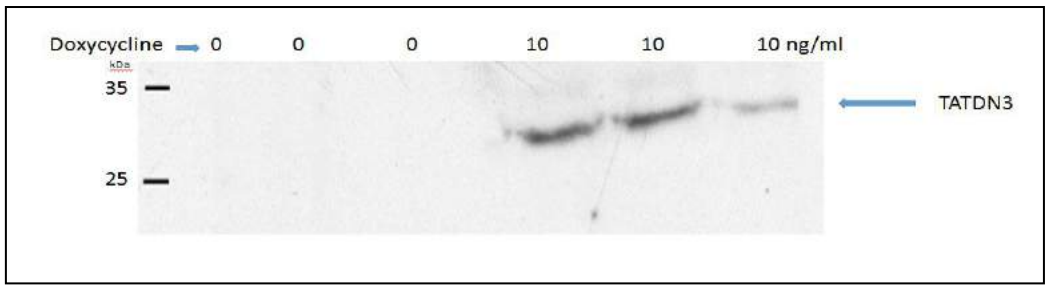


Figure 22. Western blot. Anti-FLAG-tagged polyclonal IgG1 primary antibody (see Table 2 for details) was used to detect expression of TATDN3 WT FLAG-tagged protein upon 10-ng/ml doxycycline induction for 48 hours in 293 Flp-In™ T-REx™ cells.

5.15 Topology

It was demonstrated earlier that overexpression of TATDN3 by transient transfection does not have any influence on mtDNA topology. Similarly also TATDN3-FLAG overexpression in inducible 293 Flp-In™ T-REx™ TATDN3-FLAG does not have any influence on mtDNA topology (Figure 23).

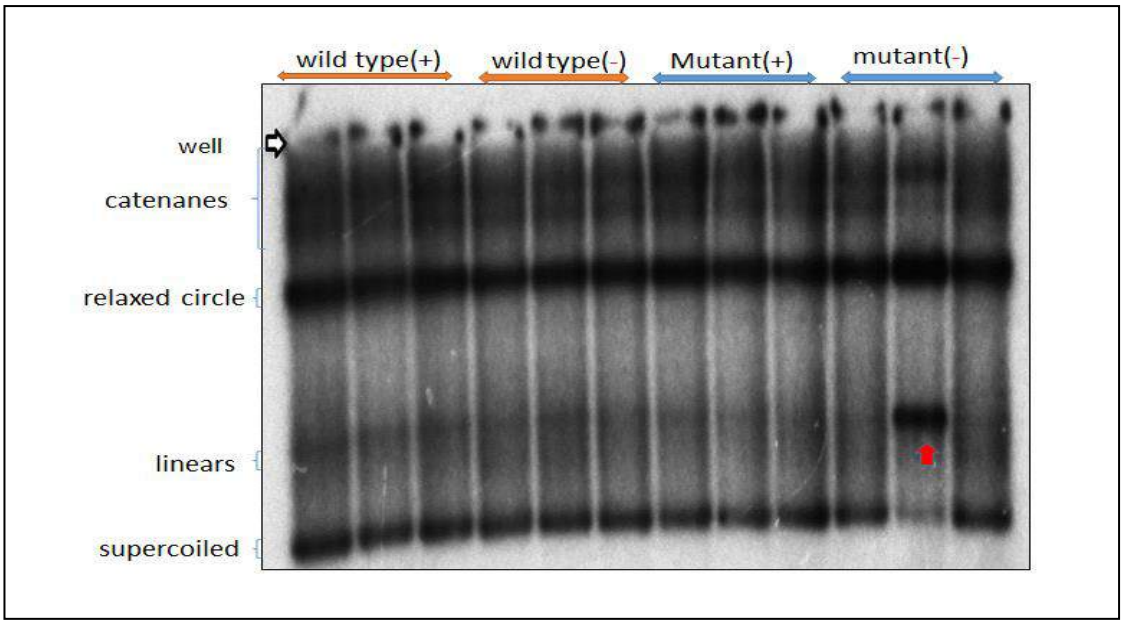


Figure 23. mtDNA topology after doxycycline-induced TATDN3 overexpression in wild-type and mutant 293 Flp-In™ T-REx™ cell lines. The increased levels of TATDN3 cause no significant changes compared to control. (+) indicates doxycycline 10 ng/ml treated cells, (-) indicates without doxycycline. Red arrow indicates abundant linear mtDNA in one sample caused by degradation, e.g. by contamination with restriction enzyme or DNase.

The typical mitochondrial topological isomers such as catenanes, relaxed circle, linears and supercoiled did not change upon overexpression of TATDN3, and this result showed clearly that the overexpressed TATDN3 is not acting as endonuclease *in vivo*.

5.16 mtDNA copy number upon TATDN3 wild-type and mutant overexpression in 293 Flp-In™ T-REx™ cells

mtDNA copy number upon transient TATDN3 wild-type overexpression in HEK293 cultured cell did not change. To confirm these results mtDNA copy number upon induction of 293 Flp-In™ T-REx™ TATDN3 WT-FLAG and 293 Flp-In™ T-REx™ TATDN3 Mutant-FLAG cells were analyzed by qPCR. The result revealed no significant changes upon overexpression of TATDN3 wild-type and mutant protein (Figure 24).

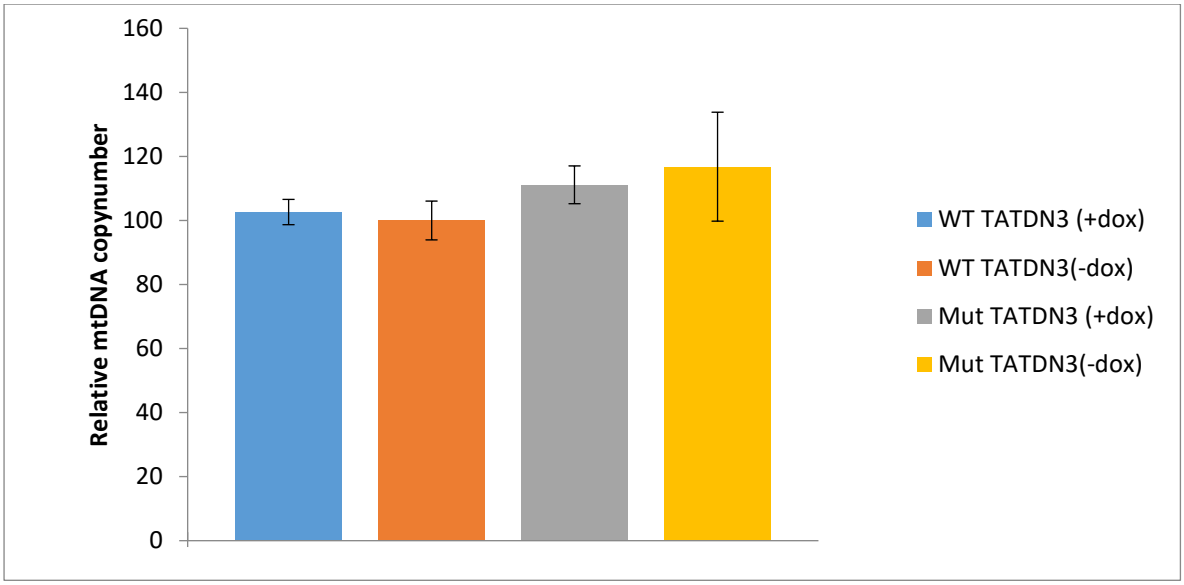
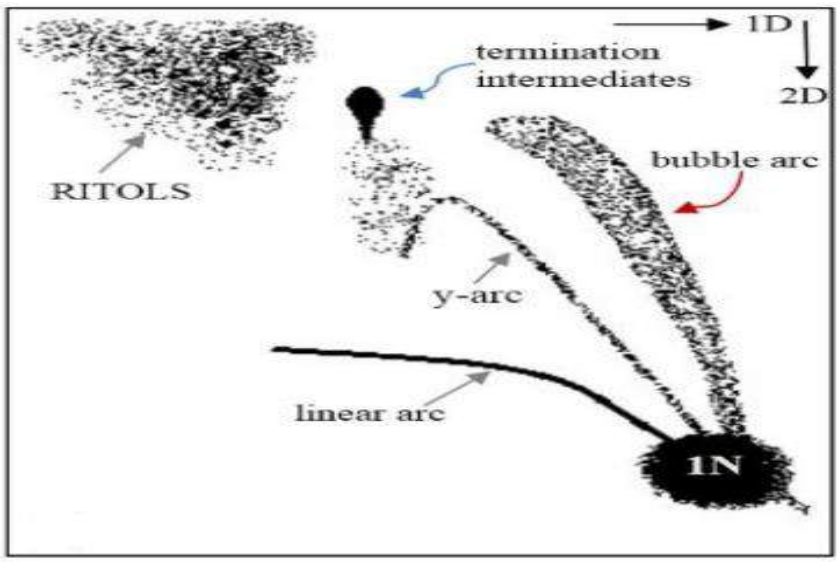


Figure 24. .mtDNA copy number analysis in induced and non-induced 293 Flp-In™ T-REx™ TATDN3 WT-FLAG and 293 Flp-In™ T-REx™TATDN3 Mutant-FLAG cells. (+dox): expression of TATDN3 was induced by 10 ng/ml doxycycline for 2 days; (-dox): without doxycycline (control). No significant differences were observed in both doxycycline-induced TATDN3 and non-induced cell lines. *P* values were insignificant. Wild-type non-induced Vs induced, *P*= 0.83; Mutation non-induced Vs induced, *P*= 0.35; (*n* = 3; two-tailed Student's t-Test; error bars represent STDEV.P).

5.17 2DNAGE

I sought whether TATDN3 overexpression had any influence on processes of mtDNA replication. The mtDNA replication pattern was visualized by 2DNAGE in 293 Flp-In™ T-REx™ TATDN3 WT-FLAG and 293 Flp-In™ T-REx™ Mutant-FLAG cell lines with and without induction of overexpression.



(B)

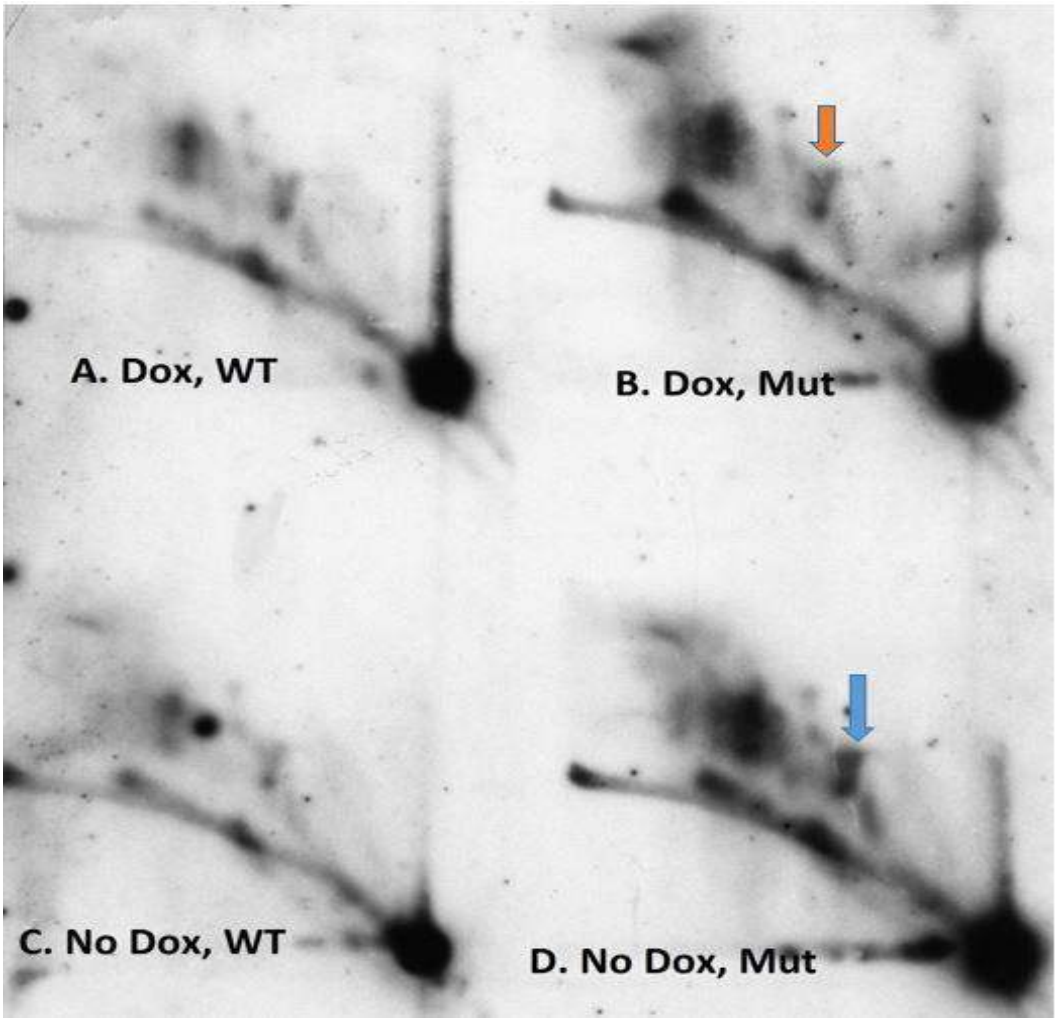


Figure 25. 2DNAGE analysis. (A) Typical formation of two-dimensional gel electrophoresis (obtained from Anu Hangas, Mitochondrial biology group, UEF, Joensuu). (B) 2D blot showing the *Hinc*II-cut O_H-containing mtDNA fragment of induced and noninduced 293 Flp-In™ T-REx™TATDN3 WT-FLAG and Mutant-FLAG cells.

Two dimensional gel electrophoresis revealed that overexpression of wild-type and mutant TATDN3 did not show major differences in replication phenotype or mtDNA content. This implies that mutation of TATDN3 gene might not have an effect on mtDNA replication.

5.18 mtDNA transcription

5.18.1 Mitochondrial transcript analysis upon induction of 293 Flp-In™ T-REx™ TATDN3 WT-FLAG and Mutant-FLAG cells

Reverse transcription and quantitative SybrGreen PCR was used to see mitochondrial transcript levels after doxycycline-induced TATDN3 overexpression in cultured 293 Flp-In™ T-REx™ cell lines. Several mitochondrial mRNA levels were analyzed (12S, ND1, ND5) and compared to the abundant nuclear 28S rRNA. The results revealed no statistical difference between wild-type or mutant TATDN3 overexpression and control (Figure 26).

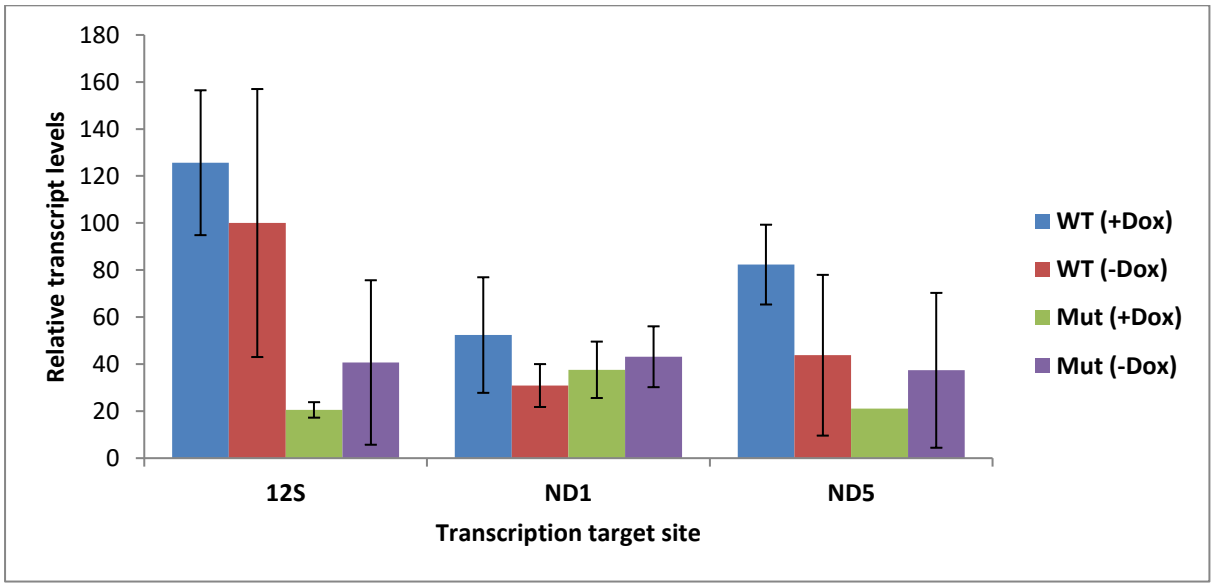


Figure 26. 12S, ND1, and ND5 mRNA transcript levels compared with nuclear rRNA 28S in both WT-FLAG and Mut-FLAG 293 Flp-In™ T-REx™ cultured cell lines. No significant differences were observed in between doxycycline-induced TATDN3 and non-induced cell lines. *P* values were insignificant. Wild-type no doxycycline Vs doxycycline-induced overexpression, *P*= 0.6, 12SrRNA; *P* = 0.3, ND1; and *P* = 0.25, ND5. Mutant no doxycycline Vs doxycycline-induced overexpression, *P* = 0.5, 12SrRNA; *P* = 0.7, ND1; and *P* =unknown, ND5 (due to expression of only one mutant doxycycline-induced sample during qPCR). (n = 3; two-tailed Student's t-Test; error bars represent STDEV.P).

5.18.2 Mitochondrial transcript analysis by Northern blot

As reverse-transcription real time PCR reaction is prone to artifacts and the results showed a rather large standard variation within the treatment groups. Northern blot analysis of mitochondrial transcript levels using the same samples as in the real time PCR was performed to confirm the described results. The analysis of same samples revealed that mutant TATDN3 overexpression in cultured cell reduced the ratio of mitochondrial transcripts to 28S rRNA, although the result was not statistically significant (*P*=0.059) (Figure 27).

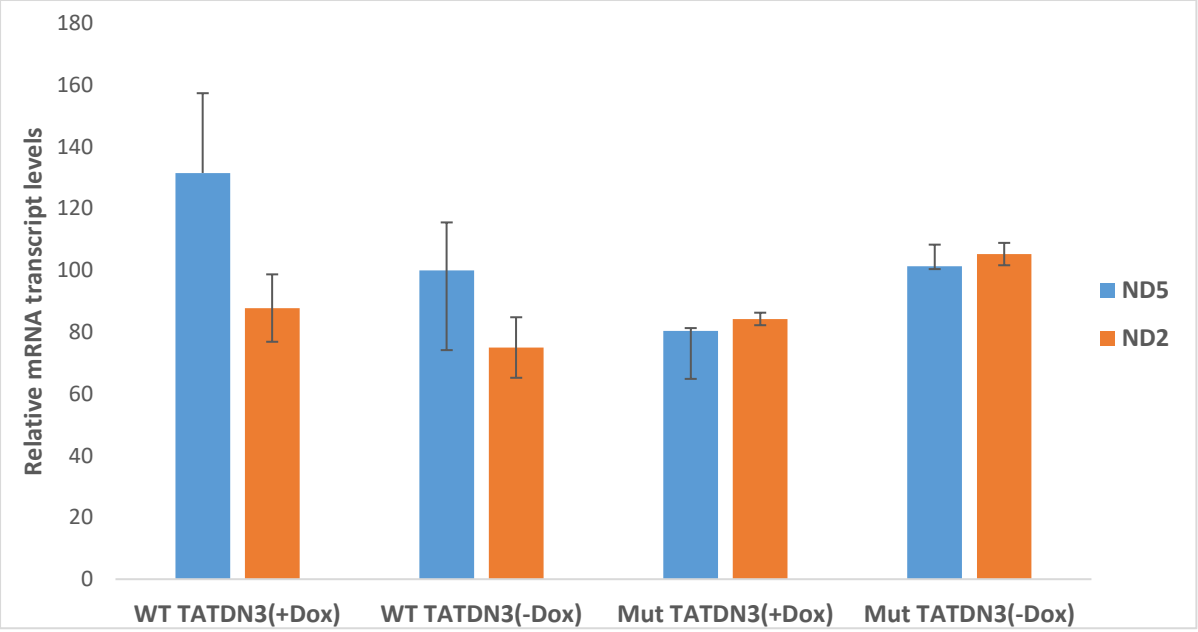


Figure 27. mRNA transcript analysis upon doxycycline-induced TATDN3 overexpression in 293 Flp-In™ T-REx™ cell lines (WT-FLAG, Mut-FLAG) by Northern blotting technique. No significant differences were observed in between doxycycline-induced TATDN3 and non-induced cell lines. In all cases *P* values were insignificant; ND5: wild-type non-induced Vs induced, *P*=0.22; mutant non-induced Vs induced, *P*=0.20; ND2: wild-type non-induced Vs induced, *P*=0.286; mutant non-induced Vs induced, *P*=0.059; (*n* =3; two-tailed Student's t-Test; error bars represent STDEV.P).

The analysis of mitochondrial transcript levels by two different techniques indicated no clear effect of TATDN3 wild-type overexpression on mitochondrial transcription rate. Upon mutant overexpression a trend towards reduced mitochondrial transcript levels was observed, suggesting a potential small influence of TATD3 function on transcript synthesis or stability. As this effect was not statistically significant, it needs to be confirmed in more detail.

6 DISCUSSION

My research hypothesis assumed that hTATDN3 acts as an endonuclease in human mitochondria, creating strand breaks in mtDNA, but my experiments in human cultured cells revealed that this protein does not cut human mtDNA randomly as it does in test tube. Studies in different species such as in *E. Coli*, *S. cerevisiae* and *C. elegans* have shown TATD family proteins are involved in DNA fragmentation during programmed cell death (details in section 2.5 with citations). The unpublished data of our collaborator supports the hypothesis also that hTATDN3 is able to cut and fragment various DNA substrates (see section 2.5). But my all experiments in human cultured cells, especially the analysis of mtDNA topology upon hTATDN3 overexpression does not give any indication for such a DNAse activity during apoptosis.

The TATD gene in *E. coli* is involved in the Tat operon system; in this operon severeal genes (TATA, TATB, and TATC) work together to form the twin-arginine translocon (Tat) system, that works in translocation of folded protein in cytoplasmic membrane in prokaryotic organisms or in plant species in thylakoid membrane. In contrast to the other genes in this operon the TATD family proteins are different, they are not part of the Tat system, but metal ion-dependent and present in all organisms. Some studies claimed that TATD family proteins are DNase domain containing proteins in all species (Chen *et al.* 2014), but from my studies in human cultured cell lines, I did not get any symptoms of mitochondrial DNA degradation due to overexpression of wild-type hTATDN3 or mutation.

Higher organisms possess three TATD genes- TATDN1, TATDN2, and TATDN3. The only studied of these proteins is TATDN1, and the research work was done in zebrafish. The researchers have shown that lack of TATDN1 protein in zebrafish hinders the eye development (Yang *et al.* 2012). TATDN1 works as an endonuclease, as it degrades supercoiled DNA topoisomers and produced circular and linear DNA molecules. The authors conclude that TATDN1 works as decatenase. Based on this study I assumed that also hTATDN3 might work as endonuclease protein, and its localization in mitochondria suggests a role in mtDNA metabolism. Surprisingly my results show that overexpression of wild-type hTATDN3, knockdown or expression of a dominant-negative mutant do not induce any alterations of mtDNA structure.

This might be in congruency with phylogenetic analysis, as TATDN1 and TATDN3 proteins are not included in the same TATD3 subfamily (Al-Furoukh *et al*. 2017 unpublished

data, Umeå University, Sweden). Therefore, TATDN3 might not be an endonuclease as TATDN1.

The *in vitro* characterization of TATDN3 showed that purified hTATDN3 protein has a preference for circular DNA molecules and degrades circular plasmids into relaxed open circles and linear molecules (shown in figure 2.4, Al-Furoukh *et al*. 2017 unpublished data). This biochemical assay suggests that hTATDN3 works as DNase, although the conditions *in vitro* do not represent the natural environment in a cell. Thus my own data from cultured cells should be more representative for the natural function of this protein. In my hands overexpression or mutation of hTATDN3 did not show any indication for effects on mtDNA.

When investigating a protein suspected to participate in mtDNA maintenance it is always effective to investigate the levels of mtDNA per cell, as disturbances in mtDNA replication or integrity should be visible as decrease of mtDNA amounts. Thus, I checked the mtDNA copy number in human cultured cells after overexpression and knockdown of hTATDN3. The rule of thumb would be that if hTATDN3 protein interacts with mtDNA, the mtDNA copy number would be altered during overexpression of wild-type hTATDN3, a dominant-negative mutant hTATDN3 or loss of hTATDN3 in cultured cell. My results did not indicate any effect of overexpression or knockdown on mtDNA copy number, thus not suggesting the idea of hTATDN3 inducing DNA degradation.

Although I did not find any indication of a mitochondrial hTATDN3 endonuclease activity, the possibility of other mtDNA damage upon hTATDN3 overexpression was investigated using a PCR-based detection assay that is based on the inhibition of a DNA polymerase by nicks, abasic sites or nucleotide dimers. My results do not indicate any mitochondrial increase in these damage types upon hTATDN3 manipulation.

To assess any influence of hTATDN3 on mtDNA replication processes I investigated mitochondrial replication by 2DNAGE. My 2DNAGE experiment revealed no effect of hTATDN3 wild-type overexpression on mtDNA replication. The 293 Flp-In™ T-REx™ TATD3 Mutant FLAG-tagged cell line showed more replication intermediates (see figure 25 (B), shown by blue arrow sign) than the wild-type cell line, but no change upon induction. Thus, it remains unclear whether the higher abundance of replication intermediates is a characteristic of this clonal cell line or connected to the TATDN3 mutant transgene.

The lack of TATDN1 protein negatively impaired the eye development of zebrafish (Yang *et al.* 2012), so the protein might be involved in cellular proliferation. Thus also TATDN3 might have some kind of effect on cell growth. My analysis of cellular proliferation

rate upon knockdown of hTATDN3 revealed that reduced levels of hTATDN3 might slow down cell division rate. Further studies are necessary to elucidate the role of TATDN3 in cell growth.

As my results did not indicate any role of hTATDN3 for mtDNA maintenance or integrity, I also analyzed the effect of hTATDN3 on mitochondrial transcription. My results suggest no major role of hTATDN3 for transcription, but a small effect of dominant mutant hTATDN3 on mitochondrial transcript levels is possible.

During my thesis I did not investigate how much overexpressed hTATDN3 protein is actually imported into mitochondrial compartment, therefore it is possible that the overexpression of hTATDN3 did not lead to an increase in mitochondrial protein levels. Also, the siRNA treatment might not have sufficiently reduced mitochondrial TATDN3 levels, as ca. 25% of TATDN3 protein remained.

To summarize, my experiments reveal that in cultured cells hTATDN3 is not working as an active endonuclease in mitochondria and does not seem to be involved in mtDNA maintenance. Knockdown of hTATDN3 reduced cellular proliferation, but the mechanism of influence remains to be investigated.

The role of hTATDN3 in mitochondria thus remains unclear. Its characterization requires further analysis, and additional *in vitro* and *in vivo* experiments in other model organisms would help to elucidate its function.

7 CONCLUSION

The research hypothesis was generated based on previous *in vitro* biochemical assays of extracted purified hTATDN3, and those experiments were studied in our collaborator's lab at Umeå University in Sweden. They confirmed that TATDN3 is a mitochondrial protein and showed also that this mitochondrial TATD protein is a metal-dependent DNase that relaxes and cuts circular DNA molecules such as plasmids, Trypanosoma kinetoplast mtDNA and human mtDNA. Along with it was assumed that it works as DNase protein in human. But all my research experiments in human cultured cells revealed that TATDN3 did not work as DNase.

In vertebrates the only studied TATD protein is TATDN1, a nuclearly localized metal-dependent DNase essential for chromosomal segregation and cell cycle progression. The name-giving *E. coli* protein TATD is a DNase involved in protein import.

To investigate the role of hTATDN3 in mammalian cells the FLAG-tagged wild-type TATDN3 (TATDN3-WT-FLAG) was overexpressed in HEK293 cells. The overexpression was confirmed by Western blot; DNA, and RNA samples were collected for further analysis after 48h and 72h.

The overexpression of an active endonuclease should change the usual topological form of mtDNA. Topoisomers of mtDNA were visualized by Southern blot; but overexpression of TATDN3- WT (TATDN3 wild-type) did not have any influence on the topological isomers of mtDNA. Also, no effect on mtDNA copy number was observed by quantitative PCR (qPCR). Also, long-range PCR, sensitive to damaged templates, did not indicate any DNase activity of TATDN3.

Immunoprecipitation (IP) research technique was used to identify interaction partners of TATDN3, but no known factor of the replication machinery was found to interact with TATDN3.

The effect of knockdown of TATDN3 gene expression on mtDNA was investigated by siRNA transient transfection. The TATDN3 transcript level in HEK293 cells was determined by reverse transcription-qPCR to get confirmation of knockdown of TATDN3 gene. A knockdown of ~75% was found with one of two siRNA constructs, but the loss of hTATDN3 had no effect on the topology of mtDNA. Also, mitochondrial transcript levels were analyzed by Northern blot, but only a small, non-significant effect was observed. On the other hand, the

cellular proliferation rate was checked after siRNA knockdown of hTATDN3 in HEK293 cells, and it was clearly shown that lack of TATDN3 reduces cell growth.

To confirm these results, the above-mentioned experiments were repeated in stable cell lines having inducible expression of wild-type (WT) and mutant (Mut) hTATDN3. 10-ng/ml doxycycline was used to induce hTATDN3 expression. Doxycycline-induced overexpression of wild-type and mutant hTATDN3 had not any effect on topoisomers of mtDNA. 2DNAGE (two- dimensional neutral-neutral agarose gel electrophoresis) was performed to visualize any effect of hTATDN3 on mtDNA replication, but no clear effect was visible.

Mitochondrial transcript levels were checked by both Northern blot and reverse transcription real-time PCR and a trend towards altered transcription was observed. Overexpression of mutant hTATDN3 reduced mitochondrial transcript levels, while hTATDN3-WT increased transcription compared to control, even though results were statistically insignificant.

In conclusion, my results show that knockdown of hTATDN3 gene reduces cell growth, slower, but it has to be confirmed by repeating the growth curve analysis in more detail. Manipulation of hTATDN3 protein levels have no effect on mitochondrial DNA maintenance and integrity, with the exception of a potential small effect on mitochondrial transcription, suggesting hTATDN3 not to be a mitochondrial endonuclease.

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