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Mitochondrial transcription regulation by mitochondrial Topoisomerase I

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Summary

Human mitochondrial DNA (mtDNA) is a closed, double-stranded DNA circle. Strand separation during transcription/replication creates topological stress that interferes with mtDNA metabolism if not released by Topoisomerases. Mitochondrial Topoisomerase I (TOP1MT) is a nuclear–encoded enzyme, resolving supercoils in mtDNA. TOP1MT$^{-/-}$ cells exhibit mitochondrial dysfunction and a nuclear mito-biogenic stress response, although TOP1MT is inessential for mtDNA transcription or replication. Thus, its role in mitochondrial function - while clearly relevant - remains unclear. The aim of this work was to investigate the role of TOP1MT in mtDNA metabolism.

We discovered that TOP1MT acts as direct negative regulator of mtDNA-transcription, a function depending on enzymatic activity. Deficiency or depletion of TOP1MT increased mitochondrial transcripts, whereas overexpression lowered mitochondrial transcripts, depleted respiratory chain complexes I, III and IV, decreased cell respiration and raised superoxide levels. Negative impact on transcription required DNA relaxation activity, which is in striking contrast to the stimulatory effect of Topoisomerase I activity on nuclear rRNA and mRNA transcription. In contrast to constitutive deficiency, acute siRNA-mediated depletion of TOP1MT did not trigger a nuclear mito-biogenic stress response and (compensatory) up-regulation of Topoisomerase IIβ, suggesting that the concomitant increase in mitochondrial transcripts was due to the release of a local effect. TOP1MT was co-immunoprecipitated with mitochondrial RNA polymerase. It selectively accumulated and rapidly exchanged at a subset of nucleoids distinguished by newly synthesized RNA and/or the presence of mitochondrial RNA polymerase. A catalytically inactive TOP1MT variant behaved similarly without affecting mitochondrial transcript abundance. TOP1MT was inactivated by oxidized glutathione and stimulated by thiol-reductive compounds (DTT), suggesting its activity is controlled by the intra-mitochondrial redox state *via* conserved cysteines in the enzyme core. Indeed, increased mitochondrial ROS levels induced *in situ* by antimycin A inactivated TOP1MT. These observations support the model of a double negative loop, by which TOP1MT directly regulates mtDNA-transcription in a redox-sensitive fashion.

Zusammenfassung

Die humane mitochondriale DNA (mtDNA) ist ein doppelsträngiges, ringförmiges Molekül. Transkription und Replikation führen zu topologischem Stress, der beseitigt werden muss um einen korrekten Ablauf der Prozesse zu gewährleisten. Dazu dient unter anderem eine nukleär kodierte mitochondriale Typ IB Topoisomerase (TOP1MT), die mtDNA-Überspiralisierung entfernen kann. TOP1MT ist für Transkription und Replikation inessentiell. Dennoch zeigen TOP1MT$^{-/-}$ Zellen mitochondriale Dysfunktionen und eine mito-biogene Stressantwort im Zellkern. In der vorliegenden Arbeit wurde die unklare Rolle von TOP1MT im mitochondrialen DNA-Stoffwechsel untersucht.

Es stellte sich heraus, dass TOP1MT als dominant negativer Regulator der mitochondrialen Transkription fungiert: TOP1MT-Defizienz stimuliert und TOP1MT-Überschuss hemmt die Expression mitochondrialer Gene. Durch die Hemmung werden die Atmungsketten Komplexe I, III, IV abgereichert, die Zellatmung gedrosselt und Superoxid-Konzentration sowie Laktatausstoß gesteigert. Dieser negative Einfluss auf die Transkription setzt katalytische Aktivität voraus und steht in auffälligem Gegensatz zur Situation im Zellkern, wo aktive Topoisomerase I die Transkription stimuliert. Im Gegensatz zur chronischen TOP1MT-Defizienz induziert ein akuter Mangel im Kern weder eine mito-biogene Stressantwort noch eine kompensatorische Hochregulation der Topoisomerase IIB. Somit ist ein lokaler intra-mitochondrialer Effekt als Ursache der erhöhten mtDNA-Expression wahrscheinlicher als ein im Zellkern induzierter Mechanismus. Durch Koimmunprezipitation konnte eine physische Interaktion zwischen TOP1MT und der mitochondrialen RNA Polymerase (POLRMT) nachgewiesen werden. Innerhalb der Mitochondrien war TOP1MT hochmobil und akkumulierte an aktiv transkribierten Nukleoiden, was durch Kolokalisation mit POLRMT und naszierender RNA gezeigt werden konnte. Eine katalytisch inaktive Mutante verhielt sich vergleichbar, zeigte jedoch keinen Einfluss auf die mitochondriale Transkription. TOP1MT konnte durch oxidiertes Glutathion inhibiert und durch thiolreduktive Substanzen wie DTT stimuliert werden, was auf eine Modulation der Aktivität durch den intra-mitochondrialen Redox Zustand hinweist. Passend dazu wurde die TOP1MT Aktivität *in situ* durch eine Antimycin A Behandlung der Mitochondrien gehemmt.

Die gewonnen Ergebnisse deuten auf einen bis *dato* unbekannten TOP1MT vermittelten Regulations-Mechanismus der mitochondrialen Transkription, der durch den Redox Status innerhalb der Mitochondrien gesteuert wird.

1. Introduction

The mitochondrion is a unique and essential cellular organelle of eukaryotic cells. It is the major component of energy metabolism in cells and plays a key role in cellular homeostasis. Processes like Krebs cycle, oxidative phosphorylation (OXPHOS), β-oxidation, synthesis of metabolites and many other take place inside the mitochondria. Important signal transduction pathways inside the cell such as apoptotic- or Ca^{2+} signaling are dependent of the mitochondria. In human cells mitochondria are the only organelles, which contain their own DNA. The first part of the introduction gives an overview of tasks, features and characteristics of the mitochondrion. The main topics of this work deal with mitochondrial DNA metabolism. Thus the focus of this introduction is on mitochondrial DNA, transcription, translation and replication, and the mitochondrial respiratory chain, which can be considered as core machinery inside mitochondria. Moreover mitochondrial dysfunction and regulation of mitochondrial biogenesis are points of this introduction. Topoisomerases play essential roles in DNA metabolism. Their structure and function is reviewed in the second part of this introduction whereby the focus is mitochondrial Topoisomerases. The introduction shall provide a basic overview of the topics important for the research project presented in this work: the biological function of mitochondrial Topoisomerase 1.

1.1 The Mitochondrion

1.1.1 The origin of mitochondria

Numerous features of mitochondria indicate that these organelles originally were independent organisms, which were engulfed or fused by a common ancestor of present eukaryotes. Theories state that the mitochondrial ancestor was up a α-proteobacterium (Rotte C 2000; Gray 2012). It is thought that the two organisms developed a symbiosis in which the eukaryotic ancestor cell supplied the α-proteobacterium with nutrients, which in return provided chemical energy in form of ATP. During evolution a lateral gene transfer from the α-proteobacterium to the cell nucleus occurred, ultimately resulting in a dependency on each other. The theory is

supported by the fact that mitochondria compared to other organelles posses a double membrane, which might be a relict of endocytosis. Additionally mitochondria still contain relics of functional DNA (mtDNA). In humans mtDNA is closed circular double stranded DNA molecule of 16 thousand base pairs encoding 13 integral proteins of the respiratory chain (RC) as well as the tRNAs and rRNAs needed for transcription of the genetic information (Kasiviswanathan *et al.* 2012). In contrast to eukaryotic nuclear DNA, mtDNA contains almost no introns. It is organized in chromosome-like protein/DNA structures termed nucleoids which resemble bacterial structures. (Garrido *et al.* 2003; Iborra *et al.* 2004). Besides these differences many more discrepancies between mitochondria and other cellular organelles are known. All point to a prokaryotic origin of mitochondria. Although today it is not exactly clarified which organisms served as symbionts to establish the eukaryotic cell (Embley and Martin 2006) there is general consent about the endosymbiontic theory itself, which has great impact on understanding mitochondrial structure and metabolism.

1.1.2 Mitochondrial role in energy metabolism

Mitochondria contribute to essential cellular processes particularly to energy metabolism. These processes are interconnected via their metabolites and form a network with complex implications (Fig 1.1). The most characteristic process in the mitochondria is oxidative phosphorylation (OXPHOS) conducted by the components of the respiratory chain (RC). OXPHOS basically creates a proton gradient, which is coupled to electron transport and drives ATP production by mitochondrial ATP synthase. Other processes of energy metabolism supply substrates, which serve as electron donors for the RC. Pyruvate derived from glycolysis or fatty acid breakdown by β-oxidation is processed by the tricarboxylic acid cycle (TCA) and finally fed into the RC via complex II (CII). Additionally $NADH+H^+$ generated by β-oxidation and the TCA-cycle feeds electrons into the RC via complex I (CI).

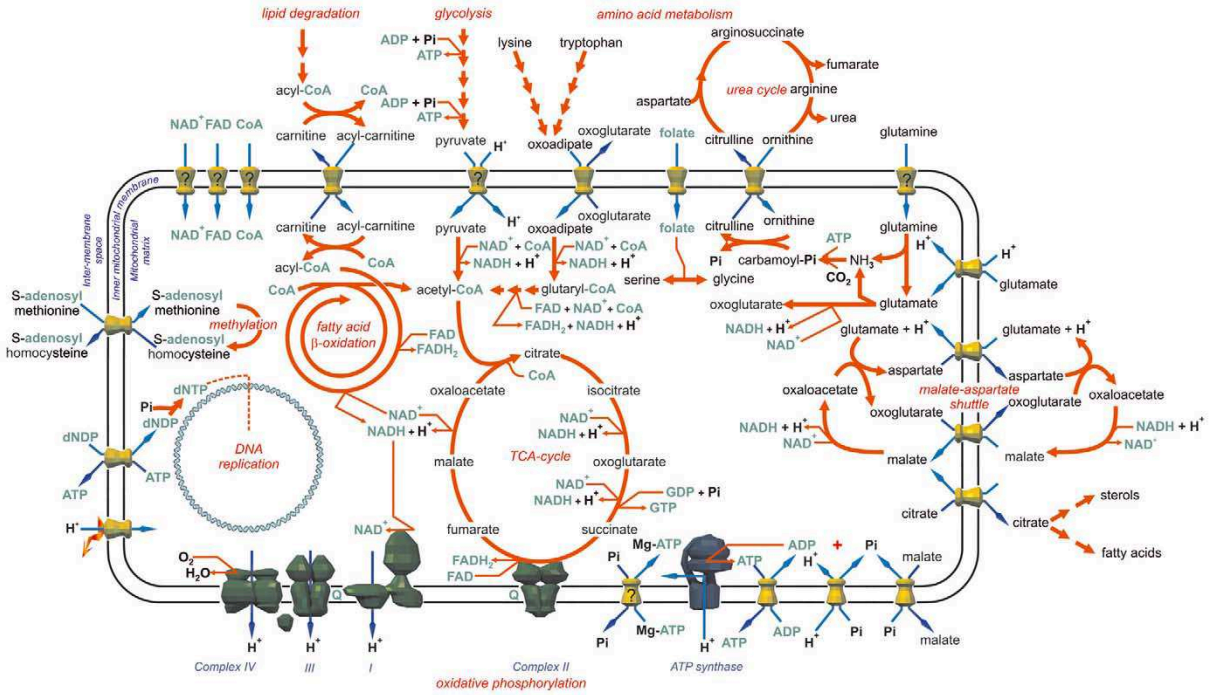

Figure 1.1: Basic mitochondrial energy metabolism. Figure adopted from (Kunji 2004).

This only depicts a small part of the connections between the different pathways but illustrates the interdependency of energy gaining processes. The RC itself is dependent on mtDNA, which encodes central components of the OXPHOS machinery. Thereby mtDNA metabolism is directly linked to energy production of mammalian cells, which emphasizes the importance of mtDNA maintenance and regulation.

1.1.3 Mitochondrial structure

Mitochondria principally can be segmented in the following compartments: outer mitochondrial membrane (OMM), intermembrane space (IMS), inner mitochondrial membrane (IMM) and mitochondrial lumen (matrix). This architecture implicates that an exchange of compounds between the mitochondrial matrix and the cytosol requires transport across two distinct membranes and the intermembrane space. Efficiency of this transport is essential, since mitochondria must not only exchange small molecules such as metabolites, but also import macromolecules such as proteins that cannot be synthesized inside (approx. 99% of mitochondrial proteins) (Wiedemann *et al.* 2004). The translocation of metabolites and other small molecules is facilitated by a number of mitochondrial carriers (Figure 1.1) (Kunji 2004). For the import of proteins, mitochondria developed a specialized transport system consisting

of multiple components. Nuclear encoded mitochondrial proteins are synthesized by common ribosomal translation in the cytosol (Koehler *et al.* 2000). The resulting pre-proteins destined for mitochondrial import contain a specific mitochondrial targeting sequence (MTS) (Rizzuto *et al.* 1989; Gebert *et al.* 2011). With assistance of chaperones the pre-protein is guided to a channel complex of the mitochondrial membrane termed *Translocase of Outer mitochondrial Membrane* (TOM) (Wiedemann *et al.* 2004). TOM recognizes the MTS and conducts import into the IMS. Precursor proteins contain positively charged sections, which - together with electrochemical gradient ($\Delta\psi_m$) created by the respiratory chain - lead to further translocation through the IMM. This process is enabled by a second channel complex called *Translocase of Inner Membrane* (TIM) (Gebert *et al.* 2011). The final step of import requires additional activity of ATP consuming motor proteins (Matouschek *et al.* 2000). Final maturation of pre-proteins includes cleavage of presequence by mitochondrial processing peptidase and protein folding by mitochondrial chaperones (Hartl and Hayer-Hartl 2002; Wiedemann *et al.* 2004)

In human cells mitochondrial shape is considerable different from the commonly depicted bean-like shape. Instead, human mitochondria are long drawn-out organelles with multiple interconnections thus forming a large network (see: Figure 4.4) (Rizzuto *et al.* 1998). Mitochondria are highly dynamic organelles, which adapt to metabolic needs of the cell. This also implies that mitochondrial shape is highly variable and subjected to permanent change. Such changes include constant movement accompanied by fusion and fission of segments of the mitochondrial network (Chen and Chan (2009)).

1.1.4 The respiratory chain

The most notable feature of mitochondria is the generation of energy in form of ATP, which is enabled by the complexes of the RC. The RC permits mitochondria to reduce oxygen (O_2) in controlled manner. Normally the chemical reaction

$$2\,H_2 + O_2 \longrightarrow 2\,H_2O$$

is strongly exothermic (explosive) and involves radical intermediates. By segmenting this reaction, the RC assures a controlled course of the reaction. The energy liberated by this reaction is finally used to generate ATP. The RC is an electron (e^-) transport chain, which couples the transfer of e^- to the translocation of protons (H^+)

from the matrix across the IMM to the IMS. An electrochemical gradient is thus generated, which is used by ATP synthase to produce ATP from ADP (Mitchell 1961). The H^+ gradient represents a reservoir of stored energy, but can also be overcharged and lead to uncontrolled reflux of e^- and the generation of reactive oxygen species (ROS) (see 1.1.5). The RC involves five major complexes. The first four complexes (CI- CIV) serve at electron transport and simultaneous H^+ translocation by numerous consecutive-connected redox reactions. Complex five (CV) is an ATP synthase which acts independent of e^- transport but couples H^+ reflux from IMS to matrix with Pi coupling to ADP, thus generating ATP (Figure 1.2).

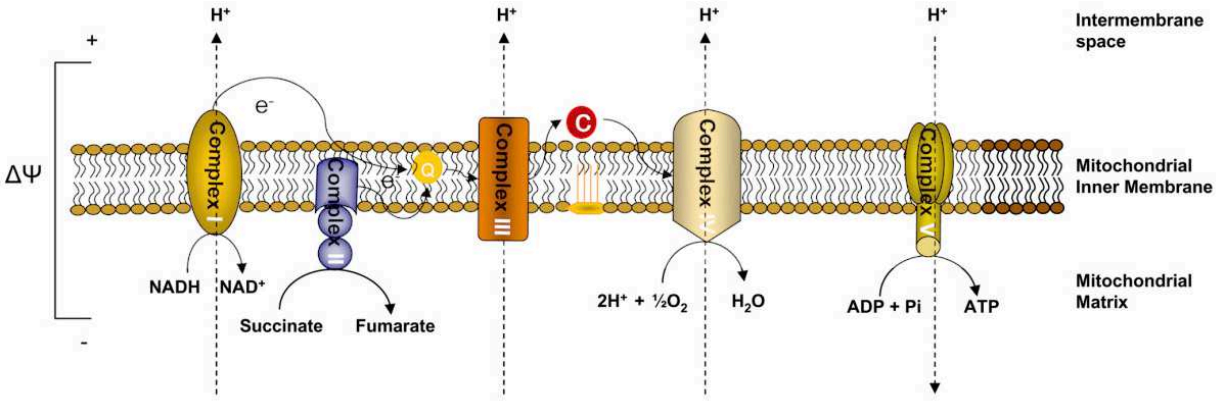

Figure 1.2: Mitochondrial respiratory chain. Schematic figure of the four complexes involved in electron transport (CI-CIV) and ATP synthase (CV) generating ATP by exploiting protongradient $\Delta\psi_m$. Figure adapted from (Bayir and Kagan 2008).

CI, also referred to as NADH dehydrogenase (ND), is a multi-subunit trans-membrane complex containing proteins of mitochondrial and nuclear origin (Lenaz and Genova 2010). It functions as entry point of e^- into the RC. NADH derived from the TCA serves as e^- donor. Each NADH molecule supplies two e^- as it is oxidized to NAD^+ during the reaction. Electron transport by CI facilitates the translocation of four H^+ to the IMS, thereby increasing $\Delta\psi_m$. After passage of CI the e^- are transferred to the mobile carrier Ubiquinone (Koopman *et al.* 2010; Hirst 2013).

CII also called Succinate dehydrogenase is the second major entrance point for electrons stemming from nutrient breakdown. It is a peripheral membrane complex at the matrix side of the IMM. CII is entirely composed of nuclear encoded proteins and does not contain any mtDNA encoded components (Lenaz and Genova 2010; Rutter

et al. 2010). CII supplies e^- from succinate oxidation, conveyed by $FADH_2$ (reduced form of Flavin Adenine Dinucleotide). These electrons are transferred to Ubiquinone. Interestingly, the oxidation of succinate by CII is also a step in the TCA cycle (Figure 1.1) thus directly links TCA cycle and OXPHOS (i.e. nutrient breakdown and energy generation).

CIII is a transmembrane complex named Cytochrome bc_1-complex. It is a multi-subunit complex composed of mitochondrial and nuclear encoded subunits. CIII oxidizes the carrier molecule ubiquinone, whereby four protons are released into the IMS contributing to H^+ gradient formation. The electrons from ubiquinone are transferred to Cytochrome C, a second mobile carrier, which becomes reduced upon e^- uptake (Lenaz and Genova 2010).

CIV is the final link of RC. Similar to CIII, CIV is a multi-subunit transmembrane complex harbouring mtDNA- and nuclear encoded subunits. It serves as acceptor for electrons from Cytochrome C and therefore is denominated Cytochrome C oxidase (COX). CIV transfers the electrons to the end-acceptor molecule, molecular oxygen (O_2). By e^- uptake O_2 is oxidized to H_2O, which represents the final step of e^- transmission through the RC. The energy released by e^- transport through CVI is used for translocation of H^+ from matrix to IMS, thus again amplifying H^+ gradient (Yoshikawa *et al.* 2011).

The two mobile carriers, Ubiquinone (also termed Co-enzyme Q) and Cytochorme C are essential for electron flux through the RC. Beside their function as “pure” carrier molecules they also constitute a pool that stores redox-energy in the form of intermediates of the RC reaction (Genova and Lenaz 2011). This capacity enables the RC to continue constant operation despite transient interruptions of substrate supplementation or temporary dysfunction of single complexes (Lenaz and Genova 2010). The pools formed by mobile carriers also prevent reverse electron transport to some degree. Another important feature entailed by their function is the ability to act as a scavenger for radicals generated by the RC (Atlante *et al.* 2000; Turrens 2003).

CV also named ATP synthase is a large transmembrane multi-subunit protein complex containing proteins of mitochondrial and nuclear origin. The ATP synthase structurally combines an ion channel with an ATP synthesizing complex arranged at the matrix side of the IMM. This complex uses the H^+ gradient to create ATP. The

power of protons passing the ion channel part of enzyme is used for the generation phosphorylation of ADP taking place in a rotating structure at the apex (Rubinstein *et al.* 2003).

1.1.5 ROS generation and clearance

The RC is one major source of endogenous ROS (Balaban *et al.* 2005). The other is NADPH oxidases (Bedard and Krause 2007). The generation of ROS by the RC is a common process, which occurs during e^- transport. ROS mostly develop when e^- escape the RC and are absorbed by O_2 (Murphy 2009). Measurements of ROS production in isolated mitochondria show that 0.12–2% of cell respiration lead to the generation of superoxide anion ($O_2^{\cdot-}$) radicals and subsequently H_2O_2 (Boveris and Chance 1973). ROS generation by the RC increases when electron transport is impaired. This can occur when e^- transport across RC complexes is inhibited by small molecules (Indo *et al.* 2007). Substances like antimycin A, cyanides or oligomycin are known inhibitors potently raising ROS levels by blocking e^- transmission or inducing accumulation of reaction intermediates (Turrens 1997). Another source for faulty e^- transport are mutations in genes contributing to RC function or mtDNA metabolism (Lagouge and Larsson 2013). Moreover disturbance of e^- transport can be induced by a high $\Delta\psi_m$ in combination with a highly reduced Cytochrome C pool. When H^+ concentration in the IMS exceeds at threshold the RC is unable to pump H^+ anymore (Adam-Vizi and Chinopoulos 2006). These conditions allow a reversal of H^+ flow across the RC, which is a major source for ROS production in mitochondria (Murphy 2009). Moreover a high $NADH/NAD^+$ ratio in the matrix can lead to ROS generation (Kussmaul and Hirst 2006).

ROS have a deleterious potential because they can oxidize lipids, proteins and DNA in a non-enzymatic fashion. RC-derived ROS are thought to act predominantly inside mitochondria and thereby harm mitochondrial function. Particularly the damage of mtDNA is crucial for mitochondrial metabolism, since essential components of RC are mtDNA-encoded. This fact provides one main argument for the theory of mitochondria-driven ageing (Harman 1972). Today ROS are considered as ambiguous agents contributing to mitochondrial malfunction but also serving as essential signalling molecules inside the cell (Balaban *et al.* 2005). This implies that ROS generation inside mitochondria must be carefully regulated to allow for ROS regulated processes without irreversibly damaging mitochondrial components. To

prevent damage a multi-component scavenging system is present inside mitochondria. The main component controlling mitochondrial redox status is the glutathione pool (Hu *et al.* 2008; Kojer *et al.* 2012; Mailloux *et al.* 2012). Reduced glutathione (GSH) can buffer oxidizing agents like H_2O_2 *via* the generation of H_2O and oxidized glutathione (GSSG). This system also relies on the concerted actions of various enzymes including GSSG reductase, thioredoxin, peroxiredoxin III, and glutaredoxin. These enzymes finally utilize NADPH to keep the mitochondrial matrix in a reduced state (Garcia *et al.* 2010).These enzymes can mediate intra-mitochondrial regulation of protein activity, when the glutathione pool is shifted to a more oxidized state due to increased ROS production. GSSG can glutathinoylate thiol groups of cysteine residues in mitochondrial proteins and thus alters protein activity (Hurd *et al.* 2005). Reduced protein activity due to glutathionylation has been reported for CI and CV (Brusque *et al.* 2002; Beer *et al.* 2004; Wang *et al.* 2011).

1.1.6 The mitochondrial Genome

Human mtDNA is circular double stranded molecule of about 16.5 kb (Shadel and Clayton 1997). The mtDNA encodes 13 mitochondrial proteins, which are all part of complexes of the RC. Among these 13 proteins seven are constituents of CI (ND1, ND2, ND3, ND4L, ND4, ND5, ND6), three are part of CIV (COX1, COX2, COX3), two contribute to CV (ATP6, ATP8) and one is a subunit of CIII (CYTB)(Falkenberg *et al.* 2007). It is striking that these proteins are core components of the respective RC complexes, which are situated inside the IMM. It has therefore been suggested that intra-mitochondrial synthesis of these highly hydrophobic components has been maintained to avoid their transport across the mitochondrial membranes (Popot and de Vitry 1990). In keeping with this notion, CII that has no transmembrane domain, contains no protein of mitochondrial origin.

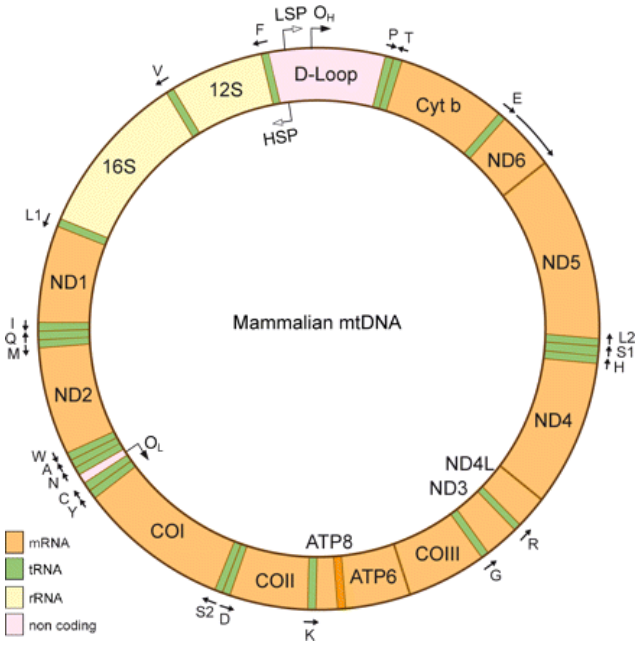

Figure 1.3: Schematic representation of mammalian mtDNA. Illustration of the circular ds mtDNA molecule. LSP: Light Strand Promoter; HSP: Heavy Strand Promoter; OH: Origin of Heavy Strand Replication; OL: Origin of Light Strand Replication. Figure adopted from (Park and Larsson 2011)

Besides proteins, mtDNA encodes two rRNAs and 22 tRNAs, components which are needed for protein synthesis inside the mitochondrion (Smits *et al.* 2010). The two strands of the circular dsDNA are denoted as Heavy- (H) and Light- (L)-strand due to their difference in mass. The mitochondrial DNA contains no introns leading to a tight arrangement of the 37 mitochondrial genes (SCARPULLA 2008). The encoded tRNAs are evenly distributed on H- and L-strand flanking the protein coding sequences. 12 of the 13 encoded proteins and both rRNAs reside on the H-strand, while the sequence coding the protein ND6 is part of L-strand. The only longer non-coding region (NCR) of mtDNA is the regulatory region consisting of the displacement loop (D-loop) and flanking promoter regions. NCR contains promoter regions for transcription start of L-strand (LSP) and two different promoters of H-strand transcription (HSP1 and HSP2). Additionally the origin of H-strand replication (O_H) is located in the NCR (Bonawitz *et al.* 2006). DNA content of human cells differs among cell types. Between 1000 and 10000 copies of mtDNA are typically found in mammalian cells (Falkenberg *et al.* 2007). MtDNA is organized in complex structures consisting of protein and DNA, which are called nucleoids. Each nucleoid holds on average one mtDNA molecule (Kukat *et al.* 2011) and a number of specific proteins (Garrido *et al.* 2003). The most prominent protein is transcription factor A (TFAM).

This protein was first identified as cofactor of mitochondrial transcription, but also shows a function in packaging of mtDNA (Alam *et al.* 2003; Kaufman *et al.* 2007). TFAM is the major component of nucleoids and therefore also referred to as a marker for nucleoid identification. Other components of the nucleoids are proteins directly involved in mtDNA metabolism. This encompasses proteins of transcription like additional transcription factors B1 (TFB1M) and B2 (TFB2M), as well as members of replication machinery such as mitochondrial DNA Polymerase γ (POLG), the mitochondrial helicase Twinkle or the mitochondrial single strand binding protein (mtSSB) (Garrido *et al.* 2003; Di Re *et al.* 2009; Gilkerson *et al.* 2013). Nucleoids were found to be attached to the IMM and ribosomes suggesting a tight spatial coupling of transcription and translation at the IMM, which offers the possibility of direct insertion of synthesized proteins into the IMM (Wang and Bogenhagen 2006; Hobbs *et al.* 2001; Fox 2012). It is likely that more components contribute to the nucleoid structure in a temporary or permanent manner than currently known.

1.1.7 Mitochondrial Transcription

The mitochondrial DNA contains essential coding sequences on both strands. Thus mtDNA also features a single promoter region for each strand, the HSP and the LSP. Both regions are part of the NCR and are fundamental for transcription initiation (Asin-Cayuela and Gustafsson 2007). Mitochondrial transcription produces polycistronic precursor RNAs (Falkenberg *et al.* 2007), which need further procession to reach mature mRNA. For transcription of the heavy strand two different promoter regions named HSP1 and HSP2 are known (Bonawitz *et al.* 2006). Transcription from HSP1 starts 16 bp upstream from the $tRNA^F$ gene and terminates downstream of the 16S rRNA gene. This leads to a transcript containing in particular the two mitochondrial rRNAs (12S and 16S rRNA) which permits an rRNA expression independent of protein expression (Scarpulla 2008). Transcription starting at HSP2 gives rise to a transcript corresponding to almost the complete H-strand. Initiation of transcription from LSP can result in two different transcripts. Regular translation produces a transcript covering nearly the entire L-strand, while a premature termination of transcription yields short RNA pieces, which are thought to function as primers in mitochondrial replication (Holt 2009). Thus the processes of transcription and replication are directly linked in mitochondria.

The basic mitochondrial transcription machinery comprises a RNA Polymerase (POLRMT), TFAM, and two related transcription factors called TFB1M and TFB2M. *In vitro* the combination of POLRMT, TFAM and TFB1M or TFB2M is sufficient to initiate transcription from DNA templates (Falkenberg *et al.* 2007). It is discussed whether two components (POLRMT and TFB2M) are sufficient for transcription initiation (Shutt *et al.* 2011). It is likley that this basic machinery is complemented by a variety of transient proteins that also contribute to transcription *in vivo*. Although it seems clear that the mitochondrial ancestor was of bacterial origin, sequence alignments revealed similarities of POLRMT to RNA polymerases in bacteriophages (Masters *et al.* 1987). POLRMT has a unique N-terminal domain whose function is not totally clear. Experiments showed that depletion of that N-terminal domain can result in mtDNA loss but the function in transcription remains unknown (Wang and Shadel 1999). The C-terminal region of POLRMT exhibits two pentatricopeptide repeat (PPR) motifs. The PPR is common motif in proteins that bind RNA and mediate interaction between RNA and other proteins. In line with this notion, POLRMT alone is unable to bind promoter regions. It depends for these actions on additional transcription factors and forms heterodimer complexes with TFB1M or TFB2M (Falkenberg *et al.* 2002). Both TFB proteins show structural similarities to a family of rRNA methyltransferases, which catalyse the dimethylation of a rRNA subunit during ribosome biogenesis (Falkenberg *et al.* 2002). Although both TFB proteins can support initiation of transcription TFB2M is the much more potent initiator, while TFB1M plays a major role in the methylation of mitochondrial rRNA (Shoubridge 2009). TFAM protein contains two common motifs named high-mobility box (Holt *et al.* 2007). These proteins are characterized by the ability to bind, unwind and bend DNA. TFAM likely plays a similar role in mitochondrial transcription. It is thought that by sequence-specific binding upstream of the promoter region the mtDNA is bend and thereby partially unwound which enables binding of the polymerase complex (Hallberg and Larsson 2011; Rubio-Cosials *et al.* 2011). Additionally TFAM plays roles in packaging of mtDNA in nucleoids and replication (Gensler *et al.* 2001; Alam *et al.* 2003).

Taken together, the above knowledge about the key players of transcription suggests the following model for mitochondrial transcription: At first TFAM binds sequence specific at a site upstream of the promoter region, thereby bending the DNA. This causes a structural change in the promoter region facilitating the binding of the

TFB2M/POLRMT complex, whereby transcription is initiated and polymerase can start RNA synthesis. This basic mechanism is supplemented by a lot of additional proteins which are required for correct progression, fine-tuning, regulation and termination of transcription. Several mitochondrial transcription termination factors (MTERF 1-4) are known to influence transcription in different ways. For example MTERF1 seemingly plays roles in initiation and termination of transcription from HSP1(Hyvarinen *et al.* 2007). MTERF 2 and 3 bind to NCR are likely involved in positive and negative regulation of transcription, respectively. MTERF 4 is suggested to participate in mitochondrial ribosome biogenesis (Roberti *et al.* 2009; Guja and Garcia-Diaz 2011). Other key-enzymes for replication processes are Topoisomerases. Topoisomerase activity is needed to control the topological state of DNA, which directly influences transcription initiation as well as progression (Parvin and Sharp 1993; Kasiviswanathan *et al.* 2012). Several mitochondrial isoforms of Topoisomerases are known, and it is certain that at least one of them contributes to mitochondrial transcription.

Currently there is little knowledge about the regulation of mitochondrial transcription. One hypothesis suggests that DNA and the production of respective proteins was retained in mitochondria to allow a rapid response to metabolic needs and redox status (Allen 1993). If this were the case it would imply that indicators of metabolic state like e.g. concentration of nutrients, ATP concentration or intra-mitochondrial redox-state must be sensed and transmitted to regulate transcription accordingly. However, until now no metabolite- or redox sensing factor regulating mitochondrial transcription is known. Furthermore, mitochondrial transcription is thought to occur in concert with nuclear synthesis of mitochondrial proteins (Scarpulla 2008), because mtDNA encoded proteins are no independently acting, but are integral components of large RC complexes mostly composed of nuclear encoded proteins. Efficient assembly of these complexes requires a correct stoichoimetry of mitochondrial and nuclear encoded subunits (Hornig-Do *et al.* 2012). This implies a communication between mitochondria and nucleus.

1.1.8 Mitochondrial replication

In mammals mitochondrial and nuclear DNA replication are not coupled (Bogenhagen and Clayton 1977). This detachment enables mitochondrial growth according to energetic demands. The exact mechanism of mitochondrial replication is

a subject of controversial debate, but some of the key players and the reactions carried out by them are known. The minimal replisome capable of DNA synthesis from a double stranded template DNA comprises the POLG and the mitochondrial helicase TWINKLE (PEO1). These two enzymes together are able to synthesize ssDNA up to 2kb. The addition of a third protein, the mtSSB, enhances replication *in vitro* and supports the synthesis of larger DNA strands of about 16kb (Falkenberg *et al.* 2007). *In vivo,* the DNA template structure and the replication process are considerably more complex and require more tools. First of all, replication initiation requires priming by short RNA pieces synthesized by POLRMT (Fuste´ *et al.* 2009). As a consequence, mitochondrial transcription and replication are stringently coupled processes and another enzyme is needed to remove the RNA primers once replication initiation and Okazaki fragment synthesis have been finished, a task in mammals performed by RNase H1 (Wu *et al.* 2013). Moreover, the replication template is a closed double stranded DNA-circle. Therefore, replication is bound to create topological problems, most notably supercoiling and catenation that need to be solved by DNA Topoisomerases (Wang 2002).

Mammalian POLG is a RNA dependent polymerase, which comprises three subunits forming a heterotrimer ($POL\gamma A_1B_2$). It is the only known polymerase in mitochondria and habours multiple faculties. The catalytic subunit POLγA shows polymerase, 3'-5' exonuclease and 5'-desoxyribose phosphate lyase activity (Graziewicz *et al.* 2006). This single subunit is already able to synthesize DNA. The association with the two smaller POLγB subunits is thought to increase processivity by facilitating a stronger binding to the template DNA (Lee *et al.* 2010). Interstingly, recent studies also revealed that POLG also can perform reverse transcription though its physiological relevance is not clear (Kasiviswanathan *et al.* 2012).

The mitochondrial Helicase TWINKLE also named PEO1 belongs to the family of RecA/DnaB enzymes. It catalyzes the unwinding of dsDNA in an ATP dependent manner (Farge *et al.* 2008). TWINKLE is found in homo- hexameric or heptameric forms. The protein alone is only able to unwind short oligonucleotides. Twinkle activity is strongly raises upon stimulation by mtSSB, enabling constant support of replication process (Korhonen *et al.* 2003). The role of TWINKLE in replication has been shown to have a strong influence on mtDNA copy number (Tyynismaa *et al.* 2004; Tyynismaa *et al.* 2005; Milenkovic *et al.* 2013).

MtSSB is small protein of about 13-16kb in different species and forms a tetramer in solution. It binds to ssDNA which is supposed to be wrapped around the protein complex. Replication is strongly enhanced by mtSSB activity and loss of mtSSB in yeast and *drosophila* results in mtDNA depletion thus demonstrating its essential function (Oliveira and Kaguni 2011).

Two different major mechanisms, concerning the course of replication have been postulated: On the one hand the 'strand-displacement model' which assumes a strand asymmetric replication and on the other hands the 'strand-coupled model', suggesting synchronous replication of H- and L-strand. The two models are based on numerous evidences which may hint to mechanism ultimately combining parts of both views. Despite the dissent, there is a consensus that replication depends on the extension of RNA primers generated by transcription machinery (Fuste´ *et al.* 2009; Kasiviswanathan *et al.* 2012). Both theories also agree in the principal mechanism of DNA synthesis by the components described above.

Strand-displacement model (SDM)

In SDM transcription is initiated by a RNA primer stemming from transcription from LSP. To generate primers transcription is terminated prematurely and the short resulting RNA sequence is further processed. At the origin of replication of the H-strand (O_H) replication machinery binds and initiates DNA synthesis of H-strand. When replication accomplishes 70% of H-strand the origin of L-strand synthesis (O_L) is revealed. Exposed OL permits initiation of L-strand replication on the displaced H-strand. In SDM therefore L-strand synthesis is delayed (Clayton 1982; Clayton 1984; Brown *et al.* 2005).

Strand-coupled model (SCM)

The strand coupled model suggests a bidirectional replication modus (Holt 2009; Holt *et al.* 2000). The initiation of replication takes place at a region near O_H and proceeds in both directions. Like in SDM a RNA primer is needed for initiation. The two emerging replication forks move simultaneously in opposite directions, whereby lagging strand synthesis is discontinuous, generating Okasaki fragments. The SCM hypothesis was refined by the discovery of large DNA-RNA hybrid regions in lagging strand synthesis. This led to RITOLS (RNA Incorporated Through out Lagging Strand) model of replication (Yasukawa *et al.* 2006). This model assumes a

transcription initiation at O_H or O_B, representing a second start position in NCR. RITOLS model suggests a bidirectional DNA synthesis like SCM, with the difference that lagging strand synthesis generates a RNA strand instead of DNA leading to formation of a DNA-RNA hybrid. The nascent RNA strand covers the lagging strand until RNA is removed (most likely by RNase H) and replaced by DNA in a second synthesis step. It is hypothesized that this switch to RNA replacement by DNA may be initiated when the proceeding replication fork reaches O_L (Yasukawa *et al.* 2006).

Recently additional putative origins of replication were discovered, which could demand even more complicated modifications of the above replication models (Reyes *et al.* 2005). Moreover, the function of the D-loop is unknown. The D-loop is a triple stranded region of mtDNA, which results from a premature termination of replication from O_H. This frequent event generates a DNA fragment of about 700bp (7S DNA) (Gensler *et al.* 2001), which stays attached to its template. The D-loop is regarded as a structure, which may somehow contribute to transcription- or replication regulation, but evidence for that is still missing (Walberg and Clayton 1981).

1.1.9 Mitochondrial transcript processing

Mitochondrial transcription creates long polycistronic pre-mRNAs which have to be processed prior to translation process. Therefore the individual gene transcripts are separated by cleavage. For this process the tRNA sequences which are flanking and separating other gene sequences play an import role. The tRNA sequences form a specific secondary structure which is recognized and cleaved out by exonucleases whereby adjacent mRNAs and rRNA are liberated. This pre-RNA processing mechanism is named the tRNA punctuation model (Ojala *et al.* 1981). The mitochondrial poly (A) polymerase catalysis polyadenylation of mRNAs to generate stop codons which may also contribute to mRNA stability. At this point RNA degradation may occur in order to regulate RNA levels and to ensure mRNA integrity (Smits *et al.* 2010). Mitochondrial tRNA maturation encompasses the attachment of a CCA triplet at 3'-end, RNA folding, and loading of the corresponding amino acid (Montoya *et al.* 2006).

1.1.10 Translation

Mitochondrial transcription, protein synthesis and protein insertion in the IMM appear to be tightly linked processes. This is reflected by spatial arrangement and seemingly dual functions of the individual components involved. Nucleoids, the units of transcription and mitochondrial ribosomes, are all found associated with the IMM. Both systems seemingly co-localize and protein interactions between both have been demonstrated (He *et al.* 2012). POLRMT in cooperation with TFB1M was shown to interact with 28S subunit of the mitochondrial ribosome, regulating ribosome assembly. In yeast it was found that NAM1, a protein which stabilizes and transports mRNA to IMM, interacts with the RNA polymerase and translational activators coupling transcription and translation procedure (Wallis *et al.* 1994). Important for complete RC-complex formations, which also consist of nuclear encoded portions, the cytoplasmatic translation machineries and protein translocations systems are thought to reside on/in OMM in close proximity to the mitochondrial ones at the IMM (Iborra *et al.* 2004). This seems meaningful for facilitating a concerted assembly of proteins of both origins.

The mammalian mitochondrial translation process by itself is complex and only partly understood, because it involves an immense number of molecules and differs from eukaryotic translation in the cell nucleus as well as from prokaryotic translation (Smits *et al.* 2010). Until now, no functional *in vitro* system for mitochondrial translation could be established. The basic translation machinery is suggested to be composed of: rRNA and tRNAs of mitochondrial origin, aminoacyl-tRNA synthetases and methionyl-tRNA formyltransferase involved in aminoacyl-tRNA synthesis mitochondrial ribosomal proteins (MRP), and numerous initiation, elongation and termination translation factors (Iborra *et al.* 2004). The mitochondrial ribosome contains two rRNAs (12S and 16S) and about 81 MRPs. The translation process can roughly be divided in initiation, elongation and termination processes. The translation is regulated and tethered to IMM by translational activation factors in yeast. In this case translational activation factors are integral membrane proteins which bind mRNAs and likely recruit mito-ribosomes, thereby enabling co-translational insertion of synthesised proteins into IMM (Smits *et al.* 2010; Christian and Spremulli 2012). In humans much less is known about translation regulation. Only few factors are known to effect translation and physiological functions remain speculative yet. Besides direct regulation, a negative feedback loop regulation was discovered. This involves an

AAA-protease which processes MRPL32, leading to MRPL32 association with IMM which seemingly is a prerequisite for protein synthesis. Other substrates (e.g. RC-proteins), especially when available in excess, compete with MRPL32 for binding to AAA-protease which then reduces processing of MRPL32, leading to less protein synthesis. This is thought to happen if unbalanced gene expression of mitochondrial and nuclear RC-genes occurs (Nolden *et al.* 2005).

1.1.11 Mitochondrial DNA repair

Even though initial experiments could not prove the existence of mitochondrial DNA repair systems (Alexeyev *et al.* 2013), today there is a lot of evidence for the availability of these systems (Liu and Demple 2010). This is not striking given that mitochondria are the main source for ROS, which are potent DNA damaging substances. Compared to the nucleus, mitochondria seem to contain a reduced less complex system of DNA repair mechanisms. Mitochondria contain systems for the repair of mismatches, generated by POLG activity or induced otherwise (Boesch *et al.* 2011). The predominant repair pathway in mitochondria is base excision repair (BER). This is in line with the fact that BER is considered the basic pathway to mend oxidative DNA lesions. Both forms, short patch BER and long-patch BER were located in mitochondria (Kazak *et al.* 2012). No major differences concerning mechanism, between nuclear and mitochondrial BER are currently known. Other nuclear repair pathways like the double-strand repair, mechanisms of homolgous recombination and nonhomlogous end joining probably exist in mitochondria but seemingly only play subsidiary roles (Alexeyev *et al.* 2013). The organization of the mitochondrial genome in plenty of copies allows another unique mechanism to cope with severe DNA damage, which is the complete depletion of DNA molecules. Since mitochondria are able to recover even from large loss of DNA, the intended depletion is a consequent method to avoid mutations (Kazak *et al.* 2012).

1.1.12 Regulation of mitochondrial biogenesis

The regulation of biogenesis in mitochondria is a complex issue, which is manipulated by many effectors on different levels. Regarding the major function of mitochondria under regular conditions, the key regulator of biogenesis should be the demand of energy in form of ATP. This implicates that mito-biogenesis should increase when energy is needed and decrease in a state of energy excess.

Additionally cells have to cope with metabolic stress conditions resulting in calorie restriction, hypoxia or elevated ROS levels, which also require adaptive reactions in mitochondrial biogenesis (Piantadosi and Suliman 2012). The major regulating proteins of mitochondrial biogenesis seem to be the co-activators of the peroxisome proliferator-activated receptors (PPAR) whose most prominent member is PPAR co-activator 1α (PGC1A) (Rohas *et al.* 2007; Finley and Haigis 2009). PGC1A is a transcription factor of nuclear mitochondrial genes and a co-activator of other regulators of mt-biogenesis. These interaction partners of PGC1A include the nuclear respiratory factors NRF1 and GABPA (GA-binding protein α chain also termed NRF2), which control the expression of OXPHOS-, mt-transcription/translation- and protein import machinery genes (Kelly and Scarpulla 2004; Roberti *et al.* 2009; Bruni *et al.* 2010). Other interaction partners are PPARγ, and estrogen related receptor α which is a nuclear receptor involved in modulating expression of genes linked to energy metabolism (Handschin and Spiegelman 2006; Rodgers *et al.* 2008). By interaction with main effectors directly manipulating the expression of mitochondrial genes in the cell nucleus, PGC1A is able to conduct complex mito-biogenesis programs and is considered as crucial master switch in regulatory process. The nuclear regulators are activated or inactivated by several pathways, which sense the different triggers of mito-biogenesis (Finley and Haigis 2009). Since many of these triggers are original mitochondrial induced and reflect mitochondrial status, the respective signaling is referred to as retrograde signaling from mitochondria to the nucleus (Finley and Haigis 2009).The mitochondrial-induced signaling-cascades at first place encompass energy dependent signaling. AMPK is the major energy sensing protein in cells (Jager *et al.* 2007). The TCA-cycle and OXPHOS dependent cytosolic NAD^+/NADH and ADP/ATP ratios are sensed and transmitted to nuclear regulators. ADP/ATP ratio is conducted by an AMPK- and mTOR dependent signal transduction, while NAD^+/NADH ratio is thought to implement general redox signaling and involves SIRT1 mediated signaling to the nucleus (Rodgers *et al.* 2008; Finkel 2009).

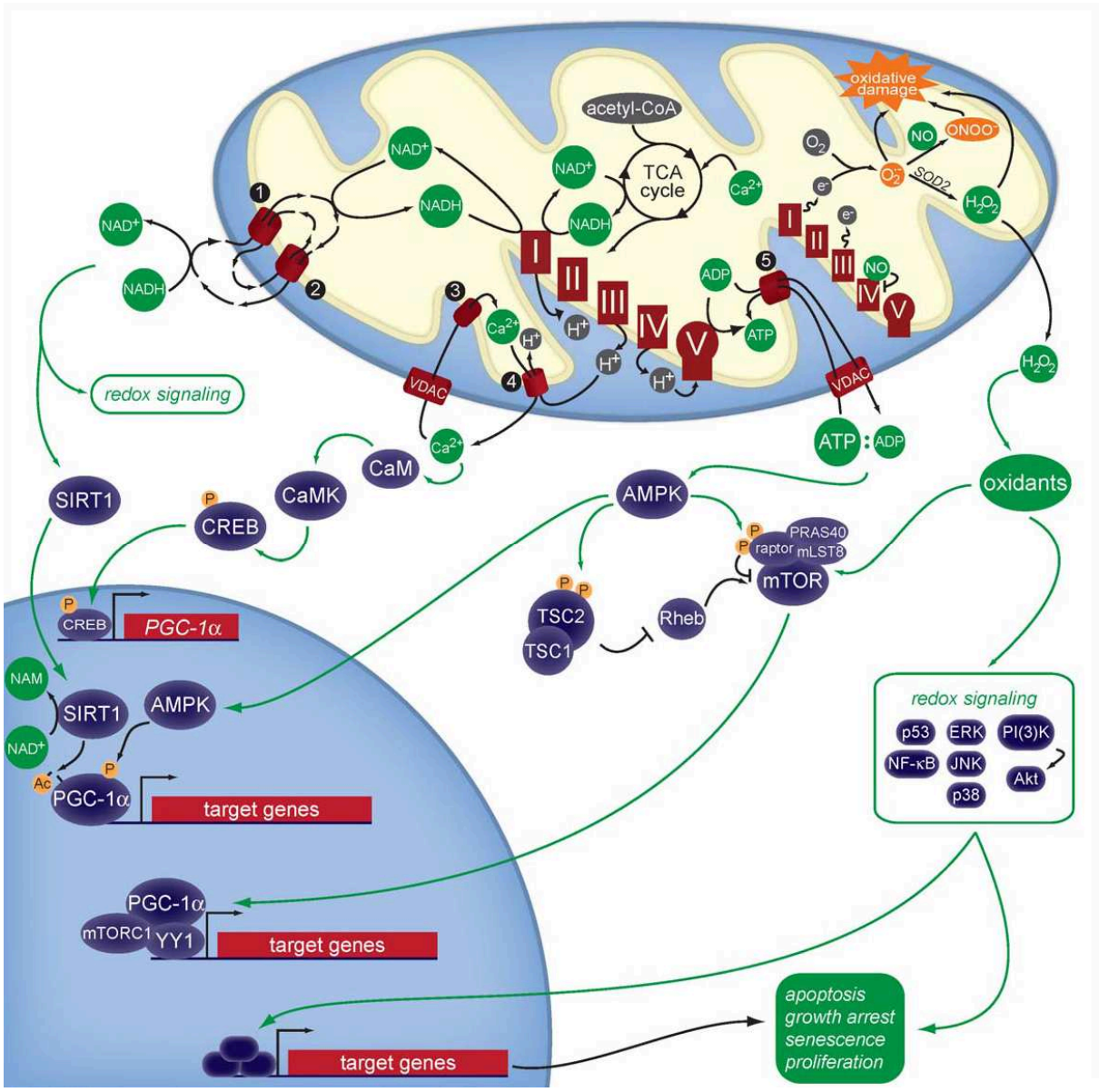

Figure 1.4: Retrograde signalling by mitochondria. Illustration of major pathways of mitochondrial signalling to the nucleus. Signalling occurs via sensing of NAD^+/NADH or ADP/ATP ratios and mitochondrial release/uptake of Ca^{2+} or release of H_2O_2. Figure adopted from (Finley and Haigis 2009).

The second important factor for retrograde signaling is the cytosolic H_2O_2 level, which mainly is a result of ROS production by the RC. Therefore H_2O_2 levels can be considered as a measure of integrity of the RC and the general redox status of the mitochondrial matrix. Thus, H_2O_2 levels reflect RC function and the supply with RC substrates (Garcia et al, 2010). Hydrogen peroxide can oxidize numerous molecules leading to the activation of multiple pathways (Finkel 2011; Finkel 2012). Oxidants have impact on mTOR mediated signaling targeting nuclear mitochondrial gene

expression but also effect general redox signaling (Schieke and Finkel 2006). The third fundamental trigger for mito-biogenesis is the Ca^{2+} concentration in cytosol and mitochondria (Gunter *et al.* 2004; Finley and Haigis 2009). A moderate raise of intra-mitochondrial Ca^{2+} has great impact on mitochondrial functions and leads to mito-biogenesis. Ca^{2+} uptake by mitochondria is dependent of $\Delta\psi_m$, which in turn depends on OXPHOS activity. Disruption of OXPHOS therefore results in raised cytosolic Ca^{2+} levels, which are sensed by calmodulins and trigger pathways resulting in CREB induced expression of PGC1A (Lee and Wei 2005). Prolonged excess of intra-mitochondrial Ca^{2+} level is known to trigger apoptosis by pore opening in OMM and IMM, breakdown of $\Delta\psi_m$ and release of cytochrome C from the mitochondria (Hajnóczky *et al.* 2006). Also relevant for mito-biogenesis is nitric oxide (NO) produced by NO synthases. NO can directly access OXPHOS by competing CIV for O_2 or activate cytosolic SIRT1 and downstream PGC1A thus add to mito-biogenesis (Yamasaki *et al.* 2001; Leary and Shoubridge 2003). NO additionally can generate ROS and thus contribute to ROS signaling. Besides the above mentioned pathways many other surly exist and all together form a large interconnected network to regulate mitochondrial biogenesis according to cellular requirements for mitochondrial products.

1.1.13 Mitochondrial diseases and aging

Mutations and other alterations of mtDNA and the nuclear DNA encoding mitochondrial components can cause mitochondrial disorders, which are known to be the basis of a broad range of human diseases. Currently about 300 point mutations, deletions and duplications of mitochondrial DNA are considered to cause diseases and syndromes in humans: Pearson syndrome, Kearns-Sayre syndrome, and progressive external encephalomyopathy are associated with large mtDNA deletions, whereas mitochondrial encephalomyopathy (MELAS), mitochondrial myopathy and Leber's hereditary optic neuropathy (LHON) result from mtDNA point mutations (Zeviani and Di Donato 2004). Some of these diseases are due to homoplasmic mtDNA alteration (all mtDNA copies are affected) as is the case in LHON, whereas others are heteroplasmic and disease symptoms appear when mutation load exceeds a certain threshold (Chinnery and Hudson 2013). The other part of mitochondria-based diseases develops as a consequence of alterations of nuclear encoded mitochondrial components. This can encompass molecules directly involved

in OXPHOS like RC components, as well as proteins contributing to mtDNA metabolism or protein translocation machinery and many others. Today about 110 mutations of nuclear genes leading to impaired OXPHOS are established (Copeland 2012; Vafai and Mootha 2012). These mutations also produce a large variety of disease patterns as for example: leukoencephalopathies, myopathies, ovarian failure or cardiomyopathies. While some diseases are highly tissue specific, others are multisystemic (Vafai and Mootha 2012). Unsurprisingly in most cases organs are concerned which have a high energy turnover like brain, liver or heart (Chinnery and Hudson 2013).

Another process associated with mitochondrial dysfunction is ageing. Ageing itself, and some of the associated diseases are thought to be triggered by mitochondrial dysfunction and the accumulation of mtDNA damage (Lagouge and Larsson 2013). For decades the “mitochondrial free radical theory of ageing” was a central dogma of ageing research (Harman 1956 ; Harman 1972). This theory states that during ageing free radicals damage mtDNA. This damage results in a defective RC, which in turn produces more radicals. This self-energizing vicious circle was considered responsible for ageing mechanisms (Lagouge and Larsson 2013). More recent findings partly contradict this classic view and suggest alternative models (Hekimi *et al.* 2011). These new models also imply the existence of systems like the mitochondrial redox defense system and mitochondrial DNA repair systems which counteract ROS dependent damage and ageing. Currently the possibility of other than ROS induced mtDNA damage, for example by replication errors is discussed. Moreover it was shown in mouse models that overexpression of oxidant defense enzymes did not significantly extend life span despite a substantial decrease in ROS levels (Pérez *et al.* 2009), while elevated ROS levels induced by an genetic impairement of ROS defense did not decrease life span (Zhang *et al.* 2009). Additionally the emerging role of ROS as an integral element of signaling has lead to a new ageing theory named “the gradual ROS response hypothesis”. This theory assumes that in a resilient (young) cell ROS are generated primary for signaling of damage, to activate the cellular stress response system to remove damage (mitohormesis) (Ristow and Zarse 2010). In this mode the moderate ROS induced damage is controlled by cellular antioxidant system. In aged cells damage accumulates and subsequently ROS levels rise. When ROS levels excel a threshold the antioxidant defense is overstrained and ROS induced damage appears (Hekimi

et al. 2011). This new hypothesis is supported by correlative evidence, but still lacks causal proof.

1.2 Topoisomerases

All biological organisms are dependent on the correct expression, maintenance and inheritance of their genetic information. In humans the complete DNA comprises 23 chromosome pairs with about 20700 protein-coding genes (Pennisi 2012) organized in chromosomes containing about six billion base pairs. Additionally, cells contain several hundred copies of the circular mitochondrial DNA inside their mitochondria, which are also essential for proper cell function (Cline 2012). The large amount of information on one single molecule implicates that efficient organization of DNA is highly important for cells. Cells achieve a high grade of packaging of the DNA molecule resulting in the highly condensed DNA form of chromosomes. DNA mostly exists in supercoiled forms (Travers and Muskhelishvili 2007), which also allow a higher grade of packaging. The handling of such complex DNA structures resembling a tangled ball of wool seems a challenging task for cells. Moreover the state of DNA conformation must permanently be shifted to enable essential processes of transcription and replication of genetic information. This DNA metabolism by transcription and replication themselves also modify DNA topology. When in transcription RNA polymerase moves along the DNA strand positive supercoils are created in front of and negative supercoils behind the transcribing complex (Wu *et al.* 1988). During replication process fork movement creates interwined nascent DNA strands behind the fork and positive supercoils in front of it. Additionally two converging replication forks or recombination of DNA can generate four-way branched structures called Holliday junctions. All these topological conditions can impede DNA metabolism and therefore must be controlled and if necessary actively adjusted. The only enzymes capable of changing DNA topology are Topoisomerases (Wang 2002). The essential role of this enzyme class is reflected in the ubiquitous appearance of Topoisomerases among species in nature (Baker *et al.* 2009). Topoisomerases basically work by binding, selective cutting and religating DNA molecule. Although Topoisomerases differ in the exact mechanism of DNA cleavage the fundamental cleavage reaction is similar in all enzymes (Vos *et al.* 2011). Thereby the OH group of a tyrosine attacks the inter-nucleoside phosphate group of

the DNA. The chemical reaction is a trans-estherfication in which the tyrosine acts as nucleophile. After cleavage the 5'- or 3'-end of DNA stays covalently bound to tyrosine generating a Topoisomerase-DNA complex.

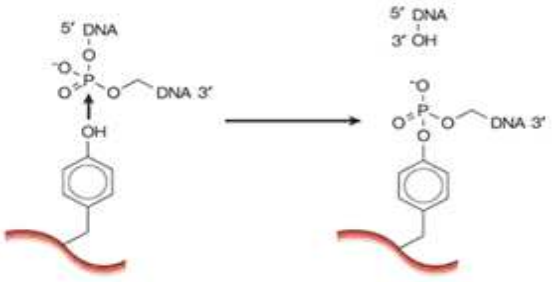

Figure 1.5: Topoisomerase mediated DNA cleavage. The substrate DNA is bound by is a trans-estherfication reaction in which the tyrosine acts as nucleophile. After cleavage the 5'- (or 3'-end in case of type IB enzyme) of DNA stays covalently bound to tyrosine generating a Topoisomerase-DNA complex. Figure adopted from (Wang 2002)

A group of Topoisomerases introduce a a single strand break whereby relaxation of the DNA by rotation of the free end is achieved. Other Topoisomerases can cleave both strands of dsDNA and even allow strand passage of a second DNA strand through the cleaved substrate strand. After designated changes of DNA topology the substrate DNA is religated and released. In the following chapters the different types of Topoisomerases are introduced. This contains a review of basic mechanistics, cellular function and differences of the individual members. The focus is on mammalian forms of Topoisomerases as this work exclusively investigates these.

1.2.1 General classification of Topoisomerases

Topoisomerases are categorized according to their properties. The two main groups are type I Topoisomerases which cleave one DNA strand during a reaction cycle and type II Topoisomerases which cleave two strands. The different members and basic features of these two groups are shown in table 1.1. In the nucleus of human cells three different families of Topoisomerases with overall five different isoforms are known and reasonably characterized (Nitiss 1998). Furthermore at least two mitochondrial Topoisomerase isoforms are currently known. These are type I (mitochondrial Topoisomerases I and IIIα) enzymes which catalyze the transient cleavage and ligation of single stranded DNA (Zhang *et al.* 2001; Wang *et al.* 2002). There is also initial evidence for a third mitochondrial Topoisomerase catalysing cleavage and ligation of both strands of the DNA double helix (mitochondrial Topoisomerase IIβ) (Low *et al.* 2003). In the following the basic types of Topoisomerases will be briefly introduced. As this work investigates the function of

mitochondrial Topoisomerases the focus is on those enzyme classes which have known mitochondrial isoforms. These are type IA (mitochondrial Topoisomerase III), type IB (mitochondrial Topoisomerase I) and type IIA (mitochondrial Topoisomerase IIβ).

Table 1.1: Types of DNA Topoisomerases. Human Topoisomerases in bold letters.

TYPE	STRUCTURE	SUBSTRATE	Catalysis	Members
IA	monomeric	(-) supercoiled DNA	**Single Strand cleavage** ssDNA passage	Eubacterial top I+II Yeast top III Drosophila topo IIIα+IIIβ **Mammalian topo IIIα+IIIβ**
IB	monomeric	(-)/(+) supercoiled DNA	**Single Strand cleavage** DNA rotation	**Eukaryotic top I + top Imt** Pox virus top I
IC	monomeric	(-)/(+) supercoiled DNA	**Single Strand cleavage** DNA rotation	*Methanopyrus kandleri* Top V
IIA	dimeric	(-)/(+) supercoiled DNA	**Double strand cleavage** dsDNA passage	Bacterial gyrase, top IV Phage T4 top Yeast top II Drosophila top II **Mammalian top IIα+IIβ**
IIB	tetrameric	(-)/(+) supercoiled DNA	**Double strand cleavage** dsDNA passage	*S. shibatae* top IV

1.2.2 Type I Topoisomerases

Type I Topoisomerases are mainly characterized by their ability to introduce a transient single DNA break. The family of type I Topoisomerases is subdivided into

type IA and type IB depending on their link to the substrate DNA. Lately a third class of type IC was proposed (Taneja *et al.* 2006) which is also considered in this chapter.

1.2.2.1 Type IA Topoisomerase

Type IA Topoisomerases are enzymes catalyzing single strand passage through a second ssDNA (Bugreev and Nevinsky 2009). Topoisomerases of this subfamily form a group of specific enzymes which can be found in all domains of life. As mentioned above they introduce single strand breaks in a substrate DNA molecule. This cleavage always involves the formation of a phoshodiester bound between the enzyme and the DNA. In the case of the IA subtype enzyme this bound is made between the protein and a 5'-phosphate of the DNA backbone. This defined binding pattern is one major difference to Topoisomerases belonging to the type IB family which bind to the 3'-phosphate of DNA. Almost all type IA enzymes need Mg(II) ions for catalytic activity (Champoux 2001). The enzymes require a single stranded region of DNA to bind. This is one reason why type IA Topoisomerases only act on negatively supercoiled DNA (Kirkegaard and Wang 1985). Negative supercoiling creates topological tension whereby DNA strand separation is supported (Drew *et al.* 1985). Type IA Topoisomerases differ in their demand for supercoiling of a DNA to serve as substrate. The enzymes of this class are monomeric. Further division of the type IA subfamily is based on structural distinction of the proteins. The subfamily of type IA consists of three subfamilies bacterial Topoisomerase I, bacterial and eukaryotic Topoisomerase III, and archaeal reverse gyrase (Baker *et al.* 2009).

Topoisomerases of subfamily type IA are composed of different structure domains (Figure 1.6). Topoisomerase IA of *E. coli* can be considered as a prototype for this enzyme class. *E. coli* Topoisomerase IA has a molecular weight of 97 kDa and consists of two major domains: Cleavage/strand passage domain and C-terminal domain containing Zn(II) binding domain.

The cleavage/strand passage domain contains the sub domains forming the active site with its catalytic active tyrosine needed for DNA cleavage. This core structure of the enzyme is formed of four domains with a characteristic molecular weight of around 67 kDa and constitutes a typical toroidal fold (Baker et al 2009).

The first domain (domain I) of 160 amino acids forms a structure known as TOPRIM (Topoisomerase-Primase) motif which resembles a Rossmann fold (Corbett and

Berger 2004). It is composed of four parallel oriented β-chains and intervening anti-parallel α-helices. This structure is typically found in proteins which bind nucleotides (Aravind *et al.* 1998). The TOPRIM domain typically contains three or four conserved acidic residues configuring an acidic cluster. This cluster is thought to bind Mg^{2+} ions and is part of the active site. The adjacent domain IV contains a CAP- (catabolite activator protein) like structure basically consisting of α-helices. This domain resembles domain III with the difference that it does not contain a catalytically active tyrosine residue. CAP-like structures are found in several DNA binding proteins (Corbett and Berger 2004). It was shown that this domain together with 5Y-CAP domain binds the DNA strand and keeps attached to the DNA 3'-end after cleavage (Changela *et al.* 2001).

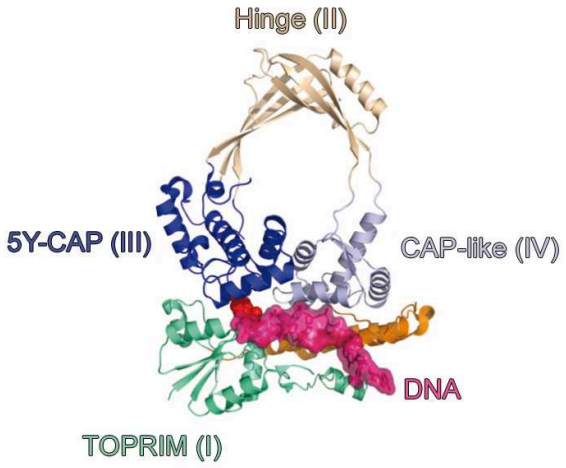

Figure 1.6: Structure model of Topoisomerase I from E. coli. The different domains are depicted in different colours, additionally a bound substrate DNA is shown in pink. Figure adopted from Chen et al, 2013.

The following domain II also known as hinge or linker domain forms the top of the toroid structure. Its organization of several anti-parallel β-sheets causes a crescent-shaped structure. The structure of the Hinge domain is mainly important to create the hole in the center of the protein which houses one DNA strand after the passage. Domain III also termed WHD- (winged helix DNA binding) or 5Y-CAP-domain is mostly composed of several α-helices (Corbett and Berger 2004). It contains the catalytic active tyrosine at position 319 (Tyr319) and other conserved residues involved in cleavage and binding of the DNA designated for gapping. Several amino acid residues of domains I, II and IV form the complete active site. All domains together give the enzyme the shape of an open toroid which can hinge away to

enable the DNA strand to enter the center hole, thereby allowing strand passage. The steps of the DNA relaxing mechanism as DNA binding, cleavage, strand passage, religation and final dissociation of the DNA require massive structural movements which are yet not fully understood (Baker *et al.* 2009). The described cleavage/strand passage domain with its subdomains shares sequence identity with all other members of subclass IA (Champoux 2001).

The C-terminal region of the *E. coli* enzyme is about 30kDa and contains five tandem zinc ribbon motifs enabling the binding of Zn. This part of the enzyme is important for DNA binding and relaxation process (Schoeffler and Berger 2008). It has been suggested that the C-terminus interacts with the "crossing" DNA strand. If this domain is absent Topoisomerase IA is still able to cleave DNA but relaxation of supercoiled structures fails (Ahumada and Tse-Dinh 2002). Notably not all type IA enzymes contain a Zn(II) binding domain.

A complete cycle of action of a type IA Topoisomerase encompasses many complex single steps in which the enzyme undergoes major structural changes (Bugreev and Nevinsky 2009). To achieve relaxation of a target DNA the substrate must be bound by multiple parts of the protein. The Tyr319 cleaves the ssDNA allowing the second strand to pass. After religation of the cleaved strand and release of the passed strand, the DNA dissociates. Thereby one reaction cycle of the type IA enzyme reduces the linking number of a processed substrate DNA by one (Dekker *et al.* 2002). Remarkably the complex reaction cycle is operated without the use of additional energy resources like ATP. That implicates that all energy needed for catalysis has to derive from substrate/enzyme interactions.

In the first step of the catalytic cycle the strands of the target DNA become partially separated and bound to the enzyme. It is thought that the strand designated for passage binds to the C-terminal domain of the enzyme. The strand destined for cleavage binds to the enzyme as a consequence of concerted interplay of domains I, III and IV. SsDNA binding requires and coincidently causes conformational changes. The exact mechanism of DNA binding is still not totally clarified. In the next step the DNA strand is cleaved by Tyr319 assisted by distinct residues of domains I, III and IV. After cleavage the 5'-end of DNA stays covalently bound to Tyr319 of domain III while the 3'-end is thought to be bound by domain I. At this state the enzyme is ready to open the gate for the strand passage. Therefore domain III lifts away from domain

I creating the gap needed for passing of the intact ssDNA. The gap is closed after entrance of the DNA by domain III hinge down. Thus strand passage is completed and the cleaved DNA is religated. In subsequent steps the gates opens again to release the incorporated DNA strand and closes again. This step marks the end of one cycle of catalysis. At this point the DNA can disassociate from the Topoisomerase or directly start another round of relaxation.

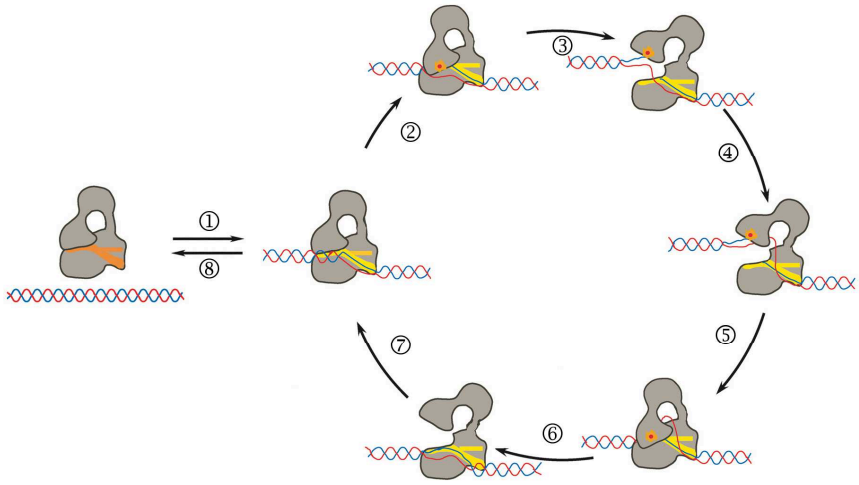

Figure 1.7: Type IA Topoisomerase mediated DNA cleavage mechanism. Cycle starts with binding of the DNA substrate (1), followed by single strand cleavage (3) and opening of DNA gate. Subsequently strand passage (4) occurs, followed by closing of gate (5) and religation of DNA substrate. Finally Topoisomerase cavity opens (6), and closes (7) again, thereby liberating 'passed' stand which allows DNA to dissolve (8). (Baker *et al.* 2009)

Many details of the catalytic cycle are still poorly understood, and additional research is necessary to fully understand the mechanism of catalysis. In particular crystal structure models of the different episodes of the relaxation process will be required to uncover the conformational changes which are the key for understanding Topoisomerase actions.

1.2.2.2 Topoisomerase III

Besides Topoisomerase I, Topoisomerase III is a prominent member of the type IA family. Particularly in eukaryotic cells Topoisomerase III plays essential roles in DNA metabolism of nuclear and mtDNA (Li and Wang 1998; Wu *et al.* 2010). Prokaryotic Topoisomerase III of *E. coli* is quite similar to human Topoisomerase I regarding the composition of its core domain. The typical constitution comprising the four domain structure can be found when the first ~600 amino acids are compared (Bugreev and Nevinsky 2009). Due to the changes in amino acid sequence the relative positioning

of the domains is different. Additionally Topoisomerase III shows two major insertions, which form two extra loops not present in Topoisomerase I. C-terminal domains of the two proteins exhibit no homology. Topoisomerase III C-terminal domain comprises no Zn(II) binding structures or other known motifs. However experiments have confirmed C-terminal domain contributes DNA binding as in other type IA enzymes (Bugreev and Nevinsky 2009). The differences in core- and C-terminal domains cause the diverse roles in cell metabolism. Unlike Topoisomerase I, Topoisomerase III functions as a decatenase in cell metabolism (DiGate and Marians 1988) and assists in resolving holliday junctions (Plank *et al.* 2006).

1.2.2.3 Reverse Gyrase

Reverse Gyrase is the most unusual member of type IA Topoisomerases. The protein has a chimera character consisting of a helicase like N-terminal domain while at C-terminus it resembles a type I Topoisomerase (Corbett and Berger 2004). Reaction mechanism involves formation of a covalent bond to the substrate DNA like the other type I enzymes. Homology to helicases is due to an ATP-binding site and further characteristic motifs. Helicases are responsible for DNA strand separation in DNA metabolic processes like replication or transcription (Gorbalenya and Koonin 1993). Reverse Gyrase was specifically found in hyperthermophiles and thermophiles revealing an essential role in organisms exposed to extreme temperatures above 90°C (Corbett and Berger 2004). Reverse Gyrase activity introduces positive supercoils in DNA strands. There are theories indicating that reverse Gyrase functions as a renaturase (Hsieh and Plank 2006). It is thought that high temperatures may lead to denaturation of the DNA by unwinding and strand separation whereby permanent single stranded bubbles of DNA can develop. At very high temperature the enzyme could also be essential to renaturate the dsDNA after passage of the transcription machinery.

1.2.3.4 Subfamily Type IB

Type IB Topoisomerases, also named “swivelases” work as swivels to relax DNA supercoils. In contrast to the type IA enzyme the relaxation does not occur stepwise. Instead the process continues to complete relaxation of the DNA substrate (Dekker *et al.* 2002). Topoisomerases of type IB can be divided into two classes: the eukaryotic Topoisomerase I and pox virus Topoisomerases.

The Topoisomerase IB enzyme is present in all eukaryotic organisms and mammalian mitochondria (Hartman Chen *et al.* 2013). In difference to type IA enzymes, IB enzymes form a covalent bound between the active site tyrosine and the 3'-phosphate of the DNA stand instead of the 5'-phosphate. The enzyme can relax positive as well as negatively supercoiled DNA. Type IB enzymes are not dependent of a single stranded DNA region for binding and do not require metal ions or Mg(II) for activity (Champoux 2001). Although viral and eukaryotic enzymes differ in size, all IB Topoisomerases share a common fold with specific highly conserved residues at the active site and a similar mechanism of action. Type IB enzymes resemble tyrosine recombinases. This is reflected in designation of the of the core part in both enzymes named IB/Int (type IB/λ Integrase) domain (Cheng *et al.* 1998). Type IB Topoisomerase plays a role in eukaryotic replication and transcription process. In the following human Topoisomerase1 (TOP1) as a representative member of type IB enzymes is described in detail.

Type IB enzymes are monomeric proteins which show a unique structure among Topoisomerases. The human TOP1 consists of four domains which can further be divided into sub domains (Leppard and Champoux 2005). The N-terminus is about 214 amino acids (AA) in size and contains four nuclear localization signals. It was shown that it is needless for the relaxation activity but seems to facilitate interaction with other proteins (Alsner *et al.* 1992; Merino *et al.* 1993; Wang and Roeder 1998) . There are also indications that N-terminal domain assists to control DNA rotation by DNA binding downstream of the cleavage site (Corbett and Berger 2004). The following 421 AA core domain contains most AA forming the active site and therefore is highly conserved. Only the active site tyrosine723 (Tyr723) is part of the 53 AA C-terminal domain. The active site is formed by a pentad of specific residues: Tyr723, Arg488, Lys532, Arg590 and His623. The C-terminal domain is connected to the core of the enzyme by a linker domain of 77 AA arranged in a coiled-coil structure. The linker domain in cooperation with parts of domains I and II is thought to influence speed and extent of DNA rotation (Redinbo *et al.* 1999). The core domain is further subdivided into subdomains I, II and III. The protein architecture resembles a clamp which can wrap-around the DNA. In this model core subdomains I and II constitute a cap structure which is connected to the base of the enzyme via a long α-helix named the connector. The connector likely forms the hinge which is needed for closing and opening of the clamp. The base encompasses core subdomain III and the C-terminal

domain. Parts of subdomains II and III are termed as “lips”. These regions on opposing parts of the enzyme get to close proximity when the clamp closes and lock this state by the formation of a salt bride and further interactions (Corbett and Berger 2004; Baker *et al.* 2009).

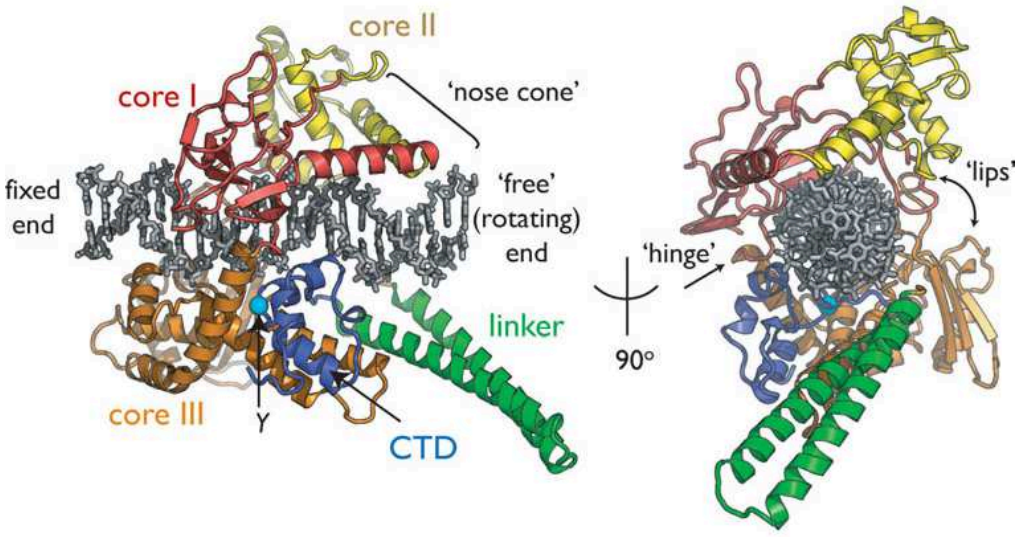

Figure 1.8: Structure model of human Topoisomerase IB. The specific domains are depicted in different colours, additionally a bound substrate DNA is shown in grey. ’Y’ marks the position of active site tyrosin. Figure adopted from (Schoeffler and Berger 2008).

The reaction mechanism of Type IB enzymes is among the best studied and understood of all Topoisomerases. Particularly the putative functions of the amminoacids which constitute the active site have been investigated in multiple studies (Champoux 2001). Type IB Topoisomerases are thought to work as swivels which allow the relaxation of torsional stress in a substrate DNA molecule. In a simplified view that is achieved by cleavage of one single strand of a dsDNA. The end of cleaved strand is bound by active site. The unbound DNA is then free to rotate and thereby to relax torsional tension. The rotation is driven by the torsional strain of the substrate DNA and proceeds until complete relaxation. The rotation itself is termed as controlled rotation because the “loose” part of the DNA molecule stays in the Topoisomerase cavity during rotation. This inflicts fricton between the rotating DNA and the protein which slows down the rotation (Redinbo *et al.* 1998; Koster *et al.* 2005). A result of the decelerated rotation is a possible gain of control thus assuring the correct course of the process.

1.2.2.5 Subfamily Type IC

Topoisomerase V from prokaryotic *Methanopyrus kandleri* is currently the only member of type IC Topoisomerase subfamily. Due to some structural analogies with type IB enzymes it was allocated to this enzyme class in the past. Like type IB enzymes Topoisomerase V forms a transient bound to the 3'-end of the processed DNA, is able to relax positive and negative supercoiled DNA, and works without Mg(II). Notwithstanding these similarities Topoisomerase V has a unique structure different from all other Topoisomerases (Taneja *et al.* 2006). The protein is structurally split: while the N-terminal part of about 30 kDa comprises the Topoisomerase domain, the C-terminus shows apurinic/apyrimidinic (AP) site processing activity, a feature known from enzymes participating in DNA repair processes (Belova *et al.* 2001). Although Topoisomerase V possesses the characteristic IB enzyme pentad of AA at the active site, the three-dimensional arrangement remarkably differs. This and the fact that an extra acidic AA belongs to the active site indicates a different mode of action for Topoisomerase V. Additionally the overall structure of the enzyme suggests that a far-reaching conformational rearrangement is required for activity. These facts were considered by introduction of a new nomenclature of type I enzymes now including subfamily IC (Baker *et al.* 2009).

1.2.3 Type II Topoisomerases

Type II enzymes are essential proteins for cells (Carpenter and Porter 2004). This is reflected in the ubiquitous appearance in all organisms (Vos *et al.* 2011). They catalyze the passage of one double strand of DNA through another. To enable this, the first dsDNA referred to as G- (gate) segment, is bound and cleaved generating a gate. In a second step another dsDNA strand termed T- (transported) segment is trapped and transported through the open G-segment. In subsequent steps the G-segment is religated and the protein dissolves from the DNA strands. All type II Topoisomerases are polymeric enzymes which bind duplex DNA. During cleavage covalent links to the 5'-ends of the DNA is formed. The reaction is dependent of Mg(II) and ATP. They are the only enzymes able to separate catenanes, which develop during replication. Therefore they are essential enzymes for replication process (Vos *et al.* 2011).

1.2.3.1 Type IIA Topoisomerases

Type IIA Topoisomerases were found in *E. coli* (Gyrase / Topoisomerase IV), bacteriophages and eukaryotes (Corbett and Berger 2004). Among eukaryotes yeast gets along with a single type IIA enzyme while vertebrates possess two isoforms: Topoisomerase IIα and IIβ (TOP2A/TOP2B). Though both isoforms show a similar structure and similar biochemical properties they differ in biological function. TOP2A plays an essential role in cell division process (Grue *et al.* 1998; Barthelmes *et al.* 2000) and is only expressed in proliferating cells where it associates with mitotic chromosomes (Linka *et al.* 2007). Besides its involvement in neuronal development (Yang *et al.* 2000; Lyu and Wang 2003) TOP2B seemingly plays a role in nuclear gene transcription (Ju *et al.* 2006). Additionally there is evidence that a mitochondrial isoform of TOP2B is present in mammalian cells (Low *et al.* 2003).

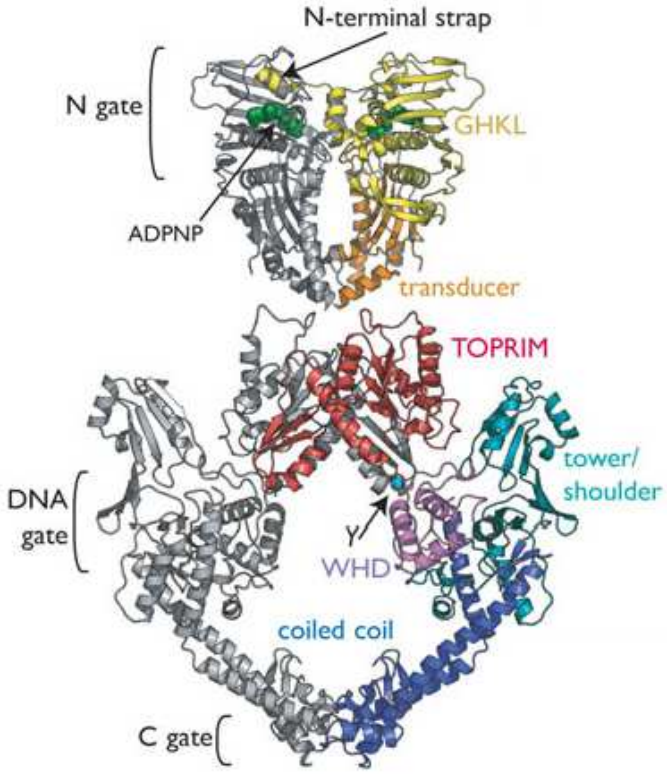

Figure 1.9: Structure model of a type IIA Topoisomerase from *S. cerevisiae*. The different domains are depicted in different colours. Figure adopted from (Schoeffler and Berger 2008).

While eukaryotic enzymes are homodimers, the bacterial proteins are heterotetramers and certain proteins of phages are heterohexamers (Champoux 2001). Type II Topoisomerases show an architecture in which the catalytically active

parts of the enzyme are linked by flexible regions which allow the conformational changes during catalysis. Sequence identities of type IIA enzymes from different organisms reveal a three domain structure (Champoux 2001). The N-terminal domain is called gyrB in *E. coli* Gyrase which is similar to the *E. coli* Topoisomerase Subunit ParE and subunit B in eukaryotic cells. The following domain respectively is named gyrA (in Gyrase), ParC in Topoisomerase IV and A-domain in eukaryotic organisms. The C-terminal tail of all enzymes is referred to a C-terminal domain (CTD). The detailed architecture of eukaryotic type IIA enzymes show the following sequence of catalytic and structural motives and subdomains: at N-terminus resides the GHKL ATPase domain. This domain is named after its s presence in diverse ATPases: Gyrases, Hsp90,CheA histidine kinases, and MutL (=GHKL), and is an important structural motif employing ATP binding to introduce conformational changes to the enzyme. GHKL domain is followed by a transducer domain, a TOPRIM domain, a WHD domain, a tower/shoulder domain, a coiled-coil domain, a scaffold domain and the CTD (Corbett and Berger 2004). In the dimeric protein several protein-protein interfaces enable the concerted action of both single proteins as well as their cohesion (Nitiss 2009). These interfaces occur between GHKL domains which are self associating to dimeric state. The WHD domains form the second interface and the scaffold domains are mainly responsible to hold both halves of the dimer together. The interaction of these domains structurally results in a protein containing two holes, which can house a DNA strand during catalysis. These two gates are named according to their position N-gate (located in proximity to N-terminus) and C-gate (located in proximity to the C-terminus).

The transport mechanism conducted by type IIA Topoisomerases is designated as a “two- gate mechanism” because T-segment is transported through the complete enzyme requiring at least two separable protein-protein interfaces (Schoeffler and Berger 2008). Catalysis starts with the binding of a G-segment DNA by a groove which is constituted by parts of WHD, TOPRIM and tower domains. That leads to a structure in which the G-segment resides in the middle of the Topoisomerase in between the N- and the G-gate. In a next step the T-segment DNA is captured in N-gate whereupon ATP binding of the two GHKL-domains leads to conformational changes including closure of N-gate.

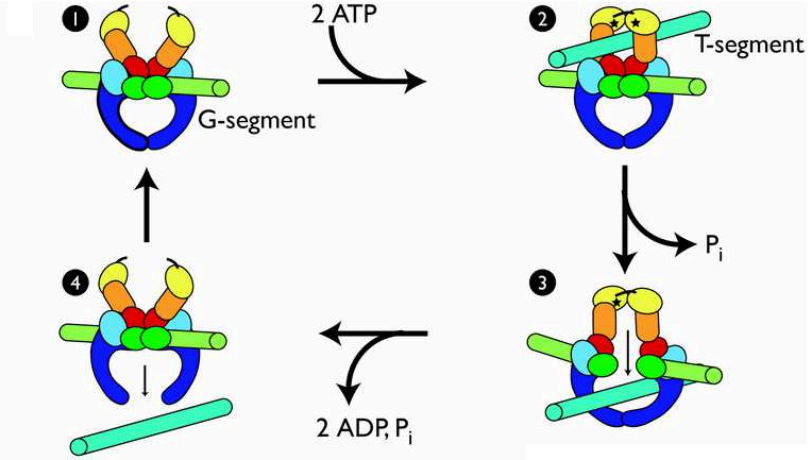

Figure 1.10: Model of Topoisomerase IIα catalyzed strand passage. The different domains are depicted in different colours, additionally substrate DNAs are shown in green (G-segment) and light blue (T-segment). 1: Binding of G-segment; 2: Captured T-segment DNA is in N-gate whereupon ATP binding of the two GHKL-domains (yellow) leads including closure of N-gate; 3: cleavage of the G-segment and passage of T-segment; 4: Release of T-segment. Figure adopted from Schoeffler and Berger, 2005.

Binding of the T-segment leads to cleavage of the G-segment and the opening of a DNA gate. This achieved by spatial separation of the two WHD domains which the two parts of the G-segment are bond to after cleavage. In the following hydrolysis of the first ATP releases the next conformational changes allowing the T-segment passage through the DNA cleavage domains to the C-gate. Subsequently closing of the DNA gate and religation of G-segment is believed to trigger the changes in structure operating C-gate opening and release of T-segment. Hydrolysis of the second ATP is likely responsible for re-opening N-gate releasing G-segment and resetting enzymes complete structure.

1.2.3.2 DNA Gyrase

A special type IIA enzyme is DNA Gyrase. This protein is able to introduce negative supercoils into DNA. Gyrase protein structure is similar to other type IIA enzymes with the exception of its C-terminus. The 33 kDa large C-terminal domain possesses an additional DNA binding domain which facilitates right-handed wrapping of the substrate DNA around the enzyme. This allows one strand of DNA to serve as G-and T-segment at once. Wrapping of substrate together with a preference for the formation of a positive crossover of G- and T-segment enables Gyrase to introduce positive supercoils in a substrate DNA (Champoux 2001; Schoeffler and Berger 2008).

1.2.3.4 Type IIB Topoisomerases

Type IIB Topoisomerases are present in most archea and higher plants but are absent in all other eukaryotes and bacteria. They are heterotertameric enzymes composed of A_2B_2 subunits. Like type IIA enzymes they catalyze the ATP dependent transport of a double-stranded T-segment of DNA through another dsDNA, the G-segment. The prototype enzyme for type IIB Topoisomerases is Topoisomerase VI. Studies on this protein on the one hand revealed the relation to type IIA proteins reflected in the use of similar catalytic domains as well as in the *modus operandi* of a two-gate mechanism. On the other hand the comparison to type IIA enzymes shows clear structural differences. Interestingly although no eukaryotic type IIB Topoisomerase was found beside that in plants a eukaryotic enzyme named Spo11 (Sporulation-specific protein 11) shows clear homology with Topoisomerase 6A. Spo11 takes part in meiotic recombination where it is needed for double strand cleavage (Bergerat *et al.* 1997; Keeney *et al.* 1997). The typical Topoisomerase related WHD and TOPRIM domains enable catalytic activity. As mentioned above, Topoisomerase 6 is composed of B- and A- subunits. The B- subunits are composed of three main structural domains: the GHKL domain, a transducer domain and a helix-two-turns-helix domain. The GHKL and transducer domains resemble architecture in type IIA enzymes. The course of strand passage catalyzed by type IIB Topoisomerases seems to be a reduced version of the two-gate mechanism applied by type IIA enzymes (Schoeffler and Berger 2008).

1.2.4 Mitochondrial Topoisomerases

As already described all activities of DNA metabolism are depending on Topoisomerase activity. During transcription the movement of the transcription machinery on the DNA strand creates tensions in form of positive supercoils ahead of the complex and negative supercoiling behind it (Wu *et al.* 1988). These torsional stress needs to be removed by Topoisomerases, otherwise increasing tension causes stalling of transcription. Also replication induces similar tensions, the moving replication complex causes positive supercoils ahead while rearward the two newly formed strands can interwine when replication complex rotates. In mitochondria the existence of interwined circular mtDNA molecules is known, and it is clear that these structures must be separated when mitochondria replicate. Furthermore DNA topology influences the initiation of transcription process (Parvin and Sharp 1993;

Travers and Muskhelishvili 2007). A certain grade of supercoiling, particularly in circular DNA molecules can promote initiation by alleviating promoter melting (Kouzine *et al.* 2008). These examples illustrate the importance of a closely regulated DNA topology and also demonstrate that DNA topology represents a tool to regulate metabolism. Topoisomerases are nature's tools to control DNA topology in the nucleus and mitochondria. In mammals the mitochondria contain at least two different Topoisomerases, Topoisomerase 3α (TOP3A) and mitochondrial Topoisomerase 1 (TOP1MT). The mitochondrial location of a type II (TOP2B) Topoisomerase notional, although few studies suggest the existence, clear evidence is currently missing.

TOP3A represents a member of type IA Topoisomerases present in the mitochondrion. Mitochondrial Topoisomerase IIIα (TOP3A) is created by alternative translation initiation of a common transcript coding for nuclear and mitochondrial representatives of the enzyme (Wang *et al.* 2002). The extended isoform contains an additional MTS sequence mediating mitochondrial location of the enzyme. The nuclear isoforms of TOP3 play a role in recombination events and are responsible for the dissolving of double holiday junctions. Nuclear TOP3A was also shown to work as decatanase (DiGate and Marians 1988). It probably also acts on DNA solenoids transiently formed during transcription (Wang 2002). Since recombination seems to be a rare event in mitochondria it is suggested that mitochondrial TOP3A may play a role in mtDNA segregation at the end of replication process (DiGate and Marians 1988). Currently TOP3A is the only known Topoisomerase essential for mtDNA metabolism. TOP3A deficiency causes a depletion of mtDNA in male germ-line stem cells of drosophila (Wu *et al.* 2010) pointing at an essential role in mtDNA maintenance which is possibly linked to its presumed interaction with Twinkle helicase. However, currently it is not clear lack of what function causes this phenotype and whether it is due to deficiency of nuclear or mitochondrial products of the TOP3A gene. Moreover, the relevance of this finding with respect to mammalian mtDNA maintenance is questionable, since in insects the machinery supporting these functions is very different.

The class of type IIA Topoisomerases is possibly also present in mammalian mitochondria (Low 2002). It was reported that a truncated form of TOP2B is present in bovine heat mitochondria (Low *et al.* 2003). The enzyme of about 150 kDa likely

results from a post-translational processing of the nuclear isoform and the truncation could serve to expose putative internal MTS. Mitochondrial TOP2B also could play a role in the decatenation of mtDNA subsequent to replication. Despite these speculations more evidence is required to confirm a role of TOP2B in mitochondrial metabolism.

In mammalian mitochondria Topoisomerases of type IB are represented by mitochondrial Topoisomerase IB (TOP1MT). TOP1MT currently is the only known mitochondrial Topoisomerase which is encoded by a separate gene on chromosome 8q24 (Zhang *et al.* 2001). The gene TOP1MT encodes 14 exons of which the last 13 exons have sizes uniformly conserved among all vertebrates (Zhang *et al.* 2004). Splitting of the TOP1 gene into nuclear and mitochondrial paralogs is highly conserved in vertebrates (Zhang *et al.* 2001; Zhang *et al.* 2004; Zhang *et al.* 2007) and reflects a functional specialisation: Nuclear TOP1 is incompatible with mtDNA transcription/replication, while TOP1MT is unable to interact with nuclear chromosomes (Dalla Rosa *et al.* 2009). Structurally TOP1MT seems closely related to its nuclear pendent and shows the common four domain structure. TOP1MT is about 70 kDa in mass and contains 601 AA. The poorly conserved NTD contains the MTS for mitochondrial targeting (Zhang and Pommier 2008). Two splicing variants were found which encode catalytically inactive proteins (Zhang *et al.* 2007). The biochemical properties of nuclear and mitochondrial TOP1 variants are similar despite some differences owing to the different conditions in nucleus and mitochondrion. Both enzymes act on similar substrates, and only differ concerning their requirements for pH-value and the availability of divalent cations (Zhang *et al.* 2001). TOP1MT catalyzes the transient cleavage and ligation of one strand of the DNA double helix and thereby provides the major activity for relaxation of mtDNA supercoils. TOP1MT DNA-cleavage activity is clustered in an mtDNA region downstream of the displacement loop (D-loop) (Zhang and Pommier 2008) that contains an additional DNA strand (7S DNA). 7S DNA is either a prerequisite or a side product of mtDNA replication (Falkenberg *et al.* 2007). Depletion of 7S DNA upon inhibition of TOP1MT (Zhang and Pommier 2008) suggests an involvement in D-loop maintenance or replication, but an essential role of TOP1MT in mtDNA maintenance seems unlikely, since *TOP1MT*$^{-/-}$ mice express mtDNA-encoded proteins (Douarre *et al.* 2012). Thus the role of TOP1MT in mitochondrial DNA metabolism remains unclear.

1.4 Aim of the work

Clearly mitochondrial DNA metabolism depends on Topoisomerase activity. Transcription and replication take place in the mitochondrial matrix. So far the existence of three mitochondrial Topoisomerases is known. Much less knowledge is available concerning the functions of these enzymes. Currently only TOP3A was shown to be cruical for mitochondrial DNA metabolism. TOP1MT apparently is inessential for mitochondrial DNA metabolism. This fact is striking as human cells afford a separate gene for the mitochondrial isoform of TOP1MT. This should implicate an important function for this enzyme in mitochondria. In this work I wanted to figure out the biological function of TOP1MT in mitochondrial metabolism. In this context it was a goal to reveal the underlying mechanistics of this function. Therefore three major experimental setups were tested:

1. The overexpression of TOP1MT and YFP-tagged TOP1MT in human cells.

2. The siRNA mediated transient knockdown of TOP1MT in human cells.

3. The chronic conditions of TOP1MT knockout in mouse cells.

2. Material

2.1 Vectors and cDNA

2.1.1 Expression vectors

Vectors for expression of mitochondrial targeted TOP1MT in human cells (see 3.1.1), were based on the bicistronic expression vector pMC-2PS-delta HindIII-P (see appendix 8.1.B) (Mielke *et al.* 2000), in which the puromycin resistance gene (pyromycin-N-acetyltransferase, pac) constitutes the second cistron, followed by the simian virus 40 (SV40) polyadenylation signal. In front, pac is linked by an IRES element (Internal Ribosomal Entry Site) to a multiple cloning site (MCS), for the insertion of the gene of interest. A cytomegalovirus promotor (CMV) fused to the myeloproliferative sarcomvirus (MPSV) LTR enhancer repeat ensures a high transcription level of the bicistronic message in various mammalian cells and the transcriptional linkage ensures a fixed simultaneous expression of pac and a gene of interest. To enable the constitutive expression of YFP-tagged proteins, the plasmids pMC-YFP-P-N (see appendix 8.1.C) and pMC-YFP-P (Christensen *et al.* 2002; Linka *et al.* 2007) were used. Cloning of the gene of interest into the MCS enabled N-terminal fusion to yellow fluorescent protein (YFP).

2.1.2 cDNA

The coding sequence of TOP1MT was provided by the plasmid pEGFP-TOP1MT (Zhang *et al.* 2001) kindly gifted by H. Zhang.

2.2 Microbiology

2.2.1 Escherichia coli strains

DH5α — Genotype: supE44 ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 (Hanahan 1983)

2.2.2 Bacterial growth media

LB-medium (1 l)	10 g Trypton, 5 g yeast extract, 10 g NaCl, adjusted to pH 7.5 (by NaOH)
LB-agar	10 g agar in 1000 ml LB medium (see above)
TB-medium (1 l)	12 g Trypton, 24 g yeast extract, 4 ml Glycerol, dissolved in 900 ml H_2O and autoclaved. 100 ml of sterile phosphate-buffer (0.17 M KH_2PO_4, 0.72 M K_2HPO_4) were added after autoclaving
SOB-medium (1 l)	20 g Trypton, 5 g yeast extract, 0.5 g NaCl, 0.184 g KCl were dissolved in 1 l H_2O, pH adjusted to 7.0 (NaOH) and autoclaved. Before use 5 ml of 2 M $MgCl_2$ and 20 ml of 1 M $MgSO_4$ were added.

For selection 50 µg/ml ampicillin was added to the media.

2.3 Cell culture

2.3.1 Cell lines

Table 3: Cell lines

HT-1080	Human fibrosarcoma cell line established from the biopsy from a 35-year-old man. DSMZ, # DSM ACC 315,Braunschweig, Germany
wt-MEF1	wild-type mouse embryonic fibroblast cell line, TOP1MT$^{+/+}$ littermates of TOP1MT $^{-/-}$, kindly supplied by Ilaria Dalla Rosa, Washington
TOP1MT $^{-/-}$ MEF	TOP1MT knockout mouse embryonic fibroblast cell line, kindly supplied by Ilaria Dalla Rosa, Washington and described in (Douarre *et al.* 2012)

wt-MEF1	wild-type mouse embryonic fibroblast cell line, TOP2B$^{+/+}$ littermates of TOP2B $^{-/-}$, kindly supplied by Yi L. Lyu and Leroy f. Liu, New Jersey described in (Lyu and Wang 2003)
TOP2B$^{-/-}$ MEF	TOP2B$^{-/-}$ knockout mouse embryonic fibroblast cell line, kindly supplied by Yi L. Lyu and Leroy f. Liu, New Jersey, described in (Lyu and Wang 2003)

2.3.2 Supplements and Antibiotics

If not otherwise specified, listed products were purchased from Gibco/Invitrogen, Carlsbad, USA.

Dulbecco's Modified Eagle Medium (DMEM) high glucose

CO_2 Independent Medium (without L-glutamine)

PBS (Ca^{2+}, Mg^{2+} free),

Foetal Bovine Serum (FCS)

Penicillin (10.000 U/ml) and Streptomycin (100 µg/ml) solution

Trypsin-EDTA solution

GlutaMAX-I Supplement, 200 mM

Puromycin (Sigma, St. Louis, USA)

Uridine (Sigma, St. Louis, USA)

DMSO (Sigma, St. Louis, USA)

2.3.3 Media

Table 4: Media for cell-culture

Growth medium	DMEM high glucose, 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin
Selection medium I	DMEM high glucose, 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.6 µg/ml puromycin
CO_2-independent medium	CO_2-independent medium, 20% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% GlutaMAX-I
Respiration medium	10 mM KH_2PO_4, 300 mM Mannitol, 10 mM KCl, 5 mM $MgCl_2$, 1 mg/ml BSA (fatty acid free)

2.4 Buffers and Stock Solutions

Table 5: Buffers and Stock Solutions

6x Agarose loading buffer	15% Ficoll type 400, 40 mM Tris-HCl (pH 8.5), 40 mM glacial acetic acid, 2 mM EDTA, 0.25% bromphenol blue
5x Laemmli buffer	156.25 mM Tris-HCl (pH 6.8), 25% glycerine, 5% SDS, 0.2% bromphenol blue
20x NuPAGE MOPS SDS Running Buffer	supplied by Invitrogen, Carlsbad, USA
10x PBS (Phosphate-Buffered Saline)	1.4 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4 $2H_2O$, 18 mM KH_2PO_4
50x TAE buffer	2 M Tris-HCl (pH 8.5), 2 M acetic acid, 0.1 M EDTA
TE buffer	10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA
10x TGS buffer	2.5 M Tris, 1.92 M glycin, 0.1% SDS

10x TBS (Tris-buffered saline)	24.2 g Tris Base, 80g NaCl, pH 7.6 with conc. HCl, filling up to 1 l ddH_2O
TBST	10x TBS, 0.1% Tween20
Transferbuffer	48 mM Tris, 39 mM glycine 20% methanol pH 9.2

2.5 Enzymes

Expand High Fidelity PCR system	Roche
DNase I free RNase	Roche, Basel, Switzerland
Quick Ligation Kit	NEB, Ipswich, USA
Restriction Enzymes:	
Apa I, Mlu I	Amersham, Little Chalfont, USA
Bam H1	Fermentas, St Leon-Roth, Germany
RNase A	Qiagen, Hilden, Germany

2.6 Chemicals

Tween20 (Polysorbate 20)	Sigma, St. Louis, USA
Polyacrylamid Rotiphorese Gel 30	Roth, Karlsruhe, Germany
TEMED	Roth
APS (Amoniumperoxidsulfat)	Roth
Ethidium bromide solution (1%) (EtBr)	Roth
Digitonin High purity	Calbiochem, Darmstadt, Germany
Antimycin A	Sigma, St. Louis, USA

Ultima Gold™	Perkin Elma, Wiesbaden, Germany
AEBSF hydrochlorid (Pefablock)	Applichem, Darmstadt, Germany
Phenylmethansulfonylfluorid (PMSF)	Applichem
Dithiothreitol (DTT)	Applichem
MitoTracker Red CMXRos	Invitrogen, Carlsbad, USA
MitoSOX Red mitochondrial superoxide indicator	Invitrogen, Carlsbad, USA
5-Bromo-2′-deoxyuridine (BrU)	Sigma
Potassium cyanide (KCN)	Sigma
Nicotinamide adenine dinucleotide (NAD)	Sigma
Adenosine Triphosphate	Sigma
Adenosine Diphosphate	Sigma
Succinate	Sigma
L-(-) Malic acid (Malate)	Sigma
L-(-)Glutamic acid (Glutamate)	Sigma
Pyruvate	Sigma
Glyceraldehyde 3-phosphate	Sigma
Oligomycin	Sigma
Rotenone	Sigma
Carbonyl cyanide 3-chlorophenylhydrazone (m-ccp)	Sigma
Oxidized Glutathione (GSSG)	Sigma

Paraformaldhyd	Sigma

2.7 Antibodies and Peptides

2.7.1 Primary antibodies

Table 2.2 Primary antibodies used in this dissertation.

Antibody	Antigen	Origin	WB/IP	Source
JL-8	GFP	Mouse	1:4000 (WB)	#632381, Clontech, Mountain View, USA
TOP1MT	TOP1MT	mouse	1:1500 (WB)	Kindly supplied by Dalla Rosa
MitoProfile Total OXPHOS Human WB Antibody Cocktail	NDUFB8 SDHB UQCRC2 Cox II ATP5A	mouse	1:200 (WB)	#ab110411, Abcam, Cambridge, USA
TFAM	TFAM	rabbit	1:2000 (IHC)	Kindly supplied by R.J. Wiesner
POLRMT	POLRMT	rabbit	1:500 (WB)	GeneTex Inc., Irvine, USA
BrdU	BrU/BrdU	mouse	1:2000 (IHC)	#clone ZBU30, Invitrogen, Karlsruhe, Germany
DNA	DNA	mouse	1:100 (IHC)	#clone AC-30-10, Progen, Heidelberg, Germany
POLRMT	POLRMT	rabbit	1µg (IP)	# sc-67350, Santa Cruz, Dallas, USA
POLRMT	POLRMT	rabbit	1:50 (IHC)	#ab32988, Abcam

2.7.2 Secondary antibodies

Table 2.3 Secondary antibodies used in this dissertation.

Name	Origin	Dilution	Source
ECL Mouse IgG, HRP-Linked Whole Ab	sheep	1:40000	Amersham, Little Chalfont, UK
ECL Rabbit IgG, HRP-Linked Whole Ab	donkey	1:10000	Amersham
Cy3™ Conjugated goat anti mouse IgG	goat	1:3200	Jackson Immune Research Europe Ltd, Suffolk, UK
Cy3™ Conjugated rabbit anti sheep	rabbit	1:4000	Jackson Immune Research
Cy2™ Conjugated rabbit anti mouse	rabbit	1:4000	Jackson Immune Research

2.7.3 Other (Blocking) Proteins

Goat-serum (GS) Sigma

Bovin serum albumin (BSA) Sigma

2.8 Consumables

Immobilon-P (PVDF) Transfer Membrane	Millipore, Bedford, USA
NuPAGE Novex 4-12% Bis-Tris Gel	Invitrogen, Carlsbad, USA
Gel cassette Novex, 1 mm	Invitrogen
1 Kb plus DNA Ladder	Invitrogen
Peq Gold Protein marker II	PeQlab, Erlangen, Germany

PeqGold Protein marker IV (prestain)	PeQlab
Protease inhibitor cocktail P2714 (Composition: AEBSF, Aprotinin, Bestatin,E-64, EDTA und Leupeptin)	Sigma, St. Louis, USA
BD vacutainer tubes for Lactate-quantification (REF 368920)	BD bioscience, Heidelberg, Germany

2.9 Kits

QIAquick Gel Extraction Kit	Qiagen, Hilden, Germany
QIAGEN Plasmid Maxi Kit	Qiagen
QuantiTect SYBR Green RT-PCR Kit	Qiagen, Hilden Germany
DNeasy Mini Kit	Qiagen
RNeasy Mini Kit	Qiagen, Hilden, Germany
Mitochondria Isolation Kit for Cultured Cells	Abcam, Cambridge, USA
Mitochondrial DNA Isolation Kit	Abcam, Cambridge, USA
Effectene Transfection Reagent	Qiagen
BCA Protein Assay Reagent	Pierce, Rockford, USA
ECL Plus Western Blotting Reagents	Amersham, Little Chalfont, UK

2.10 Instruments

Horizontal Gel Electrophoresis Apparatus Horizon 11.14	Whatman/ Biometra, Göttingen, Germany
Vertical polyacrylamid gel electrophoresis system Novex Mini-Cell Electrophoresis	Invitrogen, Carlsbad, USA
Semi-dry blot chamber Trans-Blot SD	Bio-RAD, Hercules, USA
PCR Cycler Mastercycler	Eppendorf, Hamburg, Germany
Photometer Biophotometer	Eppendorf
PH meter Calimatic 766	Knick, Berlin, Germany
Special accuracy weighing machine AE-166	Mettler Toledo, Giessen, Germany
Ultrasound Homogeniser Sonopuls	Bandelin, Berlin, Germany
Dounce homogenizer	Wheaton Inc., Millville, USA
Incubator shaker SM-30	Edmund Bühler, Tübingen, Germany
Incubator function line B12	Heraeus, Hanau, Germany
Clean bench Hera Safe	Heraeus
Thermomixer Comfort	Eppendorf
Incubator Hera Cell	Heraeus
Rotor for reaction tubes	Labor-Brand, Gießen, Germany
Flow cytometer FACS Canto2	BD bioscience, Heidelberg, Germany
Luminescent image analyzer LAS-4000	Fujifilm, Tokyo, Japan
Epifluorescent Inverse Microscope Axiovert 100	Carl Zeiss, Jena, Germany
Delta TC3 Culture Dish System	Bioptechs Inc., Butler, USA

Digital Camera Spot-RT SE Monochrom	Diagnostic Instruments, Sterling Heights, USA
Confocal Laser Scanning Microscope (LSM 510 Meta with a 63x /1.3 DIC oil immersion objective)	Carl Zeiss
Centrifuge Centrikon H-401	Heraeus
Centrifuge Rotixa / P	Hettich, Tuttlingen, Germany
Centrifuge 5417R	Eppendorf
Cell Counter Countess™	Invitrogen
Freezing box Cryo 1°C Freezing Container	Nalge Nunc, Rochester, USA
Water-bath WBT-22	P-D Industriegesellschaft mbH Medingen, Dresden, Germany
Autoclave V 150	Systec, Wettenberg, Germany
MiniMACS Separator	Miltenyi Biotec, Germany
O_2 electrode	Hansatech, Kingslynn, UK
Light Cycler 480	Roche, Mannheim, Germany

2.11 Computer software and statistic analyzing programs

GraphPad PRISM 4.0a	GraphPad Software Inc., USA
Meta Series 6.3 Software	Molecular Devices, Downingtown, USA
Zeiss Laser Scanning Mikroskop 510 Meta Software Version 3.2, Service Pack 2	Zeiss, Oberkochen, Germany
Multi Gauge	Fujifilm, Tokyo, Japan

3. Methods

3.1 Cloning

3.1.1 Plasmid construction

Most Plasmids used in this work (for expression of MY, MY-TOP1MT, MY-TOP1MT^{Y559F} and TOP1MT) were cloned and already described by Ilaria Dalla Rosa in her PhD-Thesis and in (Dalla Rosa *et al.* 2009). Thus only basic construction of these plasmids is briefly displayed in the following.

For EYFP-N (11) was extended inframe at the 5'-end with the sequence encoding the mitochondrial targeting sequence (MTS) from subunit VIII of cytochrome C oxidase mitochondrial targeting of fluorescent fusion proteins, YFP in the vector pMC- (COX) (Rizzuto *et al.* 1995) using linkerPCR, thus generating pMC-MTS-EYFP-N. Likewise, the coding sequence for TOP1MT (RefSeq NM_052963) was inserted inframe at the C-terminal end of MTS-YFP. Thus, the first cistron of the transcribed messenger encodes the mitochondrial addressed TOP1MT-YFP chimeras and the second cistron the puromycin resistance gene.
For this work TOP1MT^{Y559F} variant was sub-cloned using vector pMC-MTS-EYFP-TOP1MT^{Y559F} and vector pMC-2PS-delta HindIII-P vector to remove MTS-EYFP-tag from the construct.

3.1.2 Gel electrophoresis and recovery of DNA from agarose gels

DNA fragments were separated in an electric field according to their size by agarose gel electrophoresis. The used concentration of the agarose was dependent on the size of the DNA fragments. Agarose was melted by boiling in 1x TAE buffer. After cooling to 60°C, EtBr was added to a final concentration of 1 µg/ml and the solution was poured into a horizontal casting tray and allowed to solidify. For electrophoresis, the gel was placed in a gel chamber and was covered with 1x TAE buffer. Samples were mixed with appropriate volume of 6x DNA loading buffer, loaded onto the gel and separated. Electrophoresis was performed at 90-120 V. After gel-run, the separated DNA bands were visualized using a transilluminator at 280 nm and size was determined using DNA size marker.

3.1.3 Restriction digestion

3.1.3.1 Analytical restriction digestion

Analytical restriction digestion was used to check for the correct orientation and length of the inserted DNA fragment into the plasmid vector. 1-2 µg of plasmid DNA was digested with 5 units of restriction enzyme using the buffer recommended by the manufacturer. Digests were incubated for 1 h at 37°C. The DNA fragments were analyzed by agarose gel electrophoresis.

3.1.3.2 Preparative restriction digestion

The preparative restriction digestion was used to isolate specific DNA fragments. 1 µg of plasmid DNA were digested using 5-10 units of restriction enzyme in the appropriate buffer for 1 h at 37°C. DNA was separated by agarose gel electrophoresis. The DNA bands were detected using a transilluminator (280 nm) and cut from the gel. DNA was extracted from the gel using a gel extraction kit according to manufacturer's instruction.

3.1.4 Ligation

To insert restriction fragments into vectors, the Quick Ligation kit (NEB) was used according to manufacturer's instructions. Ligation was performed in a final volume of 10 µl for 10 min at RT and then transferred to 4°C before transformation (see 3.1.5.2).

3.1.5 Transformation and isolation of plasmid DNA

3.1.5.1 Generation of competent *E. coli* cells

E.coli cells were grown in 1 L SOB medium at 18°C and harvested (4000 x g, 20 min, 4°C), at a OD_{600} of 0.5-0.8. The cells were gently resuspended in 40 ml ice-cold TB buffer (10 mM Pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl), incubated for 20 min on ice and again sedimented (4000 x g, 20 min, 4°C). Cells were resuspended in 20 ml ice-cold TB buffer and DMSO was added gently to a final concentration of 7%. After 10 min incubation on ice, aliquots (0.2 ml) were frozen in liquid nitrogen and stored at -80°C.

3.1.5.2 Transformation of *E. coli*

An aliquot of competent cells (0.2 ml) was mixed with 2 µl ligation reaction mixture, incubated on ice for 30 min, heat shocked at 42°C for 30 sec and immediately transferred to 4°C. LB-media (0.5 ml, warm) was added to the cells, mixed and suspension was incubated for 1 h at 37°C under vigorous shaking (250 rpm). Thereafter, transformed cells were plated on LB-agar plates containing 50 µg/ml ampicillin.

3.1.5.3 Plasmid preparation at a small scale (Miniprep)

Single colonies from the selection plate were picked and inoculated in 2.5 ml TB medium containing 50 µg/ml ampicillin and incubated overnight at 37°C under shaking (250 rpm). After approximately 14 hours, 2 ml of the culture were pelleted (6800 x g, 2 min, 4°C). The cell pellet was mixed with 400 µl lysis solution (0.2 N NaOH, 1% SDS), immediately neutralized with 300 µl 7.5 M NH_4oAC, kept for 10 min on ice to precipitate genomic DNA and proteins before lysates were cleared by centrifugation (14000 x g, 10 min, 4°C). Plasmid DNA was precipitated from the supernatant with 500 µl 2-propanol and pelleted (14000 x g, 30 min, 4°C). DNA pellet was washed with 70% EtOH, dried and resuspended in 50 µl TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) supplemented with 50 µg/ml RNaseA. The plasmids sequence was finally confirmed by restriction digestions and sequencing.

3.1.5.4 Plasmid preparation at a large scale (Maxiprep)

A single colony from a selection plate was picked and inoculated in a primary culture of 3 ml selective TB medium (containing 50 µg/ml ampicillin) and incubated for approximately 8 h at 37°C under shaking (250 rpm). Afterwards the starter culture was diluted into 250 ml selection TB medium and grown overnight under the conditions mentioned above. Bacterial cells were harvest by centrifugation (6000 x g, 15 min, 4°C) and the purification of plasmid DNA was performed using QIAGEN Plasmid Maxi Kit according to the manufacturer's protocol. DNA concentration was determined spectrophotometrically at 260 nm.

3.1.5.5 Sequencing of plasmids

Sequencing of the constructs was performed by the BMFZ (Biologisch-Medizinisches Forschungszentrum) of the Heinrich-Heine-University Düsseldorf (Germany).

3.2 Cell culture

3.2.1 Maintenance of mammalian cells

HT1080- and all MEF-cells were maintained as subconfluent monolayer cultures in growth medium (see 2.3.3) at 37°C in a humidified atmosphere containing 5% CO_2. For passage, cell layers were washed once with PBS, detached by a short treatment with Trypsin-EDTA solution (see 2.3.2) and reseeded upon dilution with culture medium (1:6 every 2 days).

3.2.2 Freezing and thawing of cells

For freezing cells were detached, resuspended and centrifuged (300 x g) for 3 min. The cell pellet was resuspended in 1 ml FCS containing 10% DMSO and transferred to cryo-tubes (1.8 ml) and freezing boxes (see 2.10). After 24 h at -80°C, tubes were transferred to liquid nitrogen for long term storage.

For thawing procedure, cryo tubes were thawed in a water bath (37°C). Cells were immediately mixed with 5 ml culture medium (37°C), transferred into 15 ml tubes, centrifuged (300 x g) for 3 min and seeded to a new culture flask.

3.2.3 Transfection and selection of HT1080 cells

Confluent cells (~$1x10^6$ cells) were diluted (1:5) 24 hours before transfection. Cells were transfected with 1 µg DNA using Effectene Transfection Reagent (Qiagen) according to the manufacturer's instructions. Transient expression of YFP-fused constructs could be estimated by microscopy after 12-24 hours with a transfection efficiency varying between 20-90% depending on the used construct. 24 h after transfection, medium containing Effectene reagent were replaced by fresh medium and incubated overnight at 37°C. Thereafter cells were appropriately diluted into tissue culture dishes and stable cell clones were selected with selection medium (see 2.3.3). Stable expressing clones were isolated, expanded and subsequently cultivated in selection medium.

3.2.4. TOP1MT knockdown by siRNA transfection

Knockdown of TOP1MT was performed using commercial siRNA (ON-TARGETplus Human TOP1MT siRNA, #116447, Thermo Scientific) and transfection of cells. To

ensure an efficient siRNA pool contains four different siRNAs against different sequence sections of the enzyme. For transfection, HT-1080 cells were seeded 1:5 in small flasks the day before transfection. Transfection was carried out using Dharmafect 4 transfection reagent (Thermo Scientific) and an end concentration of 5 nM siRNA pool. As control a second parallel transfection with siRNA without any known target (On-TARGETplus Non-Targeting Pool, #D001810-10, Thermo Scientific) was conducted to monitor effect of transfection. Further investigations were carried out after 48h of incubation at 37°C.

3.2.5 Recomplementation of TOP1MT$^{-/-}$ MEF cells

Re-complementation experiments were conducted by collaboration partners (Ilaria Dalla Rosa) at the lab of Yves Pommier (NIH, Washington) and are thus only briefly described for sufficiency here.

For re-complementation the cDNA of mouse *TOP1MT* (NM_028404.2) was cloned into the retroviral vector pFB-Neo (Stratagene, La Jolla, California, USA) to generate pFB-*TOP1MT*-Neo, which was co-transfected with packaging plasmids (pVPack-GP and pVPack-VSV-G, Stratagene) in 293T cells to produce *MMLV*-based viral particles for transduction of *TOP1MT*$^{-/-}$ MEFs. Particles of empty virus similarly generated served as negative control. TOP1MT expression was confirmed by immunoblotting 24 h after transduction.

3.3 Protein analysis

3.3.1 Preparation of whole cells lysates

HT1080 cells ($3x10^6$) were pelleted washed and resuspended in 100 µl PBS. Cell lysis was performed by adding 100 µl of 2x lysis buffer (250 mM Tris-HCl, pH 6.8, 2% glycerol, 4% SDS, 20 mM DTT, 1.4 M urea, 20 mM EDTA, 2 mM PMSF, 5 mM pefa block, 0.04% bromphenol blue) and homogenizing by ultrasound (15 s at 20% power). Subsequently, samples were boiled (98°C, 5 min) and warm aliquots equivalent to ~$3x10^5$ cells were loaded onto SDS-polyacrylamide gels.

3.3.2 Preparation of Mitochondria extracts

For preparation of mitochondria $12x10^6$ were pelleted, washed and Mitochondria were isolated using Mitochondria Isolation Kit for Cultured Cells (# ab110170, abcam) according to manufacturer's instructions. Mitochondria suspension was subjected to homogenizing by ultrasound (3x 15 s at 20% power). Protein content was determined by BCA protein assay according to manufacturer's protocol. Finally mitochondrial protein was mixed with appropriate volumes of 6x lysis buffer (see 3.1.1) and inspected by western blotting.

3.3.3 Polyacrylamide gel electrophoresis

Electrophoresis was performed in 1x TGS buffer or for NuPAGE gels in 1x MOPS SDS running buffer at voltage of 50-160V.

3.3.4 Immunoblotting

3.3.4.1 Western Blot

After separation, proteins were electrophoretically transferred from the gel to a PVDF membrane by the semi-dry method. Therefore the PVDF membrane was activated in MeOH for 1min, washed in H_2O and equilibrated in transfer buffer (48 mM Tris, 39 mM glycin, 0-20 % methanol, pH ~9.2), while the gel was equilibrated for at least 20 min in transfer buffer. Two 3MM paper filters soaked in transfer buffer were stacked on the cathode side of the gel, and two were stacked on the anode side of the gel. The stack was placed between two electrodes and the protein transfer was carried out according to the manufacturer's instructions. After transfer, the PVDF membrane was incubated in PBS containing 0.05% Tween 20 and 5% milk powder overnight at 4°C or alternatively for 1h at RT. After blocking, the membrane was washed with PBS containing, 0.05% Tween 20 and incubated for 1 h with the primary antibody diluted in PBS containing, 0.05% Tween 20 and 1% BSA and washed three times (3x 10 min). The membrane was then incubated for 1 h with the secondary peroxidase conjugated antibody diluted in PBS containing 0.05% Tween 20 and 1% BSA. The membrane was again washed three times (3x 10 min) and the protein bands were visualised by chemiluminescence using the ECL Plus system (Amersham) and the luminscent image analyzer (LAS-4000 see 2.10). Band intensities were analysed using Multi Gauge software (see 2.11).

3.3.4.2 Co-Immunoprecipitation

Co-immunoprecipitations (co-IP) were carried out using µMACS^{TM} Epitope Tag Protein Isolation KIT with Anti-GFP MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) to precipitate YFP-tagged- and associated proteins. This Kit uses magnetic micro-beads (50 nm diameter) which are coated with anti-YFP antibody. Beads were incubated with mitochondrial extracts and in a second step beads were transferred onto a 2 ml column placed in a magnetic tray. This allows subsequent washing of column while bound protein is retained. Unspecific interacting molecules are washed away and the protein of interest and associated proteins can be eluated and analyzed. The kit was used according to manufacturer's instructions.

For co-IP precipitating POLRMT and associated protein µMACS^{TM} Protein A MicroBeads (Miltenyi Biotec) were applied. Therefore 1µg anti-POLRMT antibody (sc-67350, Santa Cruz) was incubated 30 min (4°C) with cell lysate before 50 µl micro-beads were added for a second incubation of 30 min (4°C) to enable protein binding to beads. All subsequent washing and elution steps followed manufacture's instructions.

3.3.5 Catalytic activity of Topoisomerase I

3.3.5.1 Mitoplast protein extracts

Cells from four confluent 175 cm^2 tissue culture flasks were harvested and washed with PBS. All subsequent steps were performed on ice. Cell pellet was resuspended in 10 ml MIB buffer supplemented with 0.1 mg/ml digitonin and incubated for 10 min.

Next, cells were homogenized with 20 strokes in a dounce homogenizer, centrifuged twice at 2600 g for 7 min at 4°C to pellet unlysed cells and nuclei. The supernatant fraction (=whole cytosolic fraction) was centrifuged at 15000 g for 10 min at 4°C to pellet mitoplasts. Pelleted mitoplasts were resuspended in 0.2 ml MIB without digitonin, aliquoted and stored at 80°C.

Mitoplast were extracted for 10 min on ice in EMIB. mitoplast extracts were cleared by centrifugation for 15000 g, 10 min, 4°C. Protein concentration was determined by BCA assay.

3.3.5.2 Relaxation assays

Mitoplast extracts (170 ng) were incubated for various times at 37°C with 0,4 µg

supercoliled pUC18 in a final volume of 20 µl assay buffer (120mM KCl, 50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1mM ATP, 0.5 mM DTT, 0.5 mM EDTA, 30 µg/ml BSA) The reaction was stopped by addition of SDS (0,5%) and subsequently digested with Proteinase K (1 mg/ml, 30 min, 50°C). To measure relaxation at different oxidative states Mitoplasts (400 µg)(1) or mitoplast extracts (500- 1000 µg)(2) were incubated for various times at 37°C with 0.4 µg supercoliled pUC18 in a final volume of 20 µl assay buffer supplemented with (1): 1mM antimycin A, 100 mM glutamate, 50mM malate and 25 mM ADP or (2): 250 nM oxidized glutathione or 0,5 mM DTT. Finally samples were subjected to agarose gel electrophoresis and separated overnight (~14h) at 0.5 V/cm in the absence of EtBr. After finishing electrophoresis, gel was stained with EtBr to illuminate DNA using a transilluminator at 280 nm.

3.3.6 Immunocytochemistry

3.3.6.1 Live cell staining

For detection of the mitochondrial compartment cells were grown on coverslips. For visualization of mitochondria, cells were incubated with 10 nM MitoTracker®Red CMXRos. After 10 min of incubation at 37°C cells were washed three times with 1ml PBS and subsequently analyzed by fluorescence microscopy.

3.3.6.2 Immunocytochemistry in fixed cells

Cells were grown on coverslips, washed with PBS, fixed with 3.7% paraformaldehyde in PBS, for 10 min, 37 °C. After washing once with PBS cells were permeabilized by applying 0.25% TX-100 in PBS for 10 min at RT. At ambient temperature fixed cells were washed with PBS (3x 10min), blocked (2% BSA and 5% GSA in PBS, 60 min). After washing once with PBS cells were incubated for 30 min with rabbit anti-human TFAM serum, rabbit anti-human mtRNA polymerase antibody, mouse anti-bromo deoxyuridine antibody and/or mouse anti-DNA antibody in PBS containing 1% blocking solution. After washing (3x 10 min) with PBS, cells were incubated for 1 h with CY2- or CY3-conjugated goat anti-rabbit IgG and/or CY3-conjugated goat anti-mouse IgM (Dianova, Hamburg, Germany). Subsequently cells were washed (3x 10 min) with PBS and mounted. To visualize mitochondrial transcripts cells were grown in 2.5 mM bromouridine (Iborra *et al.* 2004) for 60 min before immunolabeling the resulting Br-RNA.

3.4 Microscopy

3.4.1 Fluorescence microscopy

Epifluorescent images were acquired using an inverted microscope equipped with a cooled charge coupled device camera. For observation of living cells, cells were grown and inspected in CO_2-independent medium (see 2.3.3) using live-cell chambers to keep the cells at 37˚C.

3.4.2 Confocal microscopy

Confocal imaging of living cells and fixed specimen was performed at 37°C using a Zeiss LSM 510 inverted confocal laser scanning microscope equipped a 63x/1.4 NA oil-immersion objective. Cells were grown in CO_2-independent medium and Zeiss incubator XL facilitated 37 °C during live imaging microscopy.

For investigation of TOP1MT exchange rate cells were grown on coverslips and a selected spot of accumulated YFP fluorescence was bleached using an UV-laser. Fluorescence recovery was documented using confocal imaging.

3.4.3 STED microscopy

We are grateful to Dr. Olga Lévai, Leica Mikrosysteme Vertrieb GmbH, Mannheim, for making available a Leica TCS SP8 STED microscope and helping with its use. Assistance with the evaluation of super-resolution microscopy data is gratefully acknowledged to Dr. Stephanie Weidtkamp-Peters, Centre for Advanced Imaging, Heinrich Heine University, Düsseldorf.

3.5. MtDNA analysis

3.5.1 Determination of mtDNA copy number

Determination of mtDNA copy number in EtBr-treated cells was carried out by Ilaria Dalla Rosa, Düsseldorf, according to the following protocol:

3.5.1.1 DNA extraction

Total DNA from $5x10^6$ cells was isolated using the “DNeasy Mini kit” (Qiagen) according to the manufacturer’s protocol. DNA concentration was determined using spectrophotometer by absorbtion at wavelengths of 260 nm.

3.5.1.2 Quantitative Real-Time PCR (qRT-PCR)

MtDNA copy number was determined according to the quantitative TaqMan-PCR method. Template DNA was diluted to a concentration of 10 ng/µl in PCR water. 100 ng total DNA were subjected to amplification reactions performed as 25 ml duplicates in a 96-well microplate with 1X Platinum SYBR Green qPCR SuperMix-UDG with ROX (invitrogen) and forward and reverse primer. To determine the abundance of mtDNA in the sample, amplification was performed in parallel with different concentrations of standard plasmids to allow the generation of a calibration curve.

PCR setting per reaction (25 ul)

Component	**Volume**	**Final concentration**
DNA template (10 ng/µl)	10 µl	100 ng
Primer Forward	0.125 µl	500 mM
Primer Reverse	0.125 µl	500 mM
2X Platinum SYBR Green qPCR SuperMix-UDG with ROX	12.5 µl	1X
H_2O	2.25	/

Quantification of DNA copies analysis was carried out in an ABI PRISM 7000 Sequence Detector (Applied Biosystems) under the following conditions:

Denaturation	10’ 95°C	
Denaturation Annealing and Elongation	15” 94°C 1’ 60°C	40 cycles

Quantification of mtDNA copies was carried out using the primers IS-F and IS-R,

binding to unique mtDNA sequences (Table 3.4). Quantification of the common deletion (CD) and 284 deletion were performed using the primers CD-F and CD-R and D284-F and D284-R, respectively (Table 3.6). The primers amplified the sequence spanning the deletion positions. In deleted mtDNA molecules, these primers come close enough to synthesize a PCR product within the given extension time, whereas in no deleted mtDNA the distance between the primers is too long.

Table 3.6: Real Time PCR Primer used in this work

Primer	Sequence	Match
IS-F	5´- TTGACTCACCCATCAACAACC -3´	mtDNA 16091-16111
IS-R	5´- AATATTCATGGTGGCTGGCAGTA -3´	mtDNA 16130-16152
CD-F	5´- ACCCCCATACTCCTTACACTATTCC-3´	mtDNA 8417-8441
CD-R	5´- AAGGTATTCCTGCTAATGCTAGGCT-3´	mtDNA 13485-13509
D284-F	5´- TACCCCCTCTAGAGCCCACT-3´	mtDNA 8291-8310
D284-R	5´-GTAGCTTTGGCGTTTGTAT-3´	mtDNA 13541-13559

3.5.2 Analysis of mitochondrial transcripts

3.5.2.1 Total RNA isolation

Total RNA from 5x10^6 cultured cells was isolated using “RNeasy Mini Kit” (Qiagen) according to the manufacturer’s protocol. RNA concentration was determined by measuring absorption at wavelength of 260 nm.

3.5.3.2 Semiquantitative analysis of mtDNA transcripts: Northern Blotting

Northern Blotting was performed by Frank Hillebrand, Düsseldorf. For the generation of probes mitochondrial DNA isolated from HT-1080 or MEFs cells using Mitochondrial DNA Isolation Kit (# ab65321, abcam) according to manufacture’s instructions, and primers from qRT-PCR of transcript measurements (see 3.5.2.4) (human: 12S, COX1, COX2, ND2, ND5, ND6 or mouse: COX1, COX2, ND5, ND6) were supplied. Additionally isolated total RNA from HT-1080 or MEFs using RNeasy Mini Kit (see 2.9) was supplied. Northern Blotting followed published procedures (Widera *et al.* 2013).

3.5.2.4 Quantitative analysis of mtDNA transcripts: Real-Time Reverse-Transcription PCR (qRT-PCR)

Quantitative measurment of mtDNA transcripts was performed with qRT-PCR using "OneStep RT-PCR Kit" (QIAGEN) that allows both reverse transcription and PCR to take place successively in a single tube.

QRT-PCR reactions were performed as 25 ml duplicates in a 96-well microplate. To 5 ng total RNA template the Mastermix was added, consisting of RT-PCR Master Mix, for the reverse transcription of RNA into cDNA, QuantiTect SYBR Green, for the detection of PCR product accumulation, forward and reverse primer (Table 3.7).

PCR setting per reaction (25 ul)

component	**Volume**	**Final concentration**
RNA template (500 pg/ul)	10 ul	5 ng
Primer Forward	0,125 ul	500 nM
Primer Reverse	0,125 ul	500 nM
2X QuantiTect SYBR Green	12,5 ul	1X
RT-mix	0,25 ul	0,25 ul/ reaction
H_2O	2 ul	/

Background fluorescence, e.g. from residual mtDNA, was subtracted as for each sample at least one PCR reaction was performed without the RT-PCR Master Mix. RNA levels of the transcripts were normalized to the level of 18S rRNA.

The thermal cycling conditions included a reverse transcription step of 30 min at 50°C, followed by a heating step of 10 min at 95°C for the deactivation of the reverse transcriptase and the activation of DNA polymerase. PCR amplification was performed with 40 cycles under followings conditions:

Primers T_m 60°C	
Denaturation	20” 94°C
Annealing	20” 57°C
Elongation	30” 72°C

Table 3.7 Oligonucleotids for detection of human mitochondrial transcripts

Oligonucleotides used as PCR-primers T_m~60°C	
Primer	**Sequence**
18S F	5´-ATTAGAGTGTTCAAAGCAGGCCCGAGC
18S R	5´-CGTCCCTCTTAATCATGGCCTCAGTTC
12S F	5´-GGTTGGTCAATTTCGTGCC
12S R	5´-GAGTTTTTTACAACTCAGGTG
COX1 F	5´-TACCTATTATTCGGCGCATGAGCTGGA
COX1 R	5´-TGCATGGGCTGTGACGATAACGTTGTA
COX2 F	5´- ATGATGGCGCGATGTAACAC
COX2 R	5´- GGCATGAAACTGTGGTTTGCT
ND2 F	5´-CATCCGGCCTGCTTCTCCT
ND2 R	5´-CCACCTCAACTGCCTGCTAT
ND5 F	5´-GCGCCCTTACACAAAATGACA
ND5 R	5´-TGAAGAAGGCGTGGGTACAG
ND6 F	5´-GGGGGTTTAGTATTGATTGTTAGCGG
ND6 R	5´-ATCGCTGTAGTATATCAAAAGACAACCA

Table 3.8 Oligonucleotids for detection of mouse mitochondrial transcripts

Oligonucleotides used as PCR-primers T_m~60°C	
Primer	**Sequence**
18S F	5´-CCCCTCGATGCTCTTAGCTG
18S R	5´-GAACCGCGGTCCTATTCCAT
12S F	5´-CTCAAAGGACTTGGCGGTACT
12S R	5´-AGGGTTTGCTGAAGATGGCG
COX1 F	5´-TCGCAATTCCTACCGGTGTC
COX1 R	5´-CCGGTTAGACCACCAACTGT
COX2 F	5´-ACCTGGTGAACTACGACTGC
COX2 R	5´- AAGTCCTAGGGAGGGGACTG
ND2 F	5´-AGGGGCATGAGGAGGACTTA
ND2 R	5´-TTGAGTAGAGTGAGGGATGGGT
ND5 F	5´-AATTTGGCCTCCACCCATGA
ND5 R	5´-TAGTCGTGAGGGGGTGGAAT
ND6 F	5´-GGGAGATTGGTTGATGTATGAGGT
ND6 R	5´-CCCGCAAACAAAGATCACCC

3.5.2.5 Quantitative analysis of nuclear transcripts

Quantitative measurements of nuclear transcripts for mitochondrial proteins were performed with qRT-PCR as described above, using the primers listed in Table 3.8.:

Table 3.8: Human Nuclear Transcript Primer

Primer	**Sequence**
SDHB F	5´-AGTTGACTCTACTTTGACCTTCCGAAG
SDHB R	5´-GACCTTATTGAGGTTGGTGTCAATCCT
TOP1MT F	5´-GGAAATGAAGACGAGACAGC

TOP1MT R	5´-GGAGGGAACAGCAGCCCAC
TOP2B F	5´-CCAGAGCAATTTTTATATGGTACTGCA
TOP2B R	5´-GGCCAGGTTTAAAGCCATCAACAA
TOP3A F	5´-CATGGCATTGGTACGGATGCC
TOP3A R	5´-TGGAATCATAACCTTCCACAAGTCCC
PGC1A F	5´-GCCCAGGTATGACAGCTACGA
PGC1A R	5´-CGCTCTTCAATTGCCTTCTGCC
NRF1 F	5´-TCACTTATCCAGGTTGGTACGGG
NRF1 R	5´-CCCAATTTTGTTCCACCTCTCCATC
TFAM F	5´-CCTTCAGTTTTGTGTATTTACCGAGG
TFAM R	5´-TAGTTTTTGCATCTGGGTTCTGAGC
POLG F	5´-CCTAGCTCTGACTGCCCGT
POLG R	5´-TACAGCTATTACCATCCTTGTGAGGC

Table 3.8 Mouse Nuclear Transcript Primer

Primer	**Sequence**
TOP1MT F	5´-GATCCGTGCCCAGTATCAGG
TOP1MT R	5´-TCTCGCCCTCTTCCTTCTCA
TOP2B F	5´-CTGGGGTGGCTCTCAAAGTT
TOP2B R	5´-CGCCCACCTTTTGTAGTTGC
TOP3A F	5´-ATCATTGTCAGGCCCTGGTG
TOP3A R	5´-GATCTCAGAGAAGCGGGCTC
NRF1 F	5´-TCACTTATCCAGGTTGGTACGGG
NRF1 R	5´-CCCAATTTTGTTCCACCTCTCCATC
GABPA F	5´-ACTTAGCCGTGCATTACGGT
GABPA R	5´-CCAGACGGTTCAGTTCTGCT

3.6 Investigation of mitochondrial functions

3.6.1 Mitochondrial ROS production

Measuring of ROS was carried out using MitoSOX Red reagent (invitrogen), a cell-permeant fluorogenic dye for highly selective detection of superoxide in the mitochondria. Once in the cell, MitoSOX is rapidly targeted to the mitochondria, where it is oxidized by superoxide and exhibits red fluorescence.

Cells were grown till 80% confluence and loaded with 5 µM MitoSOX in medium for 30 min at 37°C. Cells were trypsinized, washed and resuspended in HBSS (see 2.4) containing 1% BSA at a density of 1 $x10^7$ cells/ml.

Fluorescence was monitored by flow cytometry. MitoSOX was excitated by laser at 488 nm and the data collected at FL2 channel. Since YFP and MitoSOX signals could not be completely separated at the chosen FACS settings, the intensity without Mitosox for each cell clone was measured and subtracted from signal with MitoSOX. Mitochondrial superoxide generation was increased by addition of 100 µM Antimycin A inhibiting the CIII of the RC.

3.6.2 Mitochondrial respiration

For measurement of cell respiration ~$24x10^6$ cells were harvested and resupended in PBS. Fresh respiration medium was filled in electrode chamber and 10-50 µl of cell suspension were added to a final volume of 250 µl. After closure of chamber measurement of global cell respiration was started. Next, cells were permeabilized by addition of 1 µl of digitonin. To investigate subsequently electron entrance via RC CI, malate/pyruvate and malate/glutamate oxidation via staggered supplementation with 1µl malate (50 mM) + 5 µl pyruvate (400 mM); 6 µl ADP (50 mM); 5 µl NAD (40 mM); 5 µl malate (500 mM) + 5 µl glutamate (500 mM) and 5 µl KCN (120 mM) was detected. A second measurement was applied to investigate electron entrance via RC CII. Therefore following permeabilization with1 µl digitonin + 0.5 µl rotenone (2 mM) + 3 µl ATP (50 mM), succinate oxidation and G3P oxidation were monitored by staggered supplementation with 5 µl succinate (500 mM); 0.5 µl oligomycin (2.5 mM); 0.5 µl CCP (1.2 mM); 5 µl malonate (500 mM); 5 µl G3P (500 mM) and 5 µl KCN (120 mM). The results were analyzed by mathematical determination of incline of oxygen

levels. The results were normalized to protein content of cell suspension as determined by BCA.

3.6.3 Lactate production

For the quantification of lactate levels $1x10^6$ cells were seeded in a 25 cm^2 culture flask. After 48 hours the medium was removed and first cleared by centrifugation then transferred to BD vacutainer tubes for lactate-quantification, contain 5 mg Na-Fluorid and 4 mg K-Oxalat. To adjust the differences in growth rate among different cell-clones, cells were counted and lactate levels were normalized to the cells number. Lactate concentration was assayed in an automated blood analyzer. The method uses an enzymatic reaction, catalyzed by the lactate oxidase, to convert lactate to pyruvate:

L-lactate + O_2 $\rightarrow$pyruvate + H_2O_2

The hydrogen peroxide produced by this reaction is then used by the enzyme peroxidase to generate a detectable colored dye

H_2O_2+ H donor + 4-aminoantipyrine$^+$$\rightarrow$ chromogen$^+$ + 2 H_2O

The intensity of the color formed is then proportional to the L-lactate concentration.

3.7 Statistics

Quantitative results are represented as mean values ± standard error of the mean of measurements carried out in duplicate or triplicate on independent cell clones expressing the same construct. Data sets were compared by an unpaired T-test and the null hypothesis was rejected on a probability level of ‹0.05 (i.e. P‹0.05 was considered significant).

4. Results

4.1 Overexpression of mitochondrial Topoisomerase 1

Although the existence of Topoisomerase 3α and Topoisomerase 1 in mitochondria has been shown and the presence of Topoisomerase IIβ is suggested, the exact roles in mitochondrial metabolism remain ambiguous. Putative functions in specific processes inside mitochondria can be deviated by the nuclear roles of the enzymes but definite evidence is missing. At least the processes of transcription and replication, which involve processing of mtDNA, are very likely dependent of Topoisomerase activity. The *in vivo* overexpression represents a strong tool to investigate functions of a certain protein. In this work the selective overexpression of TOP1MT in human cells was used to reveal more about its role in mitochondrial metabolism.

TOP1MT seems to play an important role inside mitochondria. It is present as an orthologous gene suggesting an essential role for mitochondria. This also implies a precise regulation of enzyme amount to avoid potential negative effects by oversupply or deficit of enzymatic activity. Therefore an artificial overexpression potentially discloses changes in TOP1MT dependent processes. The constructs TOP1MT, MY, MY-TOP1MT and MY-TOP1MT^{Y559F} (see below) used in this work were cloned, stably transfected and characterized by Ilaria Dalla Rosa (IDR) and already described in her PhD thesis (Dalla Rosa, 2009a) and the publication Dalla Rosa et al 2009b. For sufficiency and comprehension of my work some basic results obtained by IDR are rereleased here. All work carried out by IDR is explicitly marked as her work (see figures).

4.1.1 Expression and targeting of TOP1MT variants in HT-1080 cells

To investigate the effects of an overexpression of TOP1MT five constructs were cloned and expressed in HT-1080 cells (Figure 4.1.1). To generate a TOP1MT overexpressing cell model, human TOP1MT gene was set under control of the CMV promoter to ensure high expression rates in human cell lines. To elucidate which processes mediated by TOP1MT rely on its catalytic activity, a point mutation was introduced into the active site of TOP1MT by exchange of tyrosin (Y) at position 559

with phenylalanin (F), resulting in a catalytically inactive enzyme named TOP1MT^{Y559F}. To enable a visual tracking of TOP1MT a further series of constructs was generated where the Topoisomerase sequence was N-terminal fused in frame to the sequence of YFP from *Aequorea victoria.* The N-terminal extension of TOP1MT is supposed to interfere with mitochondrial import given that endogenous MTS is disguised by YFP sequence. To restore correct cellular localisation of the YFP-TOP1MT construct an additional MTS derived from human cytochrome C oxidase subunit VII was fused N-terminal of YFP. In this work three constructs tagged with MTS-YFP were used: MTS-YFP (MY), MTS-YFP-TOP1MT (MY-TOP1MT) and MTS-YFP-TOP1MT^{Y559F} (Figure 4.1.1). MY construct served as a control to monitor overexpression effects of a non-DNA-binding protein in the mitochondrial matrix, while the latter construct represents the YFP-tagged variant of the active site mutant. The transfections of HT 1080 cells with these constructs and following selection with puromycin gave rise to multiple clones. For each construct at least 10 clones were isolated and expanded. All isolated clones showed growth rates and morphology comparable to wild type cells.

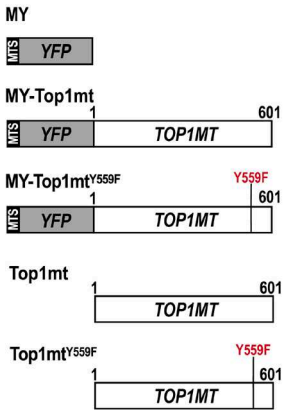

Figure 4.1.1: Constructs for constitutive overexpression of TOP1MT. "MTS"indicates the position of a mitochondrial targeting sequence derived from the human COX1 gene.

Clones containing the YFP sequence were first inspected by fluorescence microscopy and only cells holding mitochondrial located YFP fluorescence were isolated. In the next step all generated clones were screened for the expression of the transfected constructs. Therefore TOP1MT mRNA levels were detected by quantitative real time PCR (qRT-PCR). This also permitted to assess whether the untagged constructs of TOP1MT and TOP1MT^{Y559F} were overexpressed in transfected cell lines. The results showed about ~5-20 fold increased mRNA levels compared to untransfected wild type cells (Figure 4.1.2 A, B). To estimate effective protein levels and correct protein expression, a western blot analysis of cell lysates

was carried out for all designed clones. The resulting blots were probed with antibodies against TOP1MT and additionally against YFP for cell lines expressing YFP tagged constructs.

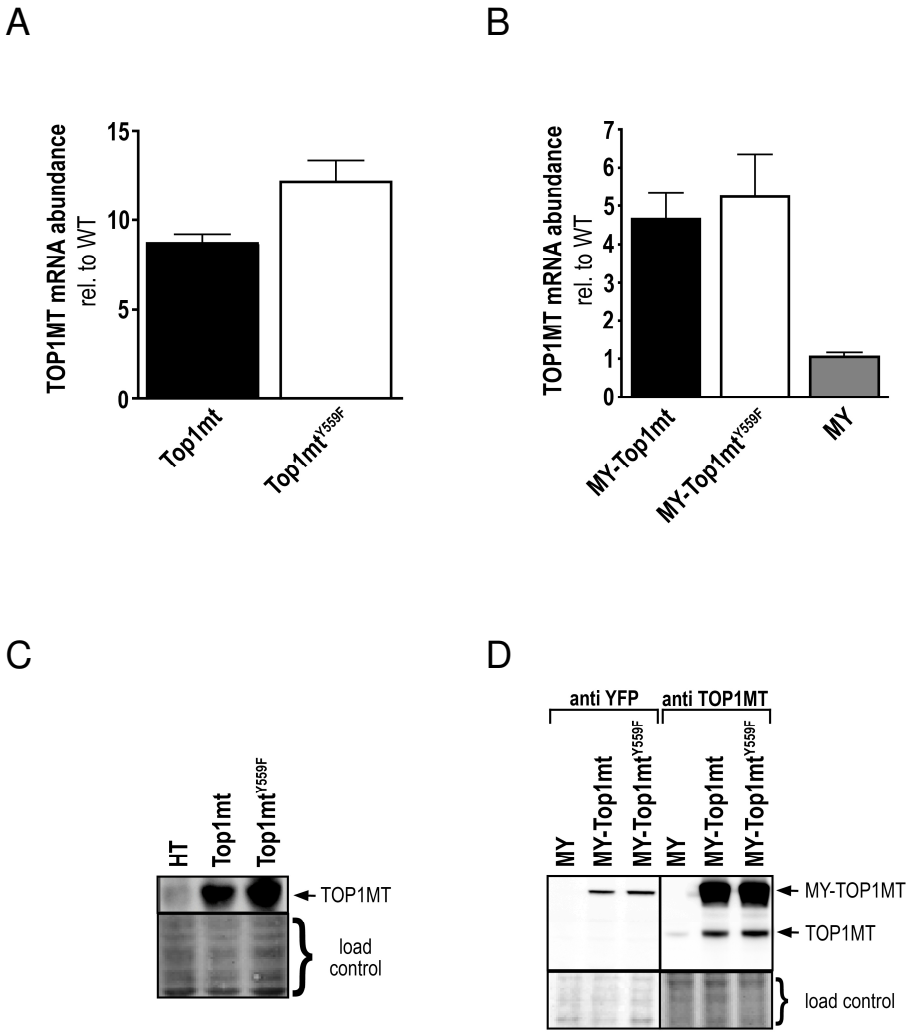

Figure 4.1.2: Overexpression of TOP1MT in HT1080 cells. A) qRT-PCR of TOP1MT-specific mRNA of cell lines expressing untagged constructs. **B)** qRT-PCR of TOP1MT-specific mRNA of cell lines expressing YFP-tagged constructs. **C)** Western Blot of TOP1MT-specific protein of cell lines expressing untagged constructs. **D)** Western Blot of proteins of cell lines expressing YFP-tagged constructs. Western blot in (D) was performed by IDR.

All cell lines expected to express TOP1MT variants (TOP1MT, TOP1MT^{Y559F}, MY-TOP1MT and MY-TOP1MT^{Y559F}) showed a strong signal of anti-TOP1MT antibody, thus confirming the overexpression (Figure 4.1.2 C, D). All detected bands corresponded to previously calculated protein sizes, approving proper protein expression. In line with this result YFP probed blots showed the anticipated expression of YFP fusion proteins, likewise of estimated sizes.

4.1.2 TOP1MT overexpression leads to increased TOP1 activity in mitochondria *in vitro*

Subsequent of affirming correct localisation and protein integrity, the catalytical activity of the overexpressed proteins was examined. Therefore relaxation activity of TOP1MT was measured (Figure 4.1.3). In the assay mitochondrial extracts of the different clones were incubated with a supercoiled DNA substrate for defined periods of time. After the termination of the reaction by inactivation of the enzyme, the topological state of the substrate DNA was subjected to gel electrophoresis, which allows the separation of the different topological DNA forms.

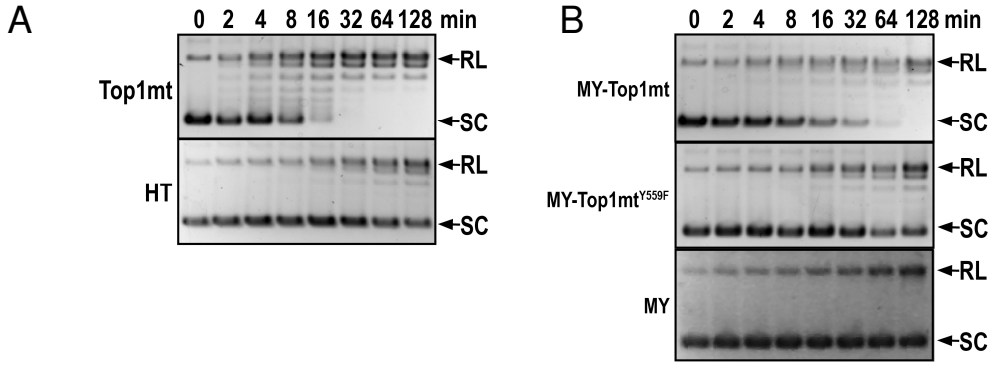

Figure 4.1.3: TOP1MT DNA relaxation activity in mitoplast extracts. A) Relaxation activity in TOP1MT overexpressing and HT cells. **B)** Relaxation activity in the denoted cells expressing the denoted protein. Shown are representative examples. Relax assays in (A) and (B) were performed by IDR.

The obtained results indicated that both cell lines overexpressing the active TOP1MT (TOP1MT and MY-TOP1MT) showed strongly increased relaxation activity, manifesting by a fast increase of relaxed DNA substrate compared to wild-type HT1080. At time points 32 min for TOP1MT and 64 min for MY-TOP1MT respectively, all detectable supercoiled DNA substrate was changed over to the relaxed form. The slight differences in relaxation activity may be due to the appended YFP-tag affecting catalytic activity. The cell lines expressing the active site mutant Topoisomerases (TOP1MT^{Y559F} and MY-TOP1MT^{Y559F}) exhibited only weak relaxation activity, which is due to the residual endogenous TOP1MT. The same was found in wild-type HT1080 cells and cell lines expressing the MY control construct. These showed only base level activity deriving from endogenous TOP1MT. It was

thus confirmed that expression of catalytically active TOP1MT inside mitochondria was attained in clones transfected with TOP1MT and MY-TOP1MT constructs.

In summary the basic characterization of the clones demonstrate that cell lines were successfully established which stably overexpressed catalytically active (TOP1MT, MY-TOP1MT) or inactive (TOP1MT^{Y559F}, MY-TOP1MT^{Y559F}) enzyme versions and one additional control construct (MY) which were targeted to the mitochondrial matrix. These generated cell lines thereby represent a suitable tool to investigate TOP1MT function.

4.1.3 MY-TOP1 variants associate with nucleoids

To investigate the sub-mitochondrial distribution of the heterologously expressed YFP tagged TOP1MT confocal fluorescence microscopy was applied. In a first set of experiments MY, MY-TOP1MT, MY-TOP1MT^{Y559F} and wild-type HT1080 cells were stained with Mito Tracker dye which is exclusively targeted to the mitochondrial matrix (Figure 4.1.4). Initially a comparison of wt-cells (bottom row) with the transfected cell lines MY (top row), MY-TOP1MT (upper middle row) and MY-TOP1MT^{Y559F} (lower middle row) showed no obvious differences of overall mitochondrial morphology. In all cases mitochondria form the interconnected network of stretched tubules typically found in mammalian cells (Rizzuto *et al.* 1989). The observation allowed excluding any severe side effects of heterologous gene expression on mitochondrial biogenesis and mitochondrial fusion/fission processes. The overlay of Mito Tracker signal (red) and YFP fluorescence (green) showed a complete overlap (Figure 4.1.4; left and middle-left column) confirming that in all cases (MY, MY-TOP1MT, MY-TOP1MT^{Y559F}) the overexpressed proteins are located exclusively inside the mitochondria. Notably the YFP induced signal obviously differs in distribution of fluorescence between the control construct of MY and the Topoisomerase expressing constructs. While MY is evenly distributed throughout the mitochondrial network (top row, middle right), the fluorescence of MY-TOP1MT and MY-TOP1MT^{Y559F} (Figure 4.1.4; middle rows, middle right) accumulates in distinct foci (arrows). Moreover cells expressing MY-TOP1MT showed brighter and more frequent foci compared to cells expressing the active site mutant enzyme version (MY-TOP1MT^{Y559F}). This may hint to a correlation between catalytic activity and accumulation of Topoisomerase constructs in focal structures.

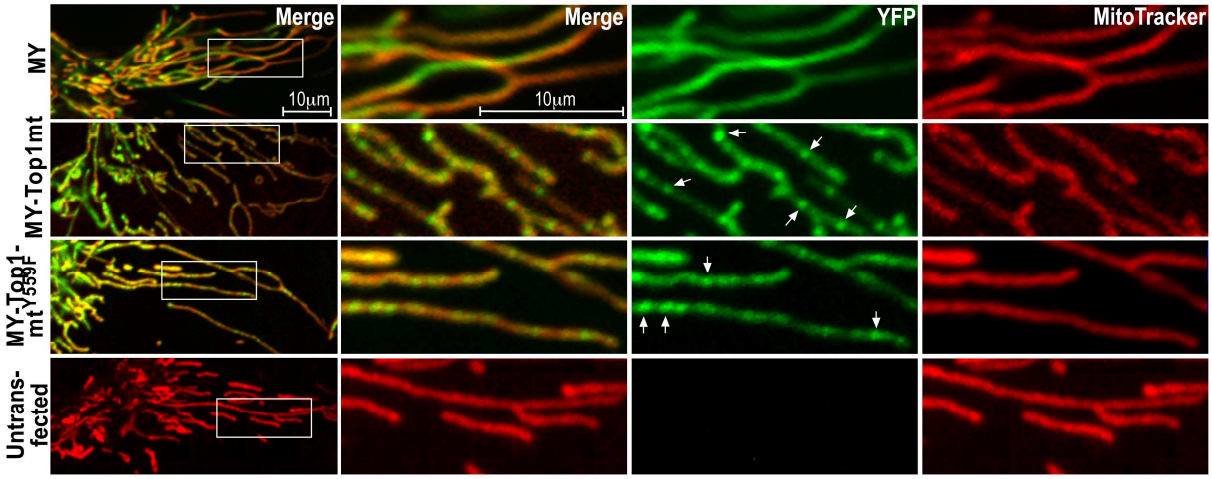

Figure 4.1.4: Mitochondrial distribution of YFP fused protein. Representative confocal microscopy images of live cells expressing the denoted constructs. Red Mitotracker fluorescence illustrates mitochondrial compartment. Arrows: focal accumulation of MY-TOP1MT and MY-TOP1MT^{Y559F}.

This punctuated pattern of distribution resembles published dyeing of mitochondrial nucleoids (Garrido *et al.* 2003). Since nucleoids represent the mitochondrial structure where mtDNA resides, it is likely that DNA-interacting proteins like Topoisomerases concentrate at these structures. To further test if the observed speckles were nucleoids immunocytochemisty (ICC) strainings were carried out. Staining with a DNA directed antibody (Figure 4.1.5) in wt HT1080 cells showed a punctuated pattern resembling the previous observed distribution of YFP-tagged Topoisomerase constructs (bottom row). Staining also reveals similar patterns in wt HT1080 cells and transfected cells, thus excluding negative effects of MY / MY-TOP1MT / MY-TOP1MT^{Y559F} overexpression on DNA abundance and distribution (right column). The overlay of the signals derived from DNA and YFP fluorescence (left and middle left column) indicated that all YFP foci (arrows) are also positive for the DNA signal. On the other hand several DNA foci are negative for the YFP signal (open arrowheads). This shows that seemingly the foci of accumulated YFP signal represent nucleoid-structures and TOP1MT co-localises with nucleoids. To further verify this result another series of ICC staining utilising an antibody against TFAM was conducted (Figure 4.1.6). TFAM is a previous described marker-protein for nucleoids (Garrido et al, 2003). It takes part in organising mtDNA and also plays a role in mitochondrial transcription.

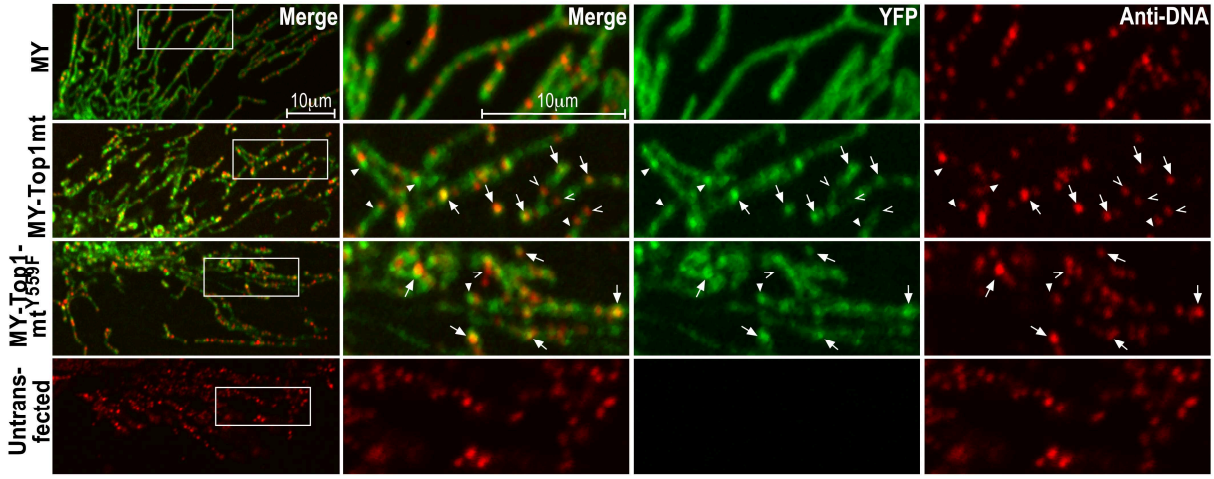

Figure 4.1.5: Co-localisation of TOP1MT and mtDNA. Representative confocal microscopy images of cells expressing the denoted constructs. Cells were fixed and stained against mtDNA (in red). Arrows: foci of mtDNA co-localized with MY-TOP1MT or MY-TOP1MT^{Y559F}, arrowheads: foci of MY-TOP1MT or MY-TOP1MT^{Y559F}foci negative for DNA, open arrowheads: DNA foci not co-localized with MY-TOP1MT or MY-TOP1MT^{Y559F}.

The results obtained by this staining strongly resembled the picture seen for the anti-DNA staining. The anti-TFAM staining showed no significant differences between wt HT1080 and the transfected cell lines, attesting overexpression of MY and MY-tagged Topoisomerase variants did not affect presence and composition of nucleoids (left column). MY-tagged Topoisomerase foci almost completly co-localize with TFAM signal (arrows) while some TFAM foci were negative for signal of TOP1MT variants (open arrowheads). This finding was in line with the results of anti-DNA staining reported above.

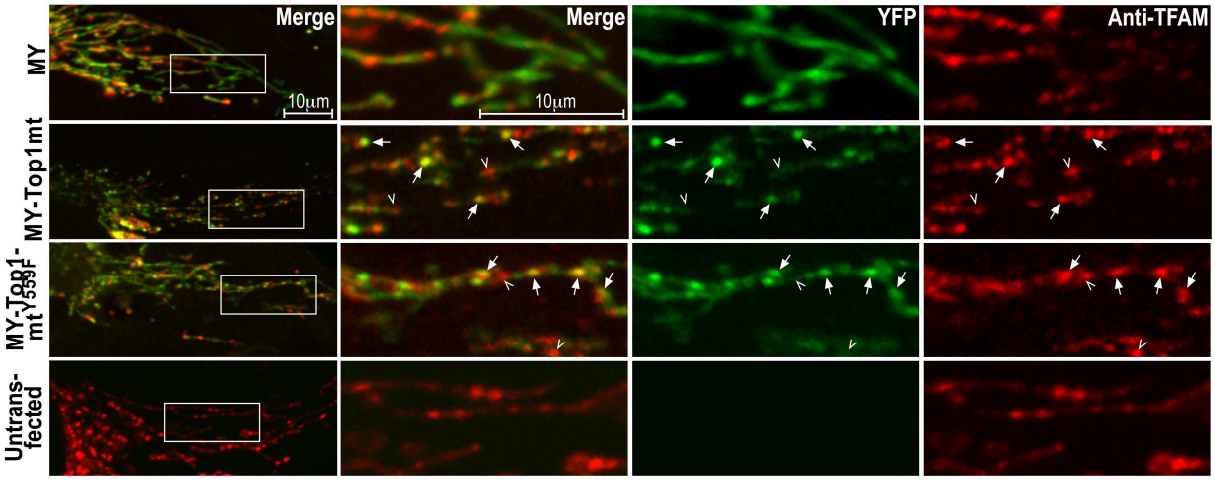

Figure 4.1.5: Co-localisation of TOP1MT and TFAM. Representative confocal microscopy images of cells expressing the denoted constructs. Cells were fixed and stained against TFAM (in red). Arrows: foci of TFAM co-localized with MY-TOP1MT or MY-TOP1MT^{Y559F}, arrowheads: foci of MY-TOP1MT or MY-TOP1MT^{Y559F}foci negative for TFAM, open arrowheads: TFAM foci not co-localized with MY-TOP1MT or MY-TOP1MT^{Y559F}.

In addition a control experiment was conducted in which wt cells were stained with antibodies against DNA and TFAM and different secondary dyes were used to distinguish both (Figure 4.1.7).

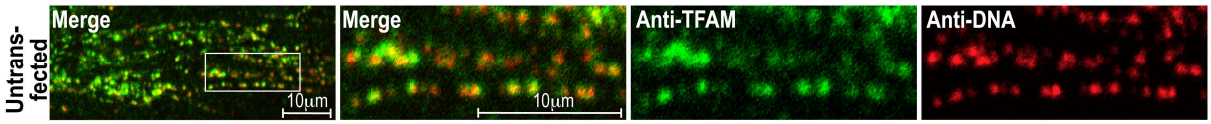

Figure 4.1.7 Co-localization of TFAM and mtDNA. Untransfected cells co-stained with antibodies against TFAM (green) and DNA (red).

In this case both signal almost perfectly co-localized (left, middle left column) thereby underlining that both antibodies bind to the same (nucleoid-) structure. We used Stimulated Emission Depletion (STED) microscopy to provide further evidence that the observed structures were actually nucleoids (Figure 4.1.8). In contrast to conventional confocal microscopy STED microscopy allows supra-optical resolution of fluorescent structures and thereby a precise determination of nucleoid size (Kurkat et al, 2011). Here we used STED to determine the true size of intra-mitochondrial foci of TOP1MT accumulation (Figure 4.1.8). Average foci sizes using STED-technology were determined 72 ± 28 nm for MY-TOP1MT and 66 ± 33 nm for MY-TOP1MT^{Y559F} overexpressing cells. These values for foci size were conforming to mammalian nucleoid size reported by other groups (Kukat *et al.* 2011).

The treatment of cultured cells with ethidium-bromide (EtBr) leads to formation of rho(0) cells characterized by total depletion of mtDNA and abnormal mitochondrial morphology (Figure 4.1.9). While EtBr treatment of MY expressing cells besides morphological changes had no influence on the even distribution of fluorescence (Figure 4.1.9 A, upper row), the treatment abolished focal accumulation of MY-TOP1MT in the according cells, indicating the requirement of an intact nucleoid structure containing mtDNA for TOP1MT attachment.

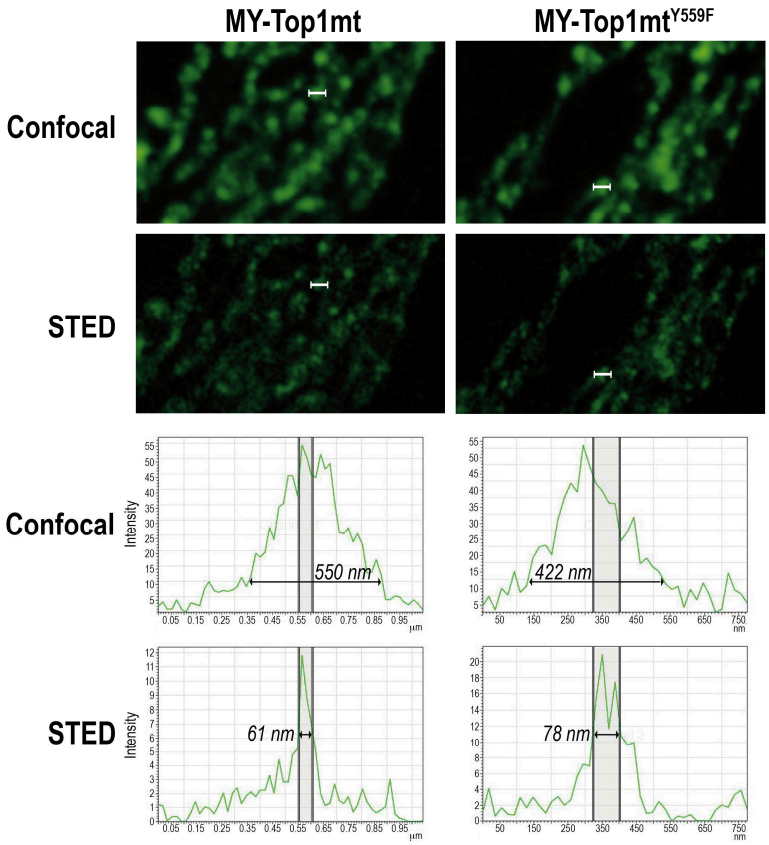

Figure 4.1.8: True size of intra-mitochondrial foci. Images of YFP fluorescence obtained by confocal microscopy (top) and STED microscopy (bottom) of cells expressing MY-TOP1MT or MY-TOP1MT^{Y559F}. Analysis at the bottom demonstrates the fluorescence intensity distribution across a single focus marked by the tracks in the fluorescent images. Estimates of the apparent size of foci in confocal (top) and STED (bottom) images are indicated by gray area. The true foci size derived from the analysis of 10 foci in various areas of the cell was 72 ± 28 and 66 ± 33 nm for foci formed by MY-TOP1MT and MY-TOP1MTY559F, respectively.

Taken together, these results demonstrated that TOP1MT variants associated with mitochondrial nucleoids. This interaction seemingly did not require, but was enhanced by catalytical activity of TOP1MT and is dependent on intact nucleoid structure.

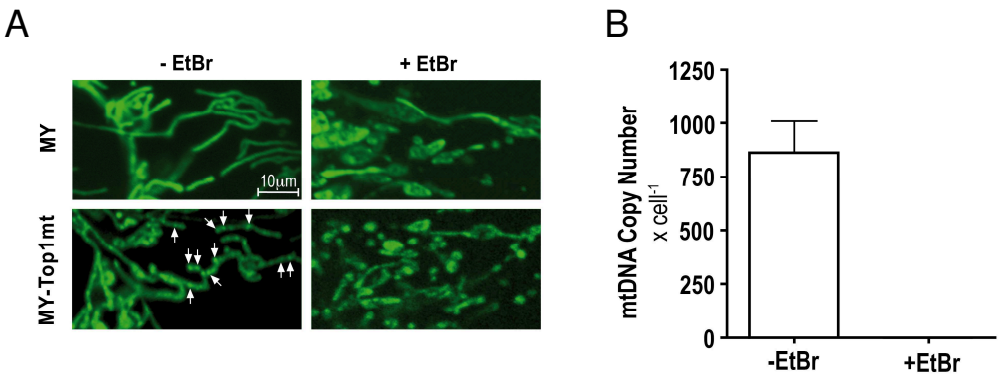

Figure 4.1.9: mtDNA-dependence of focal intra-mitochondrial MY-TOP1MT accumulation. A) Confocal images of clones expressing MY (top) or MY-TOP1MT (bottom) grown for 2 weeks in absence (left) or presence (right) of 50 ng/ml EtBr and 50 µg/ml uridine **B)** mtDNA content of the cells determined by quantitative qRT-PCR. qRT-PCR in (B) was performed by IDR.

4.1.4 TOP1MT accumulates at a subset of nucleoids containing POLRMT

The results presented above revealed that TOP1MT only associated with a certain subset of nucleoids while others were negative for TOP1MT. This implied that nucleoids differ from each other concerning their metabolic states, which means that only subsets of nucleoids undergo transcription or replication processes at the same time. To test whether the subset of nucleoids positive for MY-TOP1MT were undergoing transcription, cells expressing TOP1MT were counterstained with antibodies against POLRMT (Figure 4.1.10).

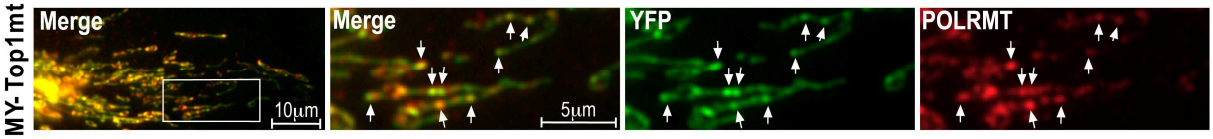

Figure 4.1.10: Co-localisation of MY-TOP1MT and POLRMT. Confocal images of YFP fluorescence in cells overexpressing TOP1MT conter-stained against POLRMT (in red). Arrows: examples of TOP1MT foci co-localized with POLRMT.

The staining revealed that TOP1MT and POLRMT signals completely co-localized (left, middle left). To confirm that such TOP1MT foci actually represented active transcription sites, cells were cultured in presence of Bromo-Uridine (BrU). BrU is a base analogue and therefore is integrated in freshly made transcripts. A specific antibody against BrU allowed staining of nascent transcripts and thereby displayed active transcription sites inside mitochondria. Microscopy analysis illustrated that all foci positive for BrU tagged RNA were coincidently positive for MY-TOP1MT (Figure

4.1.11 top, arrows). Conversely only two-thirds of MY-TOP1MT foci were positive for BrU (top, open arrowheads).

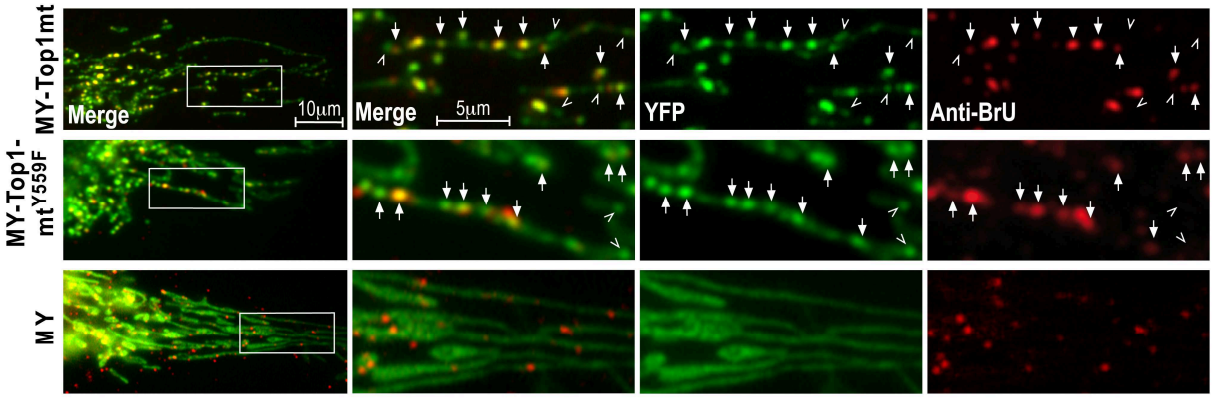

Figure 4.1.11: Co-localization of MY-TOP1MT and Bromo-uridine. Confocal images of YFP fluorescence in cells overexpressing TOP1MT conter-stained against Bru (in red). Arrows: examples of TOP1MT foci co-localized with BrU. Open arrowheads: TOP1MT foci negative for BrU fluorescence.

This suggests that TOP1MT selectively associated with nucleoids containing POLRMT. However not all of these nucleoids were actively transcribing. The same results were obtained in cell lines expressing TOP1MT^{Y559F} (Figure 4.1.11) approving catalytic activity is not an obligatory prerequisite for association with nucleoid structure. Furthermore this experiment demonstrated that TOP1MT associates with nucleoids containing POLRMT even if translation related DNA- or RNA structures are absent.

The fact that POLRMT and TOP1MT co-localize in ICC raised the question whether this is due to direct physical interaction of both proteins. This was investigated by co-immunoprecipitation (co-IP) experiments (Figure 4.1.12 A).

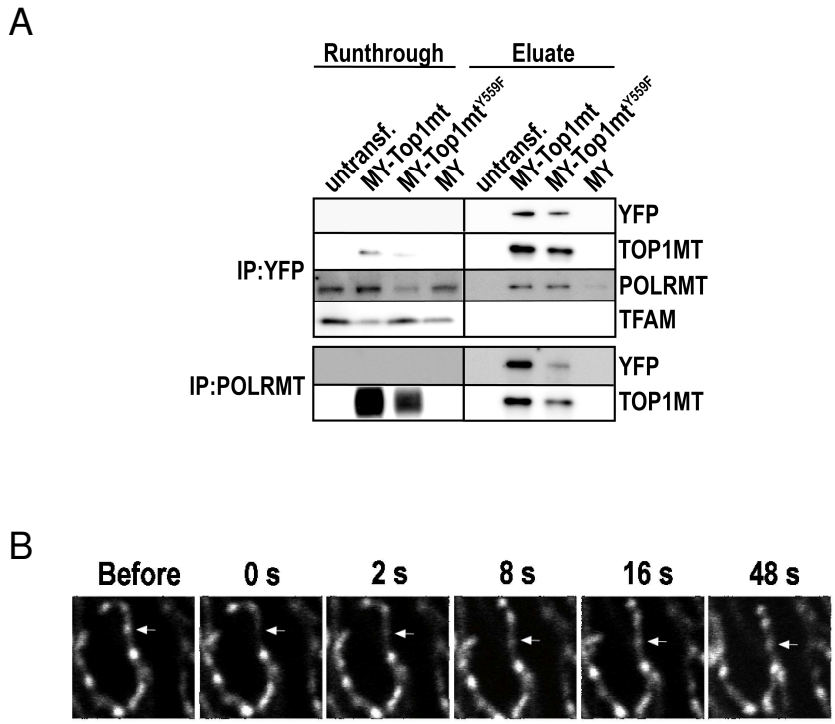

Figure 4.1.12: Interaction of TOP1MT/TOP1MT^{Y559F} with POLRMT and exchange rates of TOP1MT at nucleoids. A) Lysates of untransfected cells or cells expressing MY, MY-TOP1MT or MY-TOP1MT^{Y559F} were subjected to YFP- (top) or POLRMT-directed (bottom) immunoprecipitation. Equivalent amounts of run through (left) and immune-precipitate (right) were probed with antibodies against YFP, TOP1MT, POLRMT or TFAM as indicated on the right margin. **B)** Exchange rate of MY-TOP1MT molecules at nucleoids analysed by fluorescence recovery after photo bleaching. Images of a selected mitochondrial segment aquired before and at

In the first approach immobilised antibodies against YFP were used to immune-precipitate YFP tagged proteins and putative associated proteins (Figure 4.1.12 A IP:YFP, upper panel). The corresponding eluates were analyzed by western blot and probed with antibodies against YFP, TOP1MT, POLRMT and TFAM (as indicated on the right). The blot against YFP served as IP-control and showed bands only in cell lines expressing MY-TOP1MT and MY-TOP1MT ^{Y559F} at the expected size of about 100 kDa (right column, top box) confirming IP to be functional. The also expected band of MY migrated at 30 kDa and therefore is not visible in the compact data presentation but was observed on the corresponding membrane. Probed against TOP1MT (upper middle boxes) western blot of eluate fractions confirmed the successful precipitation of MY-TOP1MT and MY-TOP1MT^{Y559F} (right column, upper middle box). Run-through fraction displayed only weak bands, underlining effectivity of IP. In the next step the fractions were screened for co-immunoprecipitated proteins which physically interact with of TOP1MT. In contrast to TFAM (bottom row), where

protein is only detectable in the run-through fraction, POLRMT seems to co-precipitate with TOP1MT (lower middle row). Although a large amount of POLRMT is found in run-through fraction, clear bands were detected in the eluate, thus suggesting a physical interaction between TOP1MT and POLRMT. To verify this result the IP was carried out with immobilised antibodies against POLRMT (bottom panel). This setup allowed precipitating POLRMT and adherent proteins. The sustained fractions were analysed by western blot. The test revealed bands for YFP and TOP1MT at similar quantities in the eluate fraction, while only the TOP1MT antibody indicated larger amounts of TOP1MT in the run-through fraction. No bands were detected for MY expressing- and wild-type cells attesting specificity of the POLRMT – TOP1MT interaction. POLRMT co-immunoprecipitation was also observed with TOP1MT^{Y559F} and therefore was independent of TOP1MT activity. In summary co-IP experiments demonstrated a physical interaction of POLRMT and TOP1MT but not for TFAM and TOP1MT. This supported the data obtained by ICC where TOP1MT co-localized only with a subset of nucleoids containing POLRMT, but not all nucleoids.

The findings of ICC and the co-IP experiments taken together prefigure a role TOP1MT in mitochondrial transcription process. The interaction with the transcription machinery was specific as control proteins show no adhesion and seemed to be largely independent of catalytic activity as TOP1MT^{Y559F} reveals only slightly reduced accumulation at nucleoids.

There is little exchange of mtDNA between nucleoids (Gilkerson *et al.* 2008). Therefore selective association of TOP1MT with nucleoids containing POLRMT as suggested by the above data would require constant repositioning of the enzyme inside mitochondria, as individual nucleoids alter their transcriptional state. To test this prediction, MY-TOP1MT fluorescence was bleached at a selected focus and fluorescence recovery at that focus was monitored (Figure 4.1.12 B). MY-TOP1MT fluorescence at the bleached focus (arrows) mostly recovered within a minute. Since bleaching is an irreversible process, the observed recovery is due to MY- TOP1MT molecules moving in from the unbleached neighbourhood. These results indicate that TOP1MT has a sufficient mobility and exchange rate to maintain a selective association with a subset of nucleoids.

4.1.6 Overexpression of catalytically active TOP1MT reduces mitochondrial transcript and protein abundance

As hitherto obtained data (see 4.1. 3-4) strongly hint to role of TOP1MT in mitochondrial transcription, the effect of untagged enzyme overexpression on the expression of mitochondrial genes was investigated by qRT-PCR. The transcript levels of mitochondrial 12S ribosomal RNA (12S rRNA), Cytochrome C Oxidase 2 (COX2) and the nuclear encoded Succinyl Dehydrogenase Subunit B (SDHB) were measured by quantitative real-time-PCR. 12S rRNA and COX2 which is a subunit of RC-CIV represent mitochondrial encoded genes. SDHB is part of CII and representative for nuclear encoded RC proteins. Total RNA from cells overexpressing MY, TOP1MT and TOP1MT^{Y559F}, as well as from wt HT1080 was isolated and subjected to qRT-PCR (Figure 4.1.13 A).

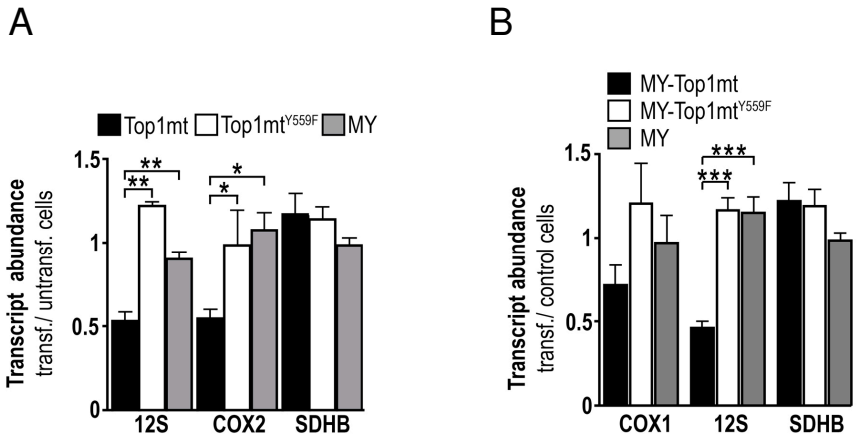

Figure 4.1.13: Impact of TOP1MT and MY-TOP1MT overexpression on mitochondrial transcription. Quantitative real-time PCR of indicated mtDNA transcripts (12S rRNA, COX2 mRNA) or nuclear encoded transcripts of mitochondrial protein (SDHB mRNA) in HT 1080 cells overexpressing in **A)** TOP1MT (black), or TOP1MT^{Y559}F (white), or mitochondrial targeted YFP serving as vector control (MY, grey); and in **B)** MY-TOP1MT (black), or MY-TOP1MT^{Y559F} (white), or mitochondrial targeted YFP serving as vector control (MY, grey); data normalized and given as mean ± SEM; n=5.

The according data uncovered the overexpression of TOP1MT causes a significant decrease of mitochondrial encoded mRNA level compared to MY and TOP1MT^{Y559F} expressing cell lines. Transcript levels of 12S rRNA and COX2 mRNA decreased about 50% compared to wt HT1080 cells while the expression of the nuclear encoded SDHB remained unchanged in TOP1MT cell lines. MY and TOP1MT^{Y559F} overexpressing cell lines show only small insignificant aberrances from wt cells for the transcript levels of 12S, COX2 and SDHB. The results illustrated that TOP1MT overexpression reduced the level of mitochondrial encoded transcripts but not

nuclear encoded transcripts. This effect was dependent of the catalytical activity since it was lacking in cell lines overexpressing TOP1MT^{Y559F}. To test whether the deranged mitochondrial transcription triggers nuclear response mechanisms by activating nuclear driven mitochondrial-biogenesis, the expression of NRF1, TFAM and the POLG were monitored by qRT-PCR (Figure 4.1.14).

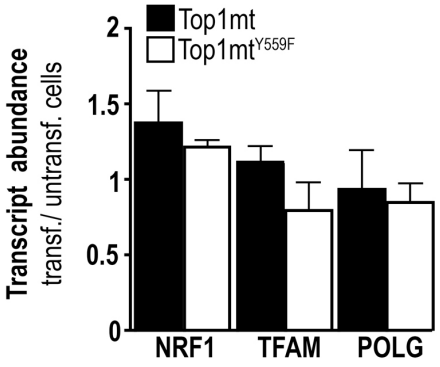

Figure 4.1.14: Impact of TOP1MT overexpression on nuclear transcription. Quantitative real-time PCR of indicated mRNA markers of nuclear mito-biogenesis program in HT 1080 cells overexpressing TOP1MT (black) or TOP1MT^{Y559F} (white); data normalized and given as mean ± SEM; n=5.

NRF1 represents a central switch for the nuclear mito-biogenesis while TFAM and POLG are downstream targets of NRF1 (Kelly and Scarpulla, 2004). Although qRT-PCR data uncovered slightly altered mRNA levels of the examined genes in TOP1MT and TOP1MT^{Y559F} cell lines, these distinctions remained insignificant compared to wt HT1080 cells. Quantitative analysis of mitochondrial protein content confirmed qRT-PCR data by detecting a decrease of mitochondrial encoded subunits of RC (Figure 4.1.14; A, B) in TOP1MT cell lines. Therefore mitochondrial extracts of wt HT1080, TOP1MT, and TOP1MT^{Y559F} cell lines were subjected to western blotting using an antibody cocktail against subunit NDUFB8 of CI, subunit 30kDa of SDHB of CII, subunit Core 1 of CIII, subunit COX II of CVI and subunit $F_{1\alpha}$ of CV for protein detection. It should be noted that these chosen subunits are only stable if the corresponding complex is correctly assembled, thus western blot only displays functional RC complexes.

In cells overexpressing TOP1MT the blots indicated reduced protein levels of CI, CIII and CIV while CII and CV are retained at levels comparable to wt HT1080 level. Seemingly assembly of CI, CII and CIV is dependent of the mitochondrial encoded subunits which represent core components of the concerned complexes. In contrast, CV core components and entire CII are devoid of mitochondrial encoded portions, reflected in unchanged quantity of these complexes in cells overexpressing

TOP1MT. As already discovered at mRNA level, the impaired formation of RC complexes is only detectable in TOP1MT cells and is lacking in cells overexpressing the active site mutant (TOP1MT^{Y559F}). TOP1MT cells showed CI, CII and CIV levels were about 2/3 less than in wt HT1080 cells and significantly lower than in TOP1MT^{Y559F} over-expressing cells (Figure 4.1.15; B). Similar results were obtained with cell lines expressing the YFP-tagged protein constructs where reduced mitochondrial transcript levels also lead to a lack of RC protein.

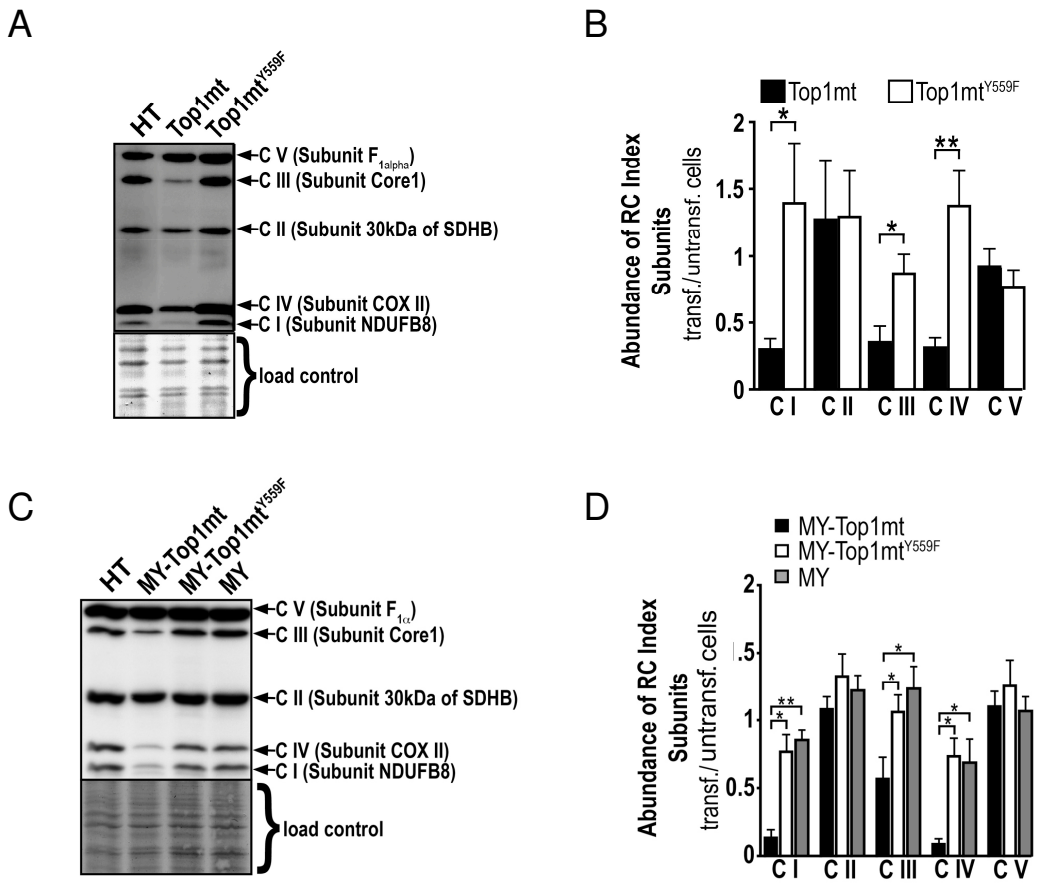

Figure 4.1.15: Impact of TOP1MT overexpression on RC-complex abundance in HT1080 cells. A) Immunoblot of index subunits of respiratory complexes (CI-CV), representative example of n=3. **B)** Abundance of index subunits determined by luminometric analysis of immunoblots as shown in (A). **C)** Immunoblot of index subunits of respiratory complexes (CI-CV) from cells expressing YFP tagged constructs and wt HT1080 cells, representative example of n=3. **D)** Abundance of index subunits determined by luminometric analysis of immunoblots as shown in (C). For quantification in (B) and (C) data in each lane were normalized to the average signal intensity within the lane and stated as mean ± SEM, n=3.

In detail 12S rRNA and COX1 mRNA declined to ~75% and 50% of wt HT1080 levels while transcript level of SDHB remained unchanged in cell lines expressing MY-

TOP1MT (Figure 4.1.13; B). The transcript levels in cells expressing MY and MY-TOP1MT^{Y559F} were significantly higher and only slightly different from levels in wt HT1080 cells. The effect of MY-TOP1MT expression also appeared on protein level where a decrease of CI to ~15%, CIII to ~50% and CIV to ~10% in relation to wt HT1080 was detected, while protein amount of CII and CV was unaffected (Figure 4.1.15; C, D). Expression of MY or MY-TOP1MT^{Y559F} apparently did not significantly influence expression of RC-Complexes, only small deviations from protein abundance in wt HT1080 cells were visible. This again emphasized that catalytic activity is crucial for the impact on mitochondrial transcription, and on the other hand ensured that MY-tagged protein was a suitable instrument to investigate TOP1MT function as the artificially tagged enzyme seemingly beard similar implications for mtDNA metabolism as the untagged version.

Additional evidence that down-regulation was exclusively due to elevated levels of active TOP1MT enzyme was obtained by a knockdown (KD) experiment (Figure 4.1.16). siRNA mediated depletion of TOP1MT in overexpressing cells resulted in reconstitution and even overcompensation (~1.4 fold compared to wt) of 12S rRNA expression level while transfection of cells with a no-target (NT) control siRNA could not rescue transcript level. The detection of SDHB transcript level revealed no changes in cells treated with siRNA or NT-siRNA compared to wt HT1080 cell line also suggestive of a specific mitochondrial mechanism of transcription manipulation.

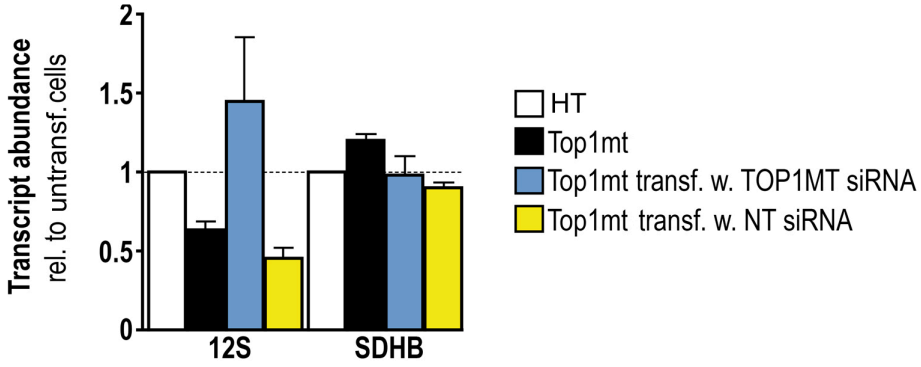

Figure 4.1.16: Reversal of TOP1MT overexpression in HT1080 by siRNA mediated TOP1MT knockdown A) Quantitative real-time PCR of indicated mitochondrial (12S rRNA, left) and nuclear (SDHB mRNA, right) transcripts in wt-cells (white), cells overexpressing TOP1MT (black), cells overexpressing TOP1MT subjected to reversal of overexpression by transfection with siRNA targeting TOP1MT or transfection with no-target control siRNA. Data was normalized and given as mean ± SEM; n=3.

The experiments presented in this article intensified the notion of an involvement of TOP1MT in mitochondrial transcription or its regulation. The overexpression studies demonstrated a negative effect on mitochondrial transcription and dependent protein synthesis emanating from an excess of catalytically active TOP1MT (or MY-TOP1MT) enzyme. Expression of mitochondrial encoded transcripts (12S rRNA) in TOP1MT cells could be restored by a siRNA mediated knockdown of TOP1MT. The expression of nuclear encoded mitochondrial protein of RC (SDHB) as well as nuclear driven mito-biogenesis (NRF1, TFAM, and POLG) was not affected by TOP1MT overexpression.

4.1.7 Overexpression of catalytically active TOP1MT impairs mitochondrial respiration

The observed decline of mitochondrial encoded transcripts and the resulting lack of RC proteins lead to an imbalance of RC complexes (Figure 4.1.15). This should also be reflected in an impaired mitochondrial respiration, thus illustrating the physiological effects of a TOP1MT overexpression. To investigate the state of respiration, oxygen consumption of TOP1MT, TOP1MT ^{Y559F} and wt HT1080 cell lines was measured under defined conditions (Figure 4.1.17). A detailed look on respiration process was achieved by selective supplementation with specific (pre-) substrates of the RC, enabling examination at different substrate entries into the RC. The results showed reduced oxygen consumption (~50%) for all investigated substrate entry points into RC in cells overexpressing TOP1MT.

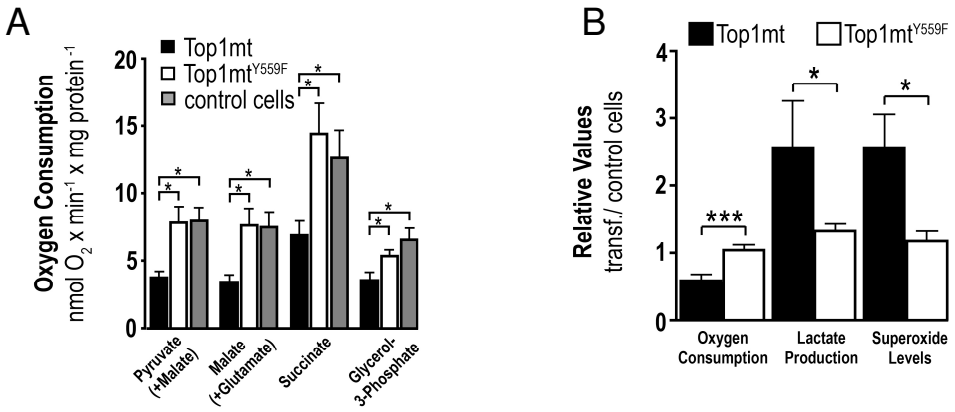

Figure 4.1.17: Respiration, Lactate and ROS production in HT1080 cells overexpressing TOP1MT. A) Oxygen consumption in control cells (grey) and cells overexpressing TOP1MT (black) or TOP1MT^{Y559F} (white) on addition of the indicated substrates; mean ± SEM, n=5-7. **B)** Oxygen consumption (endogenous substrates), lactate production and superoxide levels of cells overexpressing TOP1MT (black) or TOP1MT^{Y559F} (white); data normalized to control cells and given as mean ± SEM, n=3.

Entry via CI was detected by malate/pyruvate oxidation and malate oxidation while succinate oxidation occurs via CII. Glycerol-3-phosphate oxidation was monitored to evaluate respiration due to electron entry via mitochondrial glycerophosphate dehydrogenase (mGPDH). The determined impaired respiration process in consequence of an unbalanced expression of the RC complexes ought to be reflected in elevated ROS and enhanced lactate production. Intracellular ROS mostly arise from misguided electrons escaping the RC and thus support the formation of radicals (Murphy 2009; Jastroch *et al.* 2010). Cells overexpressing TOP1MT or TOP1MT^{Y559F} were treated with a superoxide sensitive dye which accumulates inside mitochondria (Mitosox) and analyzed by flow cytometry (Figure 4.1.17 B). While TOP1MT^{Y559F} cells did not show enhanced superoxide levels, TOP1MT cells produced about 2.5 fold more superoxide than wt HT1080 cells.

Cells tend to perform lactic acid fermentation if respiration process is disturbed (Hirschhaeuser *et al.* 2011). This can be detected by measurement of the lactate levels. The lactate levels in TOP1MT cell lines were found to be 2.5 fold higher compared to wt HT 1080 cell lines. The TOP1MT active site mutant showed unchanged levels of lactate.

The experiments presented in this chapter revealed the physiological consequences of a deregulated TOP1MT expression. The TOP1MT induced imbalance of RC complexes lead to impaired respiration an elevated superoxide and lactate production. Particularly enhanced ROS levels constitute a threat to cells by damaging proteins, lipids and most important the mtDNA. Hence TOP1MT holds a deleterious potential and therefore has to be operated and regulated carefully by cells.

4.2 Knockdown of TOP1MT leads to enhanced expression of mitochondrial encoded genes in human cells

Having figured out that overexpression of TOP1MT decreased mitochondrial transcription in a process which involved interaction with nucleoids may hint to a regulative function of TOP1MT in mitochondrial transcription. Supportive to that theory the knockdown of TOP1MT in overexpressing cell lines resulted in overcompensation of the negative impact (Figure 4.1.14). To gain more evidence for the proposed regulatory function of TOP1MT, comprehensive knockdown experiments were conducted in HT1080 cells.

Therefore the HT1080 cell line was transfected either with siRNA against TOP1MT (KD) or with a control no-target siRNA lacking any target among human mRNA (NT) to monitor implications of transfection. The knockdown with siRNA entailed a decrease of TOP1MT mRNA level of about 50% observed by qRT-PCR 48h after transfection. The decrease in transcripts was followed by an even more pronounced decline of TOP1MT protein (Figure 4.2.1).

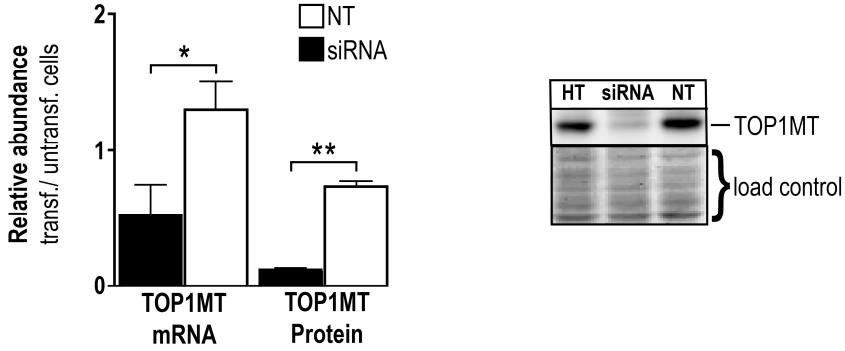

Figure 4.2.1: siRNA mediated depletion of TOP1MT mRNA and protein. A) Quantitative real time-PCR of TOP1MT-specific mRNA (left) and western blot quantification of protein (right) in HT1080 cells transfected with siRNA targeting TOP1MT (black bars) or non-targeting control siRNA (white bars); data normalized to values obtained in transfected cells are stated as mean ± SEM, n=5. **B)** Representative example of TOP1MT-directed immunoblot of untransfected cells (HT) or cells transfected with siRNA targeting TOP1MT (siRNA) or non-targeting control siRNA (NT).

Quantification of western blots using antibodies against TOP1MT attested a decline to ~10% compared to untreated cells. In contrast, in cells transfected with NT siRNA a slight increase of TOP1MT transcript level (~1.3 fold) was detected which emerged to be insignificant compared to untreated HT1080 cells. However this trend was not seen on protein level where a slight also insignificant decrease of protein amount was revealed. The differences between transcript and protein levels are most likely due to post-transcriptional events which add to decline of protein. The effect of a reduced TOP1MT expression on mitochondrial transcription was investigated by qRT-PCR monitoring mitochondrial encoded transcripts. To cover the entire size of the mitochondrial genome transcription of 12S rRNA, COX1, COX2, ND2, ND5 and ND6 mRNA was studied. Human mtDNA is about 16.5 kb whereby the chosen transcripts span: 12S rRNA ~0.6-1.6 kb, ND2 ~4.5-5.5 kb, COX1 ~5.9-7.4 kb, COX2 ~7.6-8.3 kb and ND5 ~12.3-14.1 kb of light strand. The heavy strand of mtDNA encodes only one protein coding gene which is ND6 spanning 14.1-14.7 kb. The knockdown of TOP1MT caused a significant increase in the abundance of these

transcripts (Figure 4.2.2 A) reaching from ~1.6 (ND2) to ~2.5 (COX1) fold compared to untransfected cells. The transfection with NT siRNA in some cases also slightly increased transcript levels. However, increase in all cases was insignificant compared to untransfected cells and significantly different from KD cells, thus confirming the observed effect was due to TOP1MT knockdown. This result corroborated a role of TOP1MT in the regulation of mitochondrial transcription process.

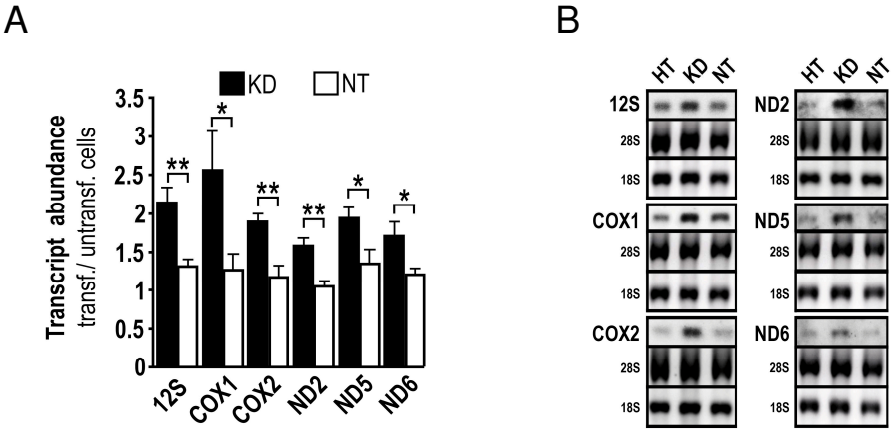

Figure 4.2.2: Impact of acute siRNA-mediated depletion of TOP1MT on mitochondrial transcript abundance. A) Quantitative RT-PCR of the indicated mitochondrial transcripts in HT1080 cells transfected with TOP1MT-directed siRNA (black bars) or no target RNA (white bars). **B)** Northern blot analysis of the indicated mitochondrial transcripts in wildtype cells (left) or cells transfected with TOP1MT-directed siRNA (middle) or no target RNA (right); 28S and 18S: loading control. A representative example of n=3 is shown.

To further exclude that enhanced transcription by TOP1MT knockdown produces aberrant transcripts by premature or defective termination of transcription northern blotting was applied. Results of northern blotting confirmed qRT-PCR data by illustrating enhanced amount of the corresponding transcript in KD cells compared to untransfected (HT) and control NT siRNA transfected cells (Figure 4.2.2 B). The observed electrophoretic mobility of transcripts was not altered and no aberrant transcripts were detected (Figure 8.2).

Next the impact of TOP1MT knockdown on nuclear genes involved in mitochondrial biogenesis was investigated. qRT-PCR illustrated transcript levels of: Peroxisome Proliferator-Activated Receptor Gamma Co-activator 1-α (PGC1A), NRF1, TFAM, POLG, Topoisomerase IIβ (TOP2B), and Topoisomerase IIIα (TOP3A) (Figure 4.2.3). PGC1A and NRF1 are nuclear transcription factors which represent two major switches of nuclear-driven mito-biogenesis and mediate the retrograde signalling.

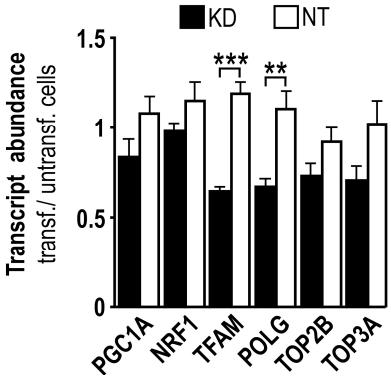

Figure 4.2.3: Impact of acute siRNA-mediated depletion of TOP1MT on nuclear transcript abundance. Quantitative RT-PCR of the indicated nuclear transcripts in HT1080 cells transfected with TOP1MT-directed siRNA (black bars) or no target RNA (white bars). All quantitative PCR data are normalized to untransfected cells and given as mean ± SEM, n=5.

TOP1MT knockdown did not significantly alter the abundance of PGC1A and NRF1 transcripts compared to untreated or NT siRNA transfected cell lines. Since only a slight decrease was found, a mito-biogenic stress response as consequence of decreased TOP1MT level can be excluded. This was also confirmed by transcript levels of TFAM and POLG which represent target genes of PGC1A and NRF1. TFAM and POLG play key roles in transcription and replication of mtDNA and are thus markers of mitochondrial biogenesis. The deficit of TOP1MT did not result in enhanced transcription of these genes, instead a significant decrease of both (to ~70% for TFAM and ~75% for POLG) was detected. This also suggests that a nuclear response in form of an up-regulation of nuclear encoded mitochondrial genes is missing. By detecting TOP2B and TOP3A transcripts in KD cells it was investigated whether these Topoisomerases may compensate for TOP1MT deficit. Compensation usually arises along with up-regulation of the involved gene. However no increase in transcript abundance could be detected for TOP2B or TOP3A, suggesting that compensation by other Topoisomerases is lacking after 48h. In contrast a small but insignificant decrease of both mRNAs was observed (to ~80% for TOP2B and ~75% for TOP3A).

In summary these data indicate that acute withdrawal leads to a global increase in mitochondrial transcript abundance without triggering a nuclear induced mito-biogenesis or an adaptive up-regulation of other Topoisomerases.

4.3 Chronic TOP1MT deficiency triggers up-regulation of mitochondrial encoded transcripts and induces nuclear driven compensatory mechanisms in MEFs

Experiments utilizing knockdown of TOP1MT in human cells revealed the main consequence is an up-regulation of mitochondrial encoded transcripts, which is not accompanied by any nuclear-driven compensatory reaction. However all analysis concerning description of KD aftermaths were carried out after 48 hours of siRNA exposure, and thus illuminating an acute phase of TOP1MT deficiency. To gain insight what happens in cells with chronic TOP1MT shortage, TOP1MT knockout mouse embryonic fibroblasts (KO-MEFs) were examined. In contrast to KD cells created by transfection, the MEF knockout model constitutes a situation without any TOP1MT activity.

The KO-MEF cell line was provided by the lab of Y. Pommier and has already been characterized (Douarre *et al.* 2012). Some of the data dealing with KO-MEFs presented in this work was produced in cooperation work by IDR of Y. Pommier's lab and is explicitly marked as her work (see Figures).

In KO-MEFs a similar pattern of enhanced expression of mitochondrial encoded transcripts as in HT 1080 KD cells was observed (Figure 4.3.1 A). The measurement of the individual transcripts of COX1, COX2, ND2, ND5 and ND6 by qRT-PCR uncovered elevated transcript levels ranging from 2 to 3 fold of wt MEFs. Integrity of the analyzed transcripts was tested by northern blot (Figure 4.2.1 B). Therefore transcripts of COX1, COX2, ND5 and ND6 of KO- and wt-MEFs were detected. The result confirmed qRT-PCR data by illustrating increased transcript levels in KO-MEFs compared to wt MEFs. No aberrant transcripts were seen on these blots (Figure 8.2) thus eliminating the possibility of premature or defective termination of transcription process. To ensure that rise in transcriptions was due to the lack of TOP1MT a re-complementation experiment was conducted. Therefore KO-MEFs were transduced with a vector containing mouse TOP1MT sequence to compensate KO. The results show that recomplementation lead to significant decline of mitochondrial encoded transcripts mostly even falling below wt MEF levels (COX2, ND2, ND5), hence attesting the changes in mitochondrial transcript abundance were dependent of TOP1MT level.

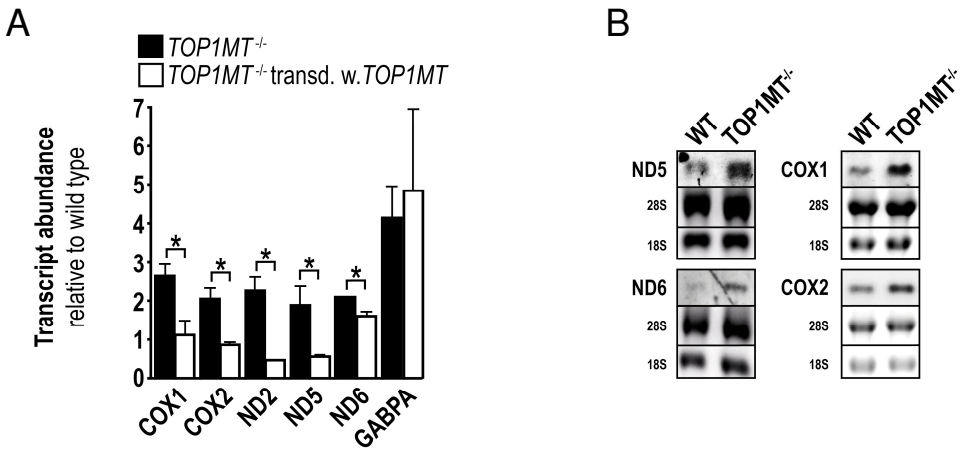

Figure 4.3.1: Impact of constitutive TOP1MT deficiency on mitochondrial transcript abundance. A) Quantitative RT-PCR of the indicated transcripts in *TOP1MT*$^{-/-}$ MEFs (black bars) optionally re-complemented with TOP1MT by retroviral transduction (white bars). **B)** Northern blot analysis of the indicated transcripts in normal MEFs (left) and *TOP1MT*$^{-/-}$ MEFs (right). 28S and 18S: loading control. A representative example of n=3 is shown. Mitochondrial transcript determination was performed by IDR, northern blots were performed by Frank Hillebrand.

It has already been shown that knockout of TOP1MT triggers a nuclear-controlled stress response assigned by a significant increase of GA-binding Protein α Chain (GABPA; also known as nuclear respiratory factor 2), TFAM and POLG expression (Douarre et al 2013). Additionally for my work GABPA level in KO-MEFs after recomplementation with TOP1MT was determined (Figure 4.3.1 A). Interestingly, in contrast to mitochondrial encoded transcripts which decline following TOP1MT supplementation, GABPA failed to normalize transcript level. This suggests mitochondrial encoded transcript level rather depends on intra-mitochondrial regulation process than on nuclear mediated regulation, which matches results obtained for KD in human cells. To exclude that recomplementation results were due to transduction effects a control transduction of KO-MEFs with particles of empty vector was conducted and transcripts of COX1 and ND2 were monitored (Figure 4.3.2). Upon transduction with empty virus no significant decline of COX1 or ND2 transcripts occurred thus ruling out the possibility of a transduction induced effect on mitochondrial transcription.

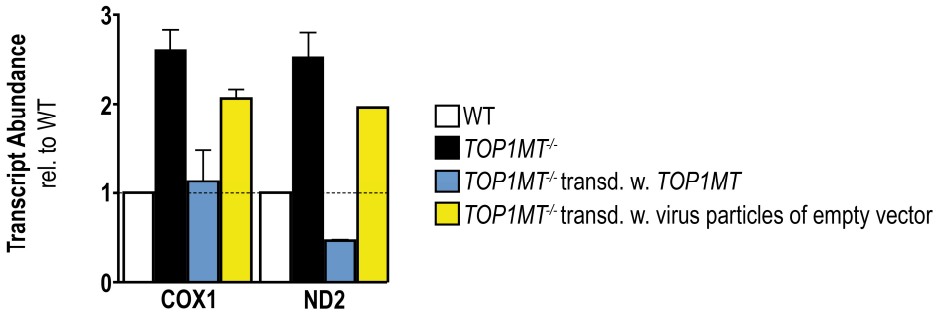

Figure 4.3.2: Vector control for retroviral recomplementation of *TOP1MT*$^{-/-}$ MEFs. Quantitative RT-PCR of COX1 mRNA (left) and ND2 mRNA (right) in normal MEFs (white bars) or *TOP1MT*$^{-/-}$ MEFs (black bars) optionally transduced with *TOP1MT* (blue bars) or empty vector (yellow bars); data normalized to values obtained in normal MEFs from *TOP1MT*$^{+/+}$littermates (dashed line) are stated as mean ± SEM, n=5, data from IDR.

Although knockdown of TOP1MT in human HT 1080 cells induced no up-regulation of other Topoisomerases to compensate the absent TOP1MT activity, the knockout in MEFs represented a different situation which may very well trigger compensation reactions. To figure out if another enzyme of the known mitochondrial targeted Topoisomerases possibly stands in for TOP1MT, transcript levels of TOP2B and TOP3A in KO-MEFs were determined by qRT-PCR (Figure 4.3.3 A).

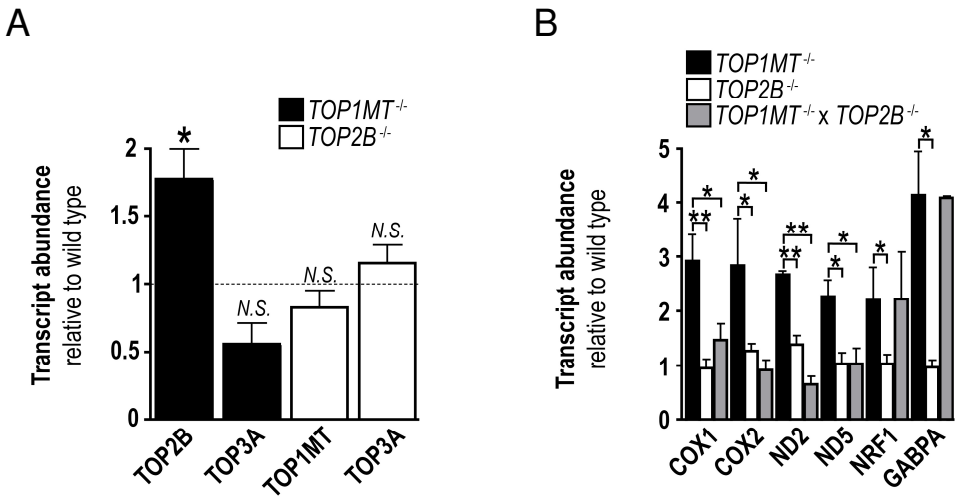

Figure 4.3.3: Impact of constitutive TOP1MT and TOP2B deficiency on nuclear transcript abundance. A) Quantitative RT-PCR of TOP1MT, TOP2B and TOP3A mRNA in TOP1$^{-/-}$ (black columns) and TOP2B$^{-/-}$ (white columns) MEFs; dotted line: levels in wild-type MEFs; asterisk, N.S.: significance versus wild-type. **B)** Quantitative RT-PCR of the indicated transcripts in TOP1MT$^{-/-}$ (black columns), TOP2B$^{-/-}$ (white columns), and TOP1MT$^{-/-}$ x TOP2B$^{-/-}$ MEFs (grey columns). All qRT-PCR data are normalized to the values in wild-type MEFs and given as mean ± SEM, n=5.

While TOP3A expression decreased and thereby fails for a compensatory function, mRNA level of TOP2B significantly increased (1.75 fold) compared to wt-MEF. This may denote that TOP2B adopted the role of TOP1MT. To investigate the correlation of mitochondrial Topoisomerases, expression level of TOP1MT and TOP3A was examined in TOP2B knockout MEFs. The used TOP2B-KO-MEFs were established and described by Y.L. Lyu (Lyu et al, 2006). qRT-PCR analysis revealed only slight changes of transcript level for both Topoisomerases. While TOP1MT mRNA level declines, TOP3A rises, though the observed changes were insignificant compared to wt-MEFs. The results suggested that TOP3A can compensate neither TOP1MT nor TOP2B deficiency as transcript levels remain nearly unchanged in both cases. Furthermore TOP2B up-regulation might compensate lack of TOP1MT action in mitochondria but otherwise TOP1MT transcript levels were not affected in TOP2B knockout cells, indicating inability of TOP1MT to substitute TOP2B.

Another important and promising tool to gain insight in role allocation of the mitochondrial Topoisomerases in transcription is the investigation of double knockout cell lines. Therefore transcript levels of COX1, COX2, ND2, ND5, NRF1 and GABPA in a TOP1MT/TOP2B double knockout MEF cell line were measured (Figure 4.3.3). Additionally the transcript levels in TOP1MT KO-MEFs and TOP2B KO-MEFs were measured to allow comparisons. While the effect of a TOP1MT knockout has already been described in Figure 4.3.1, mitochondrial encoded transcript abundance was seemingly not affected in TOP2B knockout cells (white columns). Despite minor oscillations no significant changes in expression were detectable. Also marker genes of nuclear biogenesis (NRF1 and GABPA) remained unaffected by TOP2B knockdown. This suggests TOP2B had no or only a subordinated role, but surely is dispensable in mitochondrial transcription process. The most revealing data were obtained from the TOP1MT/TOP2B double knockout cell line. Though the deficiency of TOP1MT, a raise of mitochondrial encoded transcript level was abolished. Transcript levels oscillated but a significant difference to wt MEFs was lacking. However unlike in TOP2B knockout MEF, the double knockout MEFs showed elevated abundance of NRF1 and GAPBA transcripts. The values for both transcripts were strikingly similar to those obtained in TOP1MT KO-MEFs (compare black and gray columns). This may well reflect a general nuclear reaction when confronted with a deficit of TOP1MT protein, independent of additional shortage of TOP2B. Most interesting, the double knockout MEFs revealed that neither TOP1MT nor TOP2B

were essential for mitochondrial baseline transcription. Mitochondrial encoded transcript abundance was not significantly different from wt-MEFs. The unaltered transcript levels of mitochondrial transcripts in TOP1MT/TOP2B KO-MEFs compared to TOP1MT KO-MEFs additionally may hint to role of TOP2B in mitochondrial DNA metabolism. However, in the TOP2B KO model it was impossible to distinguish direct effects on mitochondrial transcription from secondary effects induced by changes of in the regulation of nuclear encoded mitochondrial genes, as TOP2B is suggested to exert activity in both compartments.

4.4 Modulation of TOP1MT activity by oxidation/reduction

Nuclear Topoisomerase 1 (TOP1) and plant TOP1MT activity is known to be altered by redox status of the enzyme. TOP1 contains certain conserved cystein residues, which are prone to reduction/oxidation processes (Konstantinov *et al.* 2001; Tarasenko *et al.* 2008). By redox induced alterations TOP1 can adopt active and inactive conformations (Montaudon *et al.* 2007). These cystein residues are largely conserved in TOP1MT (see alignment). To investigate TOP1MT redox sensitivity and dependent catalytic activity, isolated mitochondria and mitochondrial extracts were treated with agents modulating redox conditions. In a first set of experiments mitochondrial extracts of cells overexpressing TOP1MT were treated with oxidized glutathione (GSSG) which can modify cystein by oxidation (Figure 4.4.1), or dithiothreitol (DTT) a reducing agent which prevents oxidation of cystein residues.

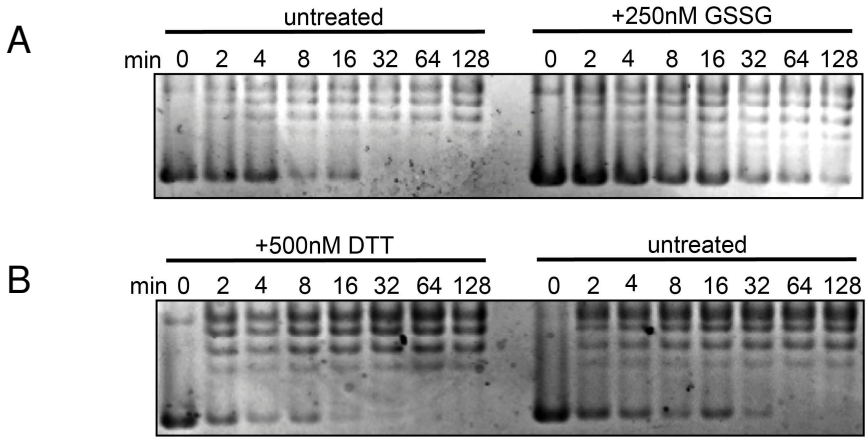

Figure 4.4.1: Redox-dependency of TOP1MT activity. A) Untreated (left) and 250 mM GSSG (right) treated mitochondrial extracts of cells overexpressing TOP1MT incubated with a supercoiled pUC18 DNA substrate. **B)** Mitochondrial extracts of cells overexpressing TOP1MT incubated with pUC 18 substrate DNA for the indicated time periods. Extracts were treated with 500mM DTT (left) or untreated (right).

Subsequently a supercoiled DNA substrate was incubated with the extract to determine TOP1MT mediated relaxation activity. The relaxation assay revealed that TOP1MT activity can be manipulated by redox conditions. Incubation of mitochondrial extracts with GSSG resulted in strongly decreased TOP1MT activity. While an oxidizing environment generated by GSSG impairs TOP1MT activity, the addition of reducing agent DTT enhances relaxation activity of TOP1MT. This basically proved that TOP1MT activity depends on surrounding redox conditions. To investigate physiological relevance of this finding extracted mitochondria were incubated with antimycin A (AntA). AntA inhibits electron transport by the RC by impeding electron transfer from CIII to cytochrome C. Thus AntA treatment leads to increasing mitochondrial ROS production by RC (Park et al, 2007). ROS represent an oxidizing agent which shifts mitochondrial redox state to a more oxidizing environment.

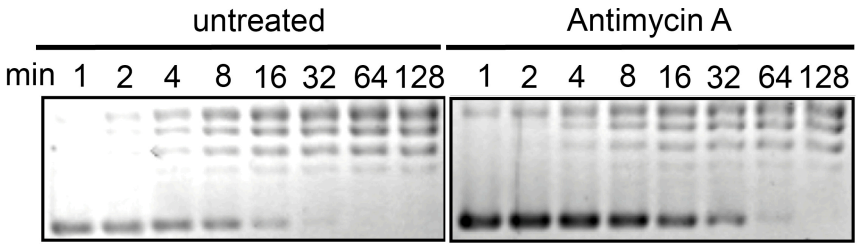

Figure 4.4.2: Antimycin A mediated modulation of TOP1MT activity. A) *In organello* effect of antinmycin A on TOP1MT activity. Untreated (left) and antimycin A (50 µM, 30 min; right) treated mitochondria of cells overexpressing TOP1MT incubated with a supercoiled pUC18 DNA substrate. Mitochondria were additionally supplemented with substrates of RC (Malate, Glutamate, ADP) to stimulate electron transport.

The *in organello* treatment with AntA resulted in a decreased activity of TOP1MT (Figure 4.4.2). This confirmed the hypothesis of TOP1MT inhibition by oxidation and displayed that RC derived oxidants also are able to modulate TOP1MT activity. The data contributed to the emerging model of mitochondrial transcription regulation by TOP1MT and provides evidence for a redox-state dependent regulatory mechanism.

5. Discussion

Human mtDNA is a closed, double-stranded DNA circle (Iborra *et al.* 2004; Lenaz and Genova 2010). Strand separation during mtDNA transcription and replication creates topological stress that interferes with these processes unless timely released. The only enzymes capable of performing this task are Topoisomerases (Wang 2002). Currently there is conclusive evidence for the existence of two enzymes catalyzing the transient cleavage and ligation of single stranded DNA (mitochondrial Topoisomerases I and IIIα) in the mitochondrial compartment. Additionally the existence of a Topoisomerase catalyzing cleavage and ligation of both strands of the DNA double helix (mitochondrial Topoisomerase IIβ) is suggested by experimental data (Low *et al.* 2003). Final evidence is still missing though. So far, very little is known about the role of these enzymes in mtDNA metabolism and maintenance. However, it appears that their functions differ markedly from those of the corresponding enzymes in the cell nucleus. This is mostly entailed by the differences between nuclear and mitochondrial DNA metabolism. In contrast to the nucleus, mitochondria are organelles with an enzymatic configuration originating from multiple ancestors. While most proteins involved in mitochondrial DNA metabolism seem to stem from bacterial ancestors (Karlberg *et al.* 2000; Marcotte *et al.* 2000) other components (e.g. POLG) were seemingly inherited from bacteriophages (Shutt and Gray 2006). Moreover the mitochondrial matrix represents an environment markedly different from nucleus. This is reflected *inter alia* in differences of pH, redox status and organization of the genome (Gray 2012).

Mitochondrial Topoisomerase I (TOP1MT) is the only known mitochondrial Topoisomerase encoded by a separate (nuclear) gene. Splitting of the TOP1 gene into nuclear and mitochondrial paralogs is highly conserved in vertebrates (Zhang *et al.* 2001; Zhang *et al.* 2004; Zhang *et al.* 2007) and reflects a functional specialisation: Nuclear TOP1 is incompatible with mtDNA transcription/replication, while TOP1MT is unable to interact with nuclear chromosomes (Dalla Rosa *et al.* 2009).

TOP1MT catalyses the transient cleavage and ligation of one strand of the DNA double helix and thereby provides the major activity for relaxation of mtDNA supercoils. An essential role of TOP1MT in mtDNA maintenance seems unlikely,

since *TOP1MT*$^{-/-}$ mice express mtDNA-encoded proteins (Douarre *et al.* 2012). In the cell nucleus, TOP1 is an essential cofactor promoting rRNA- and mRNA transcription (Zhang *et al.* 1988; Kretzschmar *et al.* 1993; Christensen *et al.* 2002).

My work mainly investigated the function of human TOP1MT. The specific role of this enzyme in mitochondrial metabolism is still unknown. Research on TOP1MT is ongoing since its discovery in 2001 (Zhang *et al.* 2001). This work studies effects of a deregulated overexpression and a knockdown/knockout of TOP1MT. The results suggest a role in transcription or rather transcription regulation for TOP1MT. Most notably results hint to the existence of a hitherto uncovered regulatory system driven by TOP1MT activity. The proposed mechanism allows direct adaption of mitochondrial transcription to mitochondrial redox state.

5.1 TOP1MT contributes to mitochondrial transcription

The deregulated expression of YFP-tagged TOP1MT variants and the employment of fluorescence microscopy represent a valuable tool to reveal TOP1MT distribution, mobility and decisive factors thereof. The YFP-tag was also used to isolate TOP1MT and interaction partners. The following three chapters address results and critical properties of experiments utilizing TOP1MT overexpression models.

5.1.1 Deregulated expression of TOP1MT variants

A general problem of transfections is that vector DNA integrates randomly into the genomic DNA of the host cell. It is also unpredictable how many copies of a vector are taken up by a cell. Among others, these two parameters strongly influence the expression rate of the heterologous DNA as well as host cell integrity. For a comparative study it is desirable to work with cell lines expressing similar amounts of the respective heterologous protein. To ensure this, HT 1080 cells overexpressing TOP1MT variants were screened for their basic properties.

A general measure for cell integrity is overall morphology and generation rate. These properties were not different from wt-cells in transfected cell lines. Cell lines used in this work showed roughly similar RNA expression levels for transfected genes ranging from a minimum of ~5 fold to a maximum of ~12 fold overexpression

compared to wt-cells. The integrity of expressed protein was confirmed by western blotting and activity assays.

No significant differences between cell lines expressing YFP-tagged and untagged TOP1MT were detected. Although the fusion to YFP likely influences TOP1MT properties somehow, all conducted experiments rule out that YFP-tagging of the protein severely alters protein function. Particularly similar results in relaxation assays (4.1.1) show that catalytic activity is comparable and *in vivo* similar effects on mitochondrial transcription (4.1.4) due to enzymatic activity underline comparability of these models.

5.1.2 TOP1MT associates with transcriptionally active nucleoids

Fluorescence microscopy uncovered a certain pattern of TOP1MT distribution inside the mitochondria, which resembled already observed patterns of nucleoids (Garrido *et al.* 2003). The co-localization with nucleoids was confirmed by immunocytochemisty staining against DNA and TFAM. This was in accordance with published results (Garrido *et al.* 2003; Bogenhagen *et al.* 2008; Zhang and Pommier 2008). Strikingly not all nucleoids were positive for TOP1MT suggesting that individual nucleoids must differ somehow. An obvious reason is that nucleoids can differ in their metabolic state. That would entail altered enzyme compositions due to temporary requirements. This hypothesis was proofed by showing virtually perfect co-localization of TOP1MT with POLRMT. This result was verified by co-immunoprecipitation experiments which indicate a direct physical interaction of TOP1MT and POLRMT. Moreover all nucleoids with ongoing transcription were shown to be positive for TOP1MT. Interestingly IHC in this case showed more TOP1MT positive foci than transcriptionally active foci. Therefore it can be stated that TOP1MT is always involved at nucleoids performing transcription (2/3 of TOP1MT foci). Additionally TOP1MT and TFAM associate with a subset of nucleoids not undergoing transcription (1/3 of TOP1MT foci). It can be speculated why the latter 1/3 of TOP1MT is linked to transcriptionally inactive nucleoids. Maybe in the course of transcription initiation these nucleoids already had recruited the components required for imminent transcription at the moment of detection. However since transcription in mitochondria is not synchronized (Bogenhagen and Clayton 1977) and transcription initiation is rapid process it seems unlikely that all foci represent transcription initiation sites. On the other hand this distribution could be due to a regulatory

function of TOP1MT in mitochondrial transcription, which is discussed in detail in the following chapters. A negative regulatory effect would likely involve an interaction between TOP1MT and nucleoids and lead to inhibition of transcription instead of initiation. In this context it would be desirable to investigate TOP1MT distribution and transcription sites at different redox states of the mitochondrial matrix. Additionally the exact function of the interaction between TOP1MT and POLRMT with a view to regulatory function in transcription should be further investigated.

In summary this work presents for the first time evidence that TOP1MT associates with transcriptionally active nucleoids and therefore likely plays a role in mitochondrial transcription. The physical interaction with POLRMT and co-localization with nascent RNA may hint to a direct participation in transcription process, while distribution data also support the idea of extended function of TOP1MT in regulation of transcription.

5.2 TOP1MT overexpression and mitochondrial transcription

This work presents data indicating a negative impact of TOP1MT overexpression on mitochondrial transcription. The implication on transcription is dependent on the catalytic activity of TOP1MT and occurred independent of the YFP-tag.

5.2.1 Transcript and Protein Abundance

The effect of chronic TOP1MT excess was studied in cell lines overexpressing TOP1MT, the active site mutant TOP1MT^{Y559F} or YFP-tagged versions of both proteins. Thereby it was possible to assess the consequences of increased catalytic activity and the YFP-tag on TOP1MT mediated mitochondrial metabolism. While the adherence of the YFP-tag had no obvious effect, catalytic activity was crucial for impeding mitochondrial transcription. The monitored mitochondrial transcripts, 12S rRNA and COXI represent a global (12S) and a RC-specific (COXI) marker of intra-mitochondrial biogenesis. 12S rRNA is essential for mitochondrial protein synthesis (Smits *et al.* 2010). Thus a decrease of 12S coincidently results in a global decline of mitochondrial encoded protein. An additional advantage is that transcription from both HSPs is illustrated by 12S transcripts. This allows a general view on HSP

induced transcription which encompasses ~74% of total human mitochondrial transcripts (tRNA, rRNA and protein). The measurement of COX1 mRNA allows a view on HSP2 dependent transcription which encompasses 12 of the 13 mitochondrial encoded proteins. The decrease in 12S and COX1 transcript abundance upon TOP1MT excess showed that transcription likely is dampened by general regulation. Moreover it can be assumed that TOP1MT dependent lowering of transcript abundance is a self-contained mitochondrial process without nuclear participation. Although it could be supposed that the consequences of TOP1MT overexpression provoke a nuclear regulation of mitochondrial biogenesis, evidence therefore was missing. Almost unchanged expression level of NRF1, TFAM, POLG and SDHB underlined that finding. This is striking in the context of the physiological consequences of impaired mitochondrial transcription discussed in the following chapter. A knockdown of TOP1MT in overexpressing cells restored transcript level of monitored 12S. Thereby it was shown that decreased transcription is truly due to TOP1MT overexpression.

5.2.2 Respiration, superoxide and lactate

As expected the lack of mitochondrial encoded RC transcripts was reflected in a decrease of RC complexes. Among the RC, CI, III and IV depend on mitochondrial encoded proteins as they are central components of these complexes (Lenaz and Genova 2010; Hornig-Do *et al.* 2012). CV also contains mitochondrial encoded protein constituting a part of the stator stalk of ATP synthase (Fearnley and Walker 1986). A lack of mitochondrial protein did not influence the abundance of CV. Hence, this auxiliary structure seemingly has low impact on cohesion and thereby abundance of CV. However it remains doubtable whether the ATP synthase is operable in this incomplete state. The second complex with unaltered abundance was CII because it entirely consists of nuclear encoded components (Rutter *et al.* 2010), whereby its assembly is independent of mtDNA-encoded protein. These findings are in line with the results of transcript determination experiments discussed above. Furthermore the noticed decrease of mitochondrial dependent RC complexes links decreased transcription to impaired mitochondrial function.

In this context an important point was to determine impact of TOP1MT overexpression on mitochondrial respiration. The observed imbalance of RC complexes resulted in a disruption of respiratory process as described previously

(Bonawitz *et al.* 2006). Besides an obvious decrease in overall oxygen consumption, respiratory impairment was also determined in detail. The results showed reduced oxygen consumption for all investigated substrate entry points into RC. Malate/pyruvate oxidation and malate oxidation represent substrate entry via CI while succinate oxidation occurs via CII. Glycerol-3-phosphate oxidation was monitored to evaluate respiration due to electron entry via mitochondrial glycerophosphate dehydrogenase (mGPDH). Independent of entry point to RC in all cases oxygen consumption decreased to ~50% of wt cells. This was corresponding to expectations as all RC components contributing to proton translocation (CI, CIII, CIV) are affected by a lack of mitochondrial protein. Due to this grave impairment of RC no substrate dependent variances of respiration rates were detected.

A defective RC is mostly related to a shift energy metabolism towards more lactic acid fermentation. This was confirmed by measurement of lactate concentration in TOP1MT overexpressing cells. Due to raised pyruvate levels lactate concentration was significantly increased in cells with impaired respiration. A second hallmark of a defective RC is an increased generation of ROS e.g. in the form of superoxide (Turrens 2003). In cells overexpressing TOP1MT a strong increase of superoxide levels was detected. Superoxide is a direct product of the impaired respiration. It is produced by mislead electrons untimely exiting from RC thereby reducing oxygen thus confirming the severe impairment of RC. ROS is major signalling molecule of mitochondria. Among others it is also involved in signalling pathways inducing mitochondrial biogenesis via NRF1 (Bruni *et al.* 2010). Interestingly an up-regulation of NRF1 and downstream targets (TFAM, POLG) was not observed in cells overexpressing TOP1MT. The reasons for this lack of nuclear answer remain unclear. It could be assumed the ROS level did not reach concentrations sufficient to trigger signal transduction.

5.3 Acute TOP1MT deficiency

A short term TOP1MT deficiency in HT1080 cells was established by siRNA mediated knockdown (KD) of TOP1MT. The KD resulted in an average decrease of 50% on transcript- and about 80% on protein level after 48h. The TOP1MT deficit was accompanied by a significant increase of mitochondrial encoded protein thus

supporting the hypothesis of a regulatory function of TOP1MT in mitochondrial transcription.

5.3.1 Knockdown of TOP1MT increases mitochondrial transcript abundance

To ensure correct assessment of KD data it was necessary to monitor more transcripts than for overexpression experiments. To determine down-regulation of mitochondrial transcription in principle one measured transcript is sufficient to display the decrease of the polycistronic transcript. In contrast the proof of an up-regulation of transcription requires evidence that transcription process yields a complete transcript. Furthermore mitochondrial DNA is dependent on transcription from the two discrete strands of mtDNA for conversion of the complete genetic information. That means besides transcript integrity, transcription from both promoters had to be monitored. The transcripts for monitoring up-regulation therefore encompassed genes located near start point HSP1 (12S), or the middle of the polycistronic transcript (ND2, COX1 and COX2), and in proximity to the termination site (ND5). Transcription from LSP was determined by measurement of the only protein coding transcript (ND6) of this strand. Moreover transcript integrity was assessed by northern blotting of the mentioned transcripts. This experimental setup provided the chance to review under the significant aspects.

Notably the obtained data gave reasonable evidence for the supposed regulatory function in transcription process by TOP1MT. The finding that deficiency of TOP1MT enhances mitochondrial transcription well fits the model initiated from overexpression experiments. Increased TOP1MT activity has negative effect on transcription while lowered activity boosts transcription. The most important finding is that TOP1MT works in both directions and hence bears the potential to be regulating component of transcription. The mechanistic principle of this regulation system is discussed in 5.6.

5.3.2 Nuclear response

For several reasons it was important to investigate nuclear driven mitochondrial biogenesis in the KD model. First, it had to be excluded that KD affects nuclear metabolism and the observed effect of mitochondrial transcript increase therefore is only a secondary effect. On the other hand up-regulation of nuclear encoded mitochondrial transcripts is a plausible event if mitochondrial-intern regulation

processes stimulates transcription. If mitochondria possess an independent regulation mechanism for transcription, a connection to the nucleus seems likely to avoid unbalanced amounts of mitochondrial and nuclear encoded protein. This signaling to the nucleus could be directly dependent on mitochondrial transcription process. Alternatively signaling might base on factors, which indicate overall status of the mitochondrion. This way of retrograde signaling dependent on general mitochondrial status is a well established model (Finley and Haigis 2009). It operates mostly by sensing levels of important mitochondrial metabolites like AMP/ADP/ATP, ROS or Ca^{2+} in the cytosol (Finley and Haigis 2009). This foregoes a more complex direct signaling, facilitating special messengers more prone to dysfunction. Monitoring PGC1A and NRF1 transcripts showed no induction of a nuclear driven mitochondrial biogenesis. As already mentioned NRF1 is a central transcription factor involved in control of many nuclear encoded mitochondrial components encompassing RC protein of all five complexes and central enzymes of transcription (Kelly and Scarpulla 2004). PGC1A is a master switch of energy metabolism. It is a transcriptional co-activator tightly connected to AMP-activated protein kinase (AMPK) the major sensor of cellular energetic status (Jager *et al.* 2007). Besides many other genes, NRF1 is a target gene of PGC1A (Joseph *et al.* 2006). Thereby PGC1A mediates retrograde signaling to the nucleus and controls mitochondrial biogenesis. Up-regulation of these genes is a hallmark of nuclear-driven mitochondrial biogenesis (Wenz 2013) which thus seems unaffected by TOP1MT KD and related induction of mitochondrial transcription. Direct nuclear transcription factors of mitochondrial genes (NRF1) as well as up-stream co-activators (PGC1A) transmitting from sensors of mitochondrial status (AMPK) were unaffected. A first glance this result may appear as a contrast to published results obtained from knockout MEFs (KO-MEFs) (Douarre *et al.* 2012). In KO-MEFs a pronounced nuclear response to TOP1MT deficit was characterized. These different effects of KD compared to KO are discussed in detail in 5.4.2. Surprisingly transcripts of downstream targets TFAM and POLG were significantly decreased in TOP1MT KD cells compared to NT transfected cells. This could be due to general effects of transfection process or may hint to a nuclear response not involving NRF1 or PGC1A. However both explanations are speculative, analysis of more transcripts (TFAM, POLG) could perhaps uncover the underlying mechanism. However, most important the reduced levels of TFAM and POLG rather represent a nuclear compensatory answer to increased mitochondrial transcription

than a reason thereof. In sum these results provide more evidence for an intra-mitochondrial regulation mechanism involving TOP1MT activity, independent of the nucleus.

In a third set of transcripts the other mitochondrial Topoisomerases (TOP2B/TOP3A) were monitored to detect a putative complementation mechanism triggered by TOP1MT deficiency. Although TOP1MT activity is reduced in KD cells a compensatory up-regulation of the alternative mitochondrial Topoisomerases was not detected. Instead a slight but insignificant decrease of transcript abundance for TOP2B and TOP3A was discovered. Thus it seems unlikely that other Topoisomerases are recruited to restore missing TOP1MT activity. This indicates a unique role of TOP1MT in mitochondrial metabolism.

It should be noted that all discussed results concerning KD illustrate the state of an acute withdrawal of TOP1MT not fully comparable to chronic deficiency produced by a knockout. This issue is further discussed in the following chapter.

5.4 Chronic deficiency of TOP1MT

Studying TOP1MT knockout MEFs opened a new perspective on cells afflicted by TOP1MT deficiency. Acute TOP1MT shortage consequences were already monitored in human KD cells. KO-MEFs constitute the different situation of a chronic deficit of TOP1MT. While a transfection with siRNA displays a transient state, suffering from inhomogeneous state of cells, in viral transduced KO all cells show a chronic and complete lack of TOP1MT. Therefore the KO system is more homogenous and also might reflect the KO mouse's properties to some extend.

5.4.1 TOP1MT Knockout effect on mitochondrial transcription

Measurement of mitochondrial encoded transcripts of RC components in TOP1MT KO-MEFs revealed similar results to KD in human cells. The chronic TOP1MT deficiency in MEFs results in an increase of all measured mitochondrial encoded transcripts. Also similar to human cells is the quantity of the observed increase ranging from two- to three-fold relative to wild-type cells. This suggests a similar TOP1MT mediated mechanism of transcription up-regulation underlying in both cases. The result also clarifies that the effect of a TOP1MT deficit apparently cannot

be compensated, even in cells suffering from permanent withdrawal. Remarkably, upon transduction of TOP1MT the levels of mitochondrial encoded transcripts in KO-MEFs normalized. This again suggests a regulation system exclusively controlled by TOP1MT activity.

5.4.2 Nuclear response

Experiments by Douarre and co-workers already described the consequences of a TOP1MT KO on the nuclear part of mitochondrial biogenesis (Douarre *et al.* 2012). The quantification of TFAM, PGC-1, NRF-1 and POLG transcripts revealed a significant increase compared to wt MEFs. My work additionally presents transcript levels of GABPA (also named NRF2) in KO-MEFS and KO-MEFs re-complemented by TOP1MT transduction. GAPBA, similar to NRF1, is a transcription factor controlling components of nuclear-driven mitochondrial biogenesis. Target genes of GABPA are for example: mTERF, POLRMT, TWINKLE, mtSSB, and Pol-γB (Bruni *et al.* 2010). Enhanced expression of GABPA was shown to up-regulate the expression of target genes (Wenz 2013). This work revealed increased expression level of GABPA which is in line with the published data (Douarre *et al.* 2012). Remarkable is the finding that GABPA transcript levels, unlike mitochondrial encoded transcripts, failed to normalize after recomplementation. This may add evidence for the proposed theory of a mitochondrial transcription regulation mechanism independent of the nucleus.

Notably the results concerning nuclear controlled mito-biogenesis of KO-MEFs are seemingly contrary to the data obtained from KD in HT 1080 cells. In cells treated with siRNA no enhanced transcription was detected, instead TFAM and POLG transcript levels significantly decreased. However this contradiction may simply reflect the different cellular status in chronic or acute TOP1MT deficiency. Moreover in a KO system TOP1MT is totally depleted while the used KD system still possessed residual TOP1MT (~20%). These facts illustrate that a comparison of the two systems is problematical. Despite differences in nuclear regulation the major effects of TOP1MT on mitochondrial transcription are identical in both systems. Some points may indicate that KD represents the more physiological system better suitable for studies of regulatory mechanism. This view is based on the fact that it is likely that a fast physiological regulation mechanism is primary dependent on TOP1MT activity and only secondary on enzyme abundance. Changes in TOP1MT activity are

presumably rapid, temporary processes, whereby the acute deficiency of TOP1MT KD should be a better model for studies of regulation process. In this context the KD model helps to prevent the formation of compensatory reactions observed in chronic TOP1MT deficiency. Furthermore in a physiological system a residual TOP1MT activity is assumable despite its down-regulation. Taken together seemingly the KD simulates a naturally occurring decrease of TOP1MT activity while the KO simulates a more pathogenic cell status lacking any activity. However KO model presents a valuable tool appropriate for more studies concerning compensation reactions and roles of the different mitochondrial Topoisomerases, as discussed in the following chapter.

5.5 TOP2B deficient and TOP1MT/TOP2B double deficient MEFs

To further determine the involvement of the three mitochondrial Topoisomerases the KO studies were extended. Therefore mitochondrial and nuclear transcript abundance and Topoisomerase expression was detected in a TOP2B KO MEF cell line. The additional investigated TOP1MT/TOP2A double knockout MEFs showed that TOP3A alone is able to maintain mitochondrial transcription. Thus a role for TOP1MT in transcription process may only encompass the proposed regulatory function.

5.5.1 Compensation of TOP1MT deficiency

Interestingly in TOP1MT KO-MEFs an Increase of TOP2B transcripts was observed while TOP3A transcripts even decreased. This could hint to a compensatory mechanism in which TOP2B adopts TOP1MT function to some extent or a general involvement of TOP2B in mitochondrial transcription. To investigate if the two Topoisomerases can substitute for each other TOP1MT expression was determined in TOP2B knockout MEFs. The result showed no significant differences of TOP1MT expression compared to wt MEFs. TOP3A the third mitochondrial Topoisomerase was not significantly affected by TOP2B KO as well. This ruled out the possibility of a substitution of TOP1MT and TOP2B function in the case of a deficiency. Generally it is difficult to assign the effect of increased transcript abundance of TOP2B in TOP1MT KO cells. Since no definitive mitochondrial isoform of TOP2B is known, a specific differentiation between nuclear and putative mitochondrial forms of TOP2B is

impossible. Therefore all analysis of TOP2B expression likely displays mostly nuclear events. The lack of evidence concerning existence of mitochondrial TOP2B isoform may also base on the fact that compared to nuclear portion only very little amounts are targeted to mitochondria. If this assumption is true differences in general TOP2B transcript abundance can hardly describe effect on mitochondria. It can be reasoned that observed enhanced transcript levels of TOP2B might primarily be due to its requirement in nuclear rather than in mitochondrial transcription. The increase in demand for TOP2B can well be triggered by the monitored nuclear response to TOP1MT deficiency. This theory is supported by a publication which showed a direct interaction of NRF1 and TOP2B (in PARP-1 DNA-PK Ku80 Ku70 Top II-containing complex) (Hossain *et al.* 2009) in NRF1 mediated gene regulation.

The fact that TOP3A transcript levels are not significantly up-regulated in TOP1 KO cells excludes a compensatory function and emphasized the unique role of TOP1MT in mitochondrial metabolism.

5.5.2 Effects on mitochondrial transcription and nuclear response

To further gain insight into the function of the different mitochondrial Topoisomerases, transcripts of mitochondrial and nuclear origin in TOP2B KO and TOP1MT/TOP2B double KO cells were compared. Interestingly data reveals completely unchanged transcript levels of mitochondrial encoded genes and nuclear markers of mitochondrial biogenesis in TOP2B KO-MEFs. Thus a role of TOP2B in mitochondrial transcription seems very unlikely. A lack of TOP2B neither has an impact on mitochondrial transcription nor a compensatory or stress induced nuclear response is triggered.

Remarkable were results obtained from the TOP1MT/TOP2B double KO cells. In these cells the previously seen up-regulation of mitochondrial encoded transcripts due to TOP1MT deficiency is missing. Seemingly the lack of TOP2B abolished up-regulation of mitochondrial transcription. Although mitochondrial transcription up-regulation fails, the nuclear transcripts of NRF1 and GABPA are elevated and similar to data from TOP1MT KO-MEFs. On the basis of the yet available data explanations to this observation are speculative. However a reason for the absence of increased transcription in mitochondria could be a lack of nuclear encoded components of mitochondrial transcription machinery. TOP2B was shown to be involved in

transcription of genes controlled by NRF1 (Hossain *et al.* 2009). Thus a shortcut of these transcripts due to TOP2B deficit seems plausible. The rise of NRF1 and GABPA transcript abundance shows the still available nuclear response to TOP1MT deficiency. It is in question whether expression of downstream targets like TFAM or TFB2M is up-regulated and should be investigated. It also could be argued that the results hint to a role for TOP2B in mitochondrial transcription process although data presented above are contraindicative.

Another important finding was that TOP1MT/TOP2B double KO cells showed unaltered mitochondrial transcript abundance compared to wt-MEFs. This revealed neither TOP1MT nor TOP2B are essential for basal mitochondrial transcription. Seemingly TOP3A is the Topoisomerase directly supporting mitochondrial transcription. This is supported by the fact that knockout of the mitochondrial isoform of TOP3A exhibits defects in fertilities in *drosophila melanogaster* due to severe mitochondrial defects (Wu *et al.* 2010). These defects derive from an impaired mitochondrial DNA metabolism leading to loss of mtDNA. Mitochondrial transcription is a requisite for replication of mtDNA (Falkenberg *et al.* 2007). Therefore a chronically impaired transcription inevitably leads to loss of mtDNA with severe defects of mitochondria. This also supports the hypothesis that TOP1MT is mainly involved in regulation of transcription than directly in transcription process.

5.6 Conclusions

It has been postulated since decades that direct regulation of mtDNA transcription is required to adjust respiratory stoichoimetry to the local redox state at the inner mitochondrial membrane, which is considered the most plausible reason for mitochondria to retain any genes at all (Allen 1993; Allen 2003; Lane 2011). However, redox-sensitive mitochondrial transcription factors that could possibly perform such a local regulatory function have not been identified.

The previously discussed results of this work suggest a role of TOP1MT in transcription process. Localization and co-immunoprecipitation data revealed the connection to sites and enzymes of transcription. Data illustrating excess and

deficiency of TOP1MT uncovered the strong influence on mitochondrial encoded transcript abundance.

Until now, neither cell based-, nor mouse knockout-models really show the expected severe deleterious consequences of an interrupted transcription (Douarre *et al.* 2012). In contrast KD and KO models surprisingly lead to an increase of mitochondrial transcription. This is striking as a TOP1MT deficiency should impair transcription process, if essential for it.

Together these facts implicate a new, alternative role of TOP1MT function in mitochondrial transcriptions process. Based on the data of this work a new model of a regulative function in mitochondrial transcription by TOP1MT activity is presented in this chapter.

5.6.1 A model for regulation of mitochondrial transcription by TOP1MT activity

The basic element suggesting a transcription-regulative function of TOP1MT was its ability to decrease or increase mitochondrial transcription. This influence was dependent on catalytic activity of TOP1MT. The proposed model for regulatory mechanism also depends on catalytic activity instead of enzyme abundance. In this context the overexpression of TOP1MT simulated conditions with increased Topoisomerase activity which leads to a down-regulation of transcription. The knockdown of TOP1MT emulates conditions of decreased Topoisomerase activity resulting in up-regulation of transcription.

It is long known that TOP1 activity is liable to attack by thiol-reactive compounds on conserved cysteine residues in the enzyme core (Konstantinov *et al.* 2001; Montaudon *et al.* 2007). These residues are also conserved in TOP1MT (Figure 8.3). Oxidative thiolation of the vicinal cysteines at positions 504 and 505 by N-ethyl-maleinemide or phenyl-arsene-oxide inactivates nuclear human TOP1 (Montaudon *et al.* 2007), suggesting that TOP1MT activity may also be regulated by direct SH-oxidation at these residues. It is well-known that nuclear TOP1 activity is dependent on its tertiary structure. TOP1MT can reversibly switch from active to inactive form by changes in tertiary structure. Responsible for these structural changes are certain cystein residues which are prone to changes by redox reactions. In human nuclear TOP1 seven cysteines (Cys-300, -341, -386, -504, -505, -630, and -733) are located

in two domains essential for TOP1 activity (Gupta *et al.* 1995) and are highly conserved between TOP1 and TOP1MT (Figure 8.3) in various species ranging from mammals to plants (Konstantinov *et al.* 2001). Experiments confirmed that TOP1MT extracted from human mitochondria can be inactivated by oxidized glutathione and stimulated by thiol-reductive compounds such as dithiotreitol (DTT). This principle offers the opportunity to modulate TOP1MT activity due to general redox status. In the environment of the mitochondrial matrix a redox sensitive protein, coincidentally regulating transcription is a very compelling concept. Redox status in mitochondria is mainly dictated by RC functionality (Balaban *et al.* 2005). A defective or overstrained RC generates ROS (Boveris and Chance 1973; Indo *et al.* 2007). Increased ROS levels can shift the mitochondrial redox status towards a more oxidized state. This is also reflected in a shift in the glutathione pool towards more oxidized glutathione (Hu *et al.* 2008; Kojer *et al.* 2012). Increased ROS or GSSG level inactivates TOP1MT resulting in enhanced mitochondrial transcription. Vice versa reduced ROS levels accompanied by an increase in reduced glutathione lead to an increase of activated TOP1MT inhibiting transcription. The proposed mitochondrial system is coupled to nuclear biogenesis by retrograde signaling via ROS (Balaban *et al.* 2005). In principle it resembles mitochondrial retrograde signaling which regulates by sensing important metabolites (Finley and Haigis 2009). This represents a simple and effective way of regulation, foregoing direct signal transduction by specialized messenger molecules.

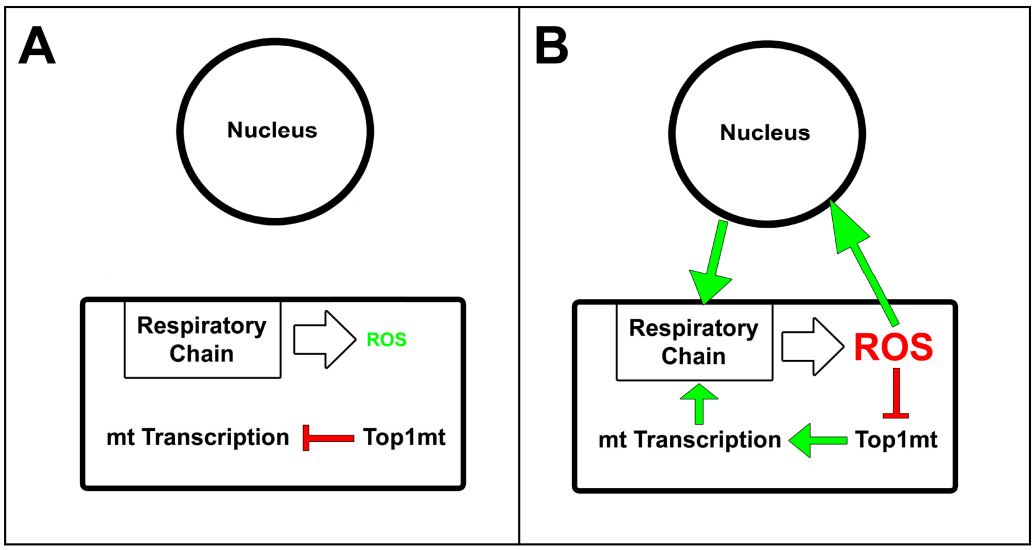

Figure 5.1: Proposed model for regulation of mitochondrial transcription by TOP1MT. A) At low ROS concentrations TOP1MT is active and inhibits mtTranscription. Signalling to the nucleus is reduced. **B)** High ROS concentrations impair TOP1MT function and mtTranscription is up-regulated. ROS simultaneously signals to nucleus and nuclear-driven mitochondrial biogenesis increases.

The ability of TOP1MT to modify mtDNA topology constitutes the mechanistic basis for the proposed model. It is known for a while that DNA topology has a strong influence on transcription (Drew *et al.* 1985). Particular supercoiling of DNA can promote initiation of transcription process. The controlled alteration of DNA supercoiling represents the major regulator of transcription in bacteria (Travers and Muskhelishvili 2005; Travers and Muskhelishvili 2007). Since mitochondria stem from bacteria (Rotte C 2000) a similar concept of transcriptional regulation seems likely. Supercoiling promotes the initiation of transcription by facilitating polymerase binding and promoter melting (Kouzine *et al.* 2008). In mitochondrial transcription regulation TOP1MT activity removes supercoils from DNA thereby restricting initiation of transcription. A reduced TOP1MT activity leads to more supercoiled DNA which in turn can promote transcription. The coupling of TOP1MT activity to redox state illustrates the significance of the proposed mechanism. It represents a feedback loop in which products of the RC (ROS) control the generation of RC transcripts at nuclear and mitochondrial level.

In summary this work for the first time provides evidence for new regulation mechanism of mitochondrial transcription process by TOP1MT. This also may contribute to finally define the currently indistinct role of TOP1MT in mitochondrial metabolism.

5.6.2 Perspective

Although this work revealed numerous evidence for regulatory function of TOP1MT parts of this work must be strengthened to definitely verify this hypothesis. At first the causal proof of the surmised regulatory loop has to be extended and verified by more experiments. The investigation of redox-state of essential cysteine residues of TOP1MT and their role in enzyme activity must be verified. Although initial publications are available (Konstantinov *et al.* 2001) a proof of principle in human cells still must be provided. In this context the co-modulation of mtDNA transcription and TOP1MT-activity by thiol-oxidative and –reductive compounds must be inspected. A second point is the characterization of components and molecular interactions in regulation process. The observed interaction with POLRMT hints to point that more components are involved in regulation process. Finally the role of regulation process in disease- and ageing-associated mitochondrial dysfunction should be investigated.

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7. List of Abbreviations

ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AMP	Adenosine monophoshate
BER	base excision repair
bp	Base pair
BSA	Bovin serum albumin
BrU	Bromo-Uridine
CAP	catabolite activator protein
CTD	C-terminal domain
CI-V	Complexes I-V
Co-enzyme Q	Ubiquinone
COX1-2	Cytochrome oxidase I-II
D-loop	displacement loop
DMSO	Dimethylsulfoxide
DTT	thiol-reductive compounds such as dithiotreitol
e.g.	Exempli gratia
EtBr	Ethidium bromide
$FADH_2$	Flavin Adenine Dinucleotide (reduced form)
FCS	Foetal bovine serum
GABPA	GA-binding protein α chain
GFP	Green fluorescent protein

GSH	Glutathione
GSSG	oxidized glutathione
GS	Goat serum
h	hour
HSP	heavy strand promoter
ICC	immunocytochemisty
IP	immunoprecipitation
IRES	Internal ribosome entry site
IMM	inner mitochondrial membrane
IMS	intermembrane space
KD	knockdown
kd	Kilo Dalton
LSP	light strand promoter
LHON	Leber's hereditary optic neuropathy
MELAS	mitochondrial encephalomyopathy
min	Minutes
mGPDH	mitochondrial glycerophosphate dehydrogenase
mtDNA	mitochondrial DNA
MTS	mitochondrial targeting sequence
mTERF 1-4	mitochondrial transcription termination factors 1-4
mtSSB	mitochondrial ssDNA-binding protein
MRP	mitochondrial ribosomal proteins
NADH	Nicotinamide adenine dinucleotide

NCR	non-coding region
ND1-6	NADH-dehydrogenase 1-6
NRF1	Nuclear Respiratory Factor 1
NT	no-target
OMM	outer mitochondrial membrane
OXPHOS	oxidative phosphorylation
pac	Pyromycin-N-acetyltransferase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGC1A	PPAR co-activator 1α
POLG	mitochondrial DNA Polymerase γ
PEO1	mitochondrial helicase TWINKLE
PPAR	peroxisome proliferator-activated receptors
PPR	pentatricopeptide repeat
qRT-PCR	quantitative real time PCR
RC	respiratory chain
ROS	endogenous radical oxygen species
s	seconds
SCM	Strand-coupled model
SDM	Strand-displacement model
SDHB	Succinyl Dehydrogenase Subunit B
SEM	Standard error of the mean
SDM	Strand-displacement model

Spo11	Sporulation-specific protein 11
TCA	tricarboxylic acid cycle
TFAM	transcription factor A
TFB1M	transcription factors B1
TFB2M	transcription factors B2
TIM	Translocase of Inner Membrane
TOM	Translocase of Outer mitochondrial Membrane
TOPRIM	Topoisomerase-Primase
TOP1	Topoisomerase 1
TOP1MT	mitochondrial Topoisomerase 1
Top2A	Topoisomerase 2α
Top3B	Topoisomerase 3α
YFP	Yellow fluorescent protein
WHD	winged helix Domain

8. Appendix

8.1 Plasmid maps

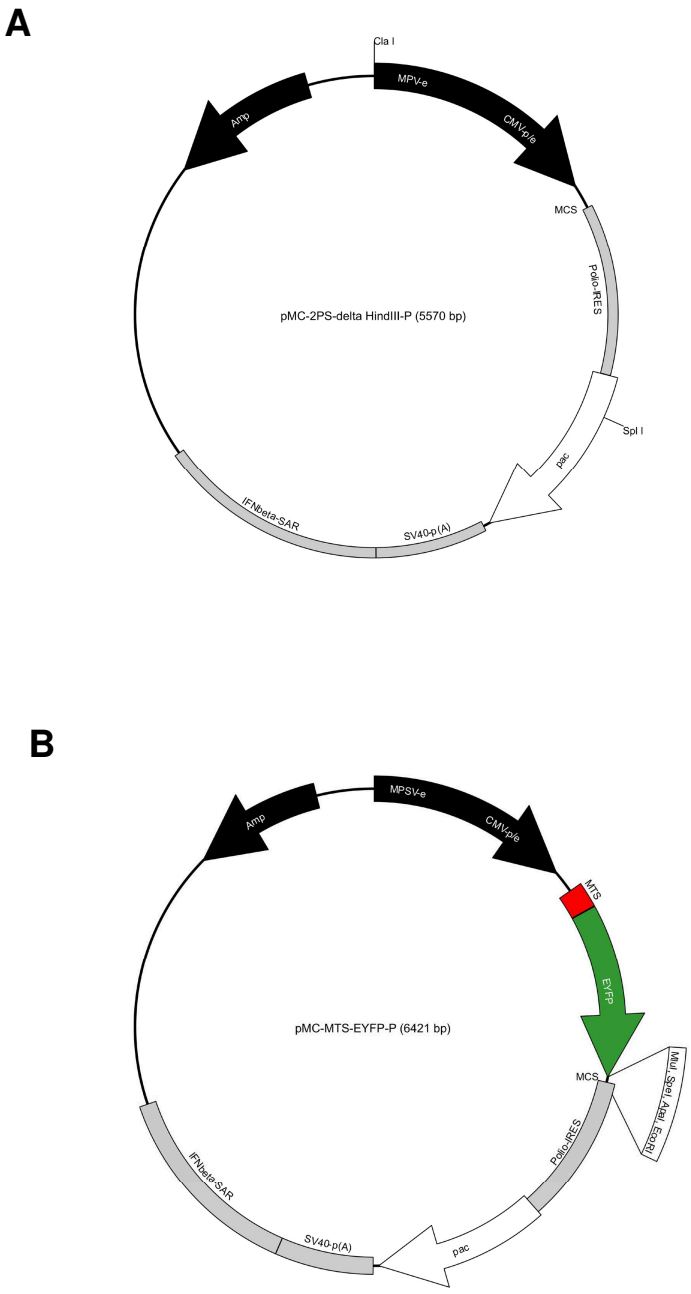

Figure 8.1: A Basic bicistronic expression plasmid pMC-2PS-delta HindIII-P (Mielke et al, 2000). B Bicistronic expression plasmid pMC-MTS-EYFP-P. YFP (green) is fused at the 5'end with the MTS from subunit VIII of COX.

8.2 Full length Northern Blots of mitochondrial transcripts

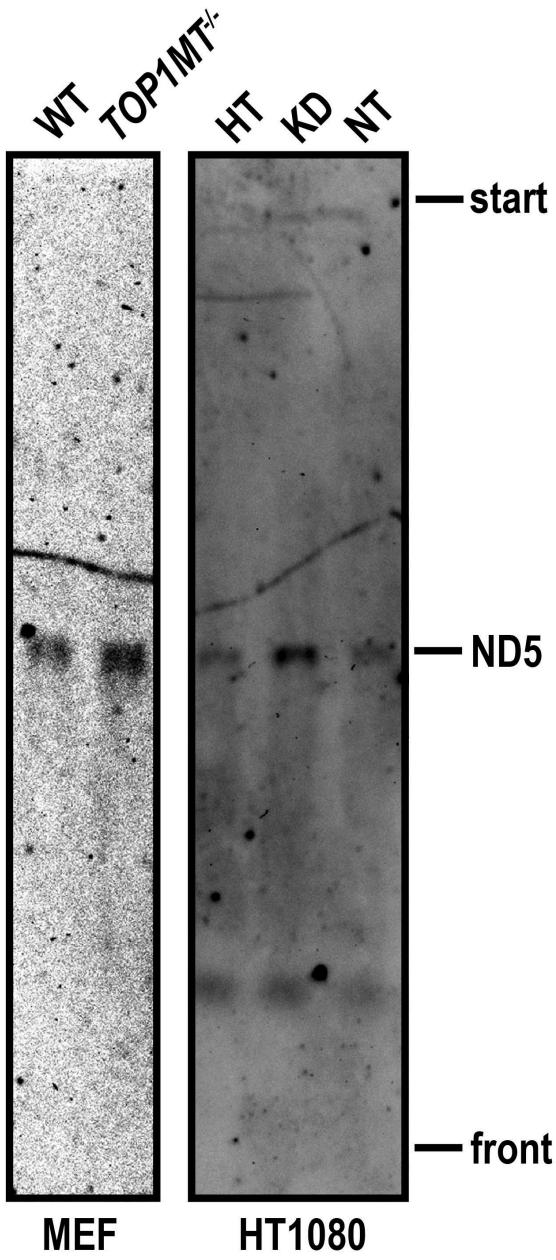

Figure 8.2: Representative Full length Northern Blots of Mitochondrial transcript of human cells and mouse cells.

8.3 Alignment of human Type IB Topoisomerases

Alignment nuclear Top1 / mitochondrial Top1mt

```
Query  206  WEEERYPEGIKWKFLEHKGPVFAPPYEPLPENVKFYYDGKVMKLSPKAEEVATFFAKMLD  265
            WE+E++ +G+KW+ LEHKGP FAPPYEPLP+ V+F+Y+G+ ++LS  AEEVATF+ +MLD
Sbjct  42   WEKEKHEDGVKWRQLEHKGPYFAPPYEPLPDGVRFFYEGRPVRLSVAAEEVATFYGRMLD  101

Query  266  HEYTTKEIFRKNFFKDWRKEMTNEEKNIITNLSKCDFTQMSQYFKAQTEARKQMSKEEKL  325
            HEYTTKE+FRKNFF DWRKEM  EE+ +I +L KCDFT++ +YF  +  ARK +S+EEK
Sbjct  102  HEYTTKEVFRKNFFNDWRKEMAVEEREVIKSLDKCDFTEIHRYFVDKAAARKVLSREEKQ  161

Query  326  KIKEENEKLLKEYGFCIMDNHKERIANFKIEPPGLFRGRGNHPKMGMLKRRIMPEDIIIN  385
            K+KEE EKL +E+G+CI+D H+E+I NFKIEPPGLFRGRG+HPKMGMLKRRI PED++IN
Sbjct  162  KLKEEAEKLQQEFGYCILDGHQEKIGNFKIEPPGLFRGRGDHPKMGMLKRRITPEDVVIN  221

Query  386  CSKDAKVPSPPPGHKWKEVRHDNKVTWLVSWTENIQGSIKYIMLNPSSRIKGEKDWQKYE  445
            CS+D+K+P PP GH+WKEVR DN VTWL +WTE++Q SIKYIMLNP S++KGE  WQK+E
Sbjct  222  CSRDSKIPEPPAGHQWKEVRSDNTVTWLAAWTESVQNSIKYIMLNPCSKLKGETAWQKFE  281

Query  446  TARRLKKCVDKIRNQYREDWKSKEMKVRQRAVALYFIDKLALRAGNEKEEGETADTVGCC  505
            TARRL+  VD+IR+QYR DWKS+EMK RQRAVALYFIDKLALRAGNEKE+GE ADTVGCC
Sbjct  282  TARRLRGFVDEIRSQYRADWKSREMKTRQRAVALYFIDKLALRAGNEKEDGEAADTVGCC  341

Query  506  SLRVEHINLHPELDGQEYVVEFDFLGKDSIRYYNKVPVEKRVFKNLQLFMENKQPEDDLF  565
            SLRVEH+ LHPE DG ++VVEFDFLGKD IRYYN+VPVEK V+KNLQLFMENK P DDLF
Sbjct  342  SLRVEHVQLHPEADGCQHVVEFDFLGKDCIRYYNRVPVEKPVYKNLQLFMENKDPRDDLF  401

Query  566  DRLNTGILNKHLQDLMEGLTAKVFRTYNASITLQQQLKELTAPDENIPAKILSYNRANRA  625
            DRL T  LNKHLQ+LM+GLTAKVFRTYNASITLQ+QL+ LT  +++I AKILSYNRANR
Sbjct  402  DRLTTTSLNKHLQELMDGLTAKVFRTYNASITLQEQLRALTRAEDSIAAKILSYNRANRV  461

Query  626  VAILCNHQRAPPKTFEKSMMNLQTKIDAKKEQLADARRDLKSAKADAKVMKDAKTKKVVE  685
            VAILCNHQRA P TFEKSM NLQTKI AKKEQ+A+AR +L+ A+A+ K   D K++ V+E
Sbjct  462  VAILCNHQRATPSTFEKSMQNLQTKIQAKKEQVAEARAELRRARAEHKAQGDGKSRSVLE  521

Query  686  SKKKAVQRLEEQLMKLEVQATDREENKQIALGTSKLNYLDPRITVAWCKKWGVPIEKIYN  745
             K++ +++L+EQL +L VQATD+EENKQ+ALGTSKLNYLDPRI++AWCK++ VP+EKIY+
Sbjct  522  KKRRLLEKLQEQLAQLSVQATDKEENKQVALGTSKLNYLDPRISIAWCKRFRVPVEKIYS  581

Query  746  KTQREKFAWAIDMADEDYEF  765
            KTQRE+FAWA+ MA ED+EF
Sbjct  582  KTQRERFAWALAMAGEDFEF  601
```

Figure 8.3: Alignment of human Type IB Topoisomerases.

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10. Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation eigenständig und ohne unerlaubte Hilfe angefertigt habe. Ich habe diese Dissertation in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den

Stefan Sobek