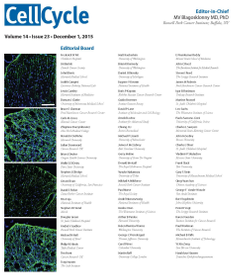

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Beyond the unwinding: Role of TOP1MT in mitochondrial translation

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Abstract

Mitochondria contain their own genome (mtDNA), encoding 13 proteins of the enzyme complexes of the oxidative phosphorylation. Synthesis of these 13 mitochondrial proteins requires a specific translation machinery, the mitoribosomes whose RNA components are encoded by the mtDNA, whereas more than 80 proteins are encoded by nuclear genes. It has been well established that mitochondrial topoisomerase I (TOP1MT) is important for mtDNA integrity and mitochondrial transcription as it prevents excessive mtDNA negative supercoiling and releases topological stress during mtDNA replication and transcription. We recently showed that TOP1MT also supports mitochondrial protein synthesis, and thus is critical for promoting tumor growth. Impaired mitochondrial protein synthesis leads to activation of the mitonuclear stress response through the transcription factor ATF4, and induces cytoprotective genes in order to prevent mitochondrial and cellular dysfunction. In this perspective, we highlight the novel role of TOP1MT in mitochondrial protein synthesis and as potential target for chemotherapy.

Keywords: mitochondrial topoisomerase I, mitochondria, mitochondrial translation, mitoribosome, mitochondrial targeted therapy, cancer

Mitochondrial topoisomerases

Mitochondria are central hubs for numerous metabolic pathways, providing cellular energy, supplying macromolecules for cellular proliferation, and controlling redox homeostasis [1]. Likely derived from a bacterial ancestor, mitochondria possess their own circular double-stranded genome of about 16.6 kb. Human mitochondrial DNA (mtDNA) encodes 2 mitochondrial ribosomal RNAs, 22 mitochondrial transfer RNAs and 13 mitochondrial messenger RNAs encoding a small but essential fraction of the subunits of the electron transport chain [2]. MtDNA lacks introns, and contains only one noncoding region (NCR), which harbors the promoters for transcription of both strands and the replication origin for the heavy (H) strand (O_H) [3, 4]. Replication of mtDNA from this origin is usually strand-synchronous, with the displaced lagging strand template being covered with RNA, protein or a combination of the two [5] until second-strand DNA synthesis begins, typically from a site located 11 kbp downstream of O_H, known as O_L [6].

Mitochondrial transcription proceeds from the H-strand promoters (HSP1 and HSP2) and L-strand promoter (LSP) for the heavy and light strand, respectively. The resulting polycistronic transcripts are processed according to the 'tRNA punctuation model' whereby 22 interspersed tRNAs are excised to concomitantly release rRNAs and mRNAs [7, 8]. Translation of the mitochondrial encoded genes, requires further RNA processing by polyadenylation, methylation, or pseudouridinylation, and in case of tRNAs CCA addition and aminoacylation [9].

Due to its circular structure and attachment to the mitochondrial inner membrane [10, 11], mtDNA is readily supercoiled as transcription and replication generate torsional stress by unwinding the DNA. Critical factors in releasing topological stress are topoisomerases, which transiently cleave the DNA-backbone, allowing DNA untwisting and passage of a DNA segment through another [12]. Among the six human topoisomerases, three possess dual localizations in the nucleus and the mitochondria: topoisomerase IIα (TOP2A) [13], topoisomerase IIβ (TOP2B) [14] and topoisomerase IIIα (TOP3A) [15]. Mitochondrial topoisomerase I (TOP1MT) is the only topoisomerase exclusively devoted to mitochondria [16]. TOP1MT is critical for limiting mtDNA negative supercoiling and for the maintenance of mtDNA integrity after doxorubicin treatment [13, 17]. In *Top1mt* knockout cells mtDNA is in an underwound state, which might cause the observed increase in steady state levels of mitochondrial RNA [18, 19].

In contrast to TOP2A, TOP2B and TOP3A, TOP1MT is dispensable in mice with no obvious phenotype of *Top1mt* knockout mice under basal conditions, suggesting that its function can be compensated by other topoisomerases [13, 17, 20]. Yet, loss of TOP1MT attenuates cell proliferation by limiting mtDNA expansion in cardiomyocytes after doxorubicin treatment and under high-energy demand during liver regeneration [17, 21].

Role of TOP1MT in mitochondrial protein synthesis

We recently reported that, in mouse models of colon and liver cancer, TOP1MT promotes tumor growth and frequency by sustaining cancer cell proliferation in a metabolically compromised environment [22]. This conclusion tempers the Warburg effect hypothesis by demonstrating the importance of mitochondria for cancer cell proliferation. Consistent with this possibility, analyses of The Cancer Genome Atlas (TCGA) databases show elevated expression of TOP1MT in hepatocellular carcinomas [22]. Besides its role in enabling mtDNA replication and expansion, we demonstrated that TOP1MT possesses a non-canonical function in facilitating mitochondrial translation [22].

Mammalian mitoribosomes consist of a large 39S subunit (mtLSU) and a small 28S (mtSSU) subunit, containing the 16S and 12S mitochondrial rRNA, and mt-tRNA (Val), respectively [23, 24]. Approximately 80 nuclear-encoded proteins are imported to mitochondria assembled in the mitoribosome with the RNA components as scaffolds [25]. Based on steady-state rRNA levels, it has been estimated that there are 100 mitoribosomes per mitochondrion in rat hepatocytes [3]. Similar to cytosolic ribosomes, mitochondrial ribosomes carry out translation, which encompasses initiation, elongation, termination and recycling. These processes depend on the expression, synthesis and import of nuclear-encoded proteins [26, 27]. Thus, protein synthesis in the cytosol and mitochondria warrants temporal and spatial coordination. While the molecular details of mitoribosome biogenesis are currently limited [28], the initial steps of mitochondrial assembly have been suggested to occur at mitochondrial nucleoids [25, 29, 30]. Recent advances indicate that initial and partly co-transcriptional RNA processing is organized in distinct foci, called mitochondrial RNA granules (MRGs) that form in close proximity to mitochondrial nucleoids [31, 32]. MRGs contain proteins involved in RNA processing, mitochondrial ribosomal proteins and translation-associated factors, indicating that the early steps of the mitoribosome subunit assembly might take place in these foci. It has been

proposed that newly transcribed rRNAs and/or early mitoribosome assembly intermediates are transferred from nucleoids to the MRGs where mitoribosome assembly is completed [28]. However, the proteome of MRGs varies within different subpopulations suggesting that these are dynamic structures [28, 33]. TOP1MT has been found to localize to MRGs [34, 35] and pulldown experiments have shown that TOP1MT associates with mitochondrial ribosomal proteins [22]. Consistent with these results, we observed that mitochondrial protein synthesis is significantly decreased in *Top1mt* KO murine embryonic fibroblasts (MEF) and in tumor models lacking TOP1MT [22].

Recent experiments further elaborate on the role of TOP1MT in mitochondrial translation.

Figure 1 shows that transient depletion of mtDNA with ethidium bromide significantly diminishes the steady state protein levels of the electron transport chain and that the rate of mtDNA copy number expansion in the recovery phase is decreased in cells lacking TOP1MT (Fig. 1A), which is in line with our previous studies [36]. Despite elevated mitochondrial transcript levels (Fig. 1B), the recovery rate of the electron transport chain proteins was markedly delayed in cells lacking TOP1MT as compared to WT controls. While the electron transport chain subunits fully recovered in the WT cells 6 days after ethidium bromide release, protein levels only started to reappear in the TOP1MT KO cells after 3 additional days (Fig. 1C). Several possibilities may explain how TOP1MT impacts mitochondrial translation. First, defects in mitochondrial RNA processing could account for the downstream defects in mitochondrial protein synthesis. However, maturation and polyadenylation of the mitochondrial transcriptome occurred normal in TOP1MT KO MEFs [19]. Second, TOP1MT might play a role in the temporal and spatial coordination of the mitoribosomal assembly through its interaction with both the transcription machinery and mitoribosomal proteins. Another possibility is that mitochondrial translation is regulated by a long noncoding RNA spanning the D-loop, which was strongly upregulated in the absence of TOP1MT [19]. Notably, the steady state levels of the same RNA were found strongly increased in cells where mitochondrial protein synthesis was inhibited [37]. Further investigations are warranted to elucidate these possibilities.

Mitochondrial translation and coupling with cellular proliferation

Mitochondria fulfill essential cellular functions ensuring bioenergetic supply and metabolic processes that are essential for cell growth and survival as well as cell death (cytochrome C release for apoptosis). Consequently, mitochondria are in constant crosstalk with the nucleus and the cytosol to regulate cellular homeostasis and adaption to mitochondrial stress [38]. Proteostatic stress in the mitochondria can initiate feedback responses to block cell proliferation, such as the mitochondrial unfolded protein response, the proteolytic stress response and the heat shock response [39]. Stalling of mitochondrial translation has been demonstrated to trigger antiproliferative signals by activating mitochondrial release factors with concomitant signaling to halt cell proliferation, rather than loss of respiratory function, which has been suggested to be a downstream effect [40, 41]. Failure of this initial rescue attempt triggers the mitochondrial ribosomal and RNA decay pathway, ensuring degradation of the majority of mitoribosomes and mitochondrial rRNA and mRNA [40].

A multiomics approach identified activating transcription factor 4 (ATF4) as the main regulator of the mitonuclear stress response [42]. ATF4 activation reduces cytosolic translation and induces cytoprotective genes, with a concomitant decrease of mitochondrial ribosomal protein levels induced by mitochondrial stress. Thus, ATF4 and mitochondrial ribosomal proteins are the main effectors in mitonuclear stress pathways [42]. Notably, we identified ATF4 as upstream regulator in the transcriptome analysis of chemically induced WT and *Top1mt* KO liver tumors[22] suggesting that impaired mitochondrial translation activates ATF4, which in turn diminishes total protein synthesis and therefore halts cell proliferation in *Top1mt* deficient tumors.

Figure 2 summarizes our model linking TOP1MT with cellular proliferation for tissue regeneration and tumor growth [21, 22]. Coupling of TOP1MT with cellular proliferation is exemplified by our prior finding that MYC regulates the transcription of *TOP1MT* [43].

Mitochondrial translation as therapeutic target

Mitochondria are essential for cancer cell growth, to meet their anabolic and bioenergetic needs in challenging tumor microenvironments (Figure 2) [44-47]. Hence, mitochondria represent potential targets for the development of novel anticancer agents [48, 49]. Recent studies indicate that inhibition of mitochondrial protein synthesis is a potential therapeutic strategy [50], particularly against quiescent cancer stem cells that strictly depend on mitochondrial biogenesis

[51]. The FDA-approved antibiotic tigecycline has been demonstrated to sensitize non-small cell lung cancer [52], hepatocellular carcinoma [53], high-grade B-cell lymphoma [54] and renal cell carcinoma to chemotherapy [55]. In line with these findings, tigecycline treatment in combination with daunorubicin and cytarabine displayed additive or syntergistic effects in primary human acute myeloid leukemia (AML) cells [56]. Similarly, the FDA-approved therapeutic doxycycline has been shown to inhibit mitochondrial protein translation and biogenesis in cancer stem cells [51, 57]. Oral doxycycline treatment decreased the expression of cancer stem cell markers in a clinical pilot study in early breast cancer patients [58]. In line with these findings, a mitochondrial gene signature has been proposed to stratify patients with high mitochondrial markers to receive mitochondrial-targeted therapies in addition to conventional therapy [57].

A genome-wide CRISPR and shRNA screen revealed that K-Ras mutant tumors are sensitive to mitochondrial translation inhibitors, and combinational therapy with BRAF and MEK inhibitors together with tigecycline might be a sustainable strategy against K-Ras tumors [59]. Thus, the survival of a subpopulation of dormant cells, responsible for relapse of K-Ras mutant pancreatic ductal adenocarcinoma, depends on mitochondrial activity [60] (Figure 2). MYC has also been shown to directly activate TOP1MT [43]. Mouse models of MYC-driven lymphoma showed that MYC activation sensitizes cancer cells to inhibition of mitochondrial translation with tigecycline without affecting normal tissue [61]. Notably, treatment with mitochondria-targeted drugs might enhance mitophagy and TOP1MT-deficient MEFs have been found to activate autophagy [20]. Similar to authophagy, the role of mitophagy in carcinogenesis depends on the cellular context and stage of tumorigenesis [62, 63]. Thus, autophagy has been shown to possess both pro- and anti-tumorigenic effects [62]. In a KRAS mutant colorectal cancer cell model, mitochondrial targeted treatment induces autophagy, thereby suppressing cancer cell proliferation [64]. However, autophagy facilitates survival of Ras-driven lung tumor cells by preventing nucleotide depletion and energy crisis in a nutrient deprived environment [65]. Thus, the contextual role of mitophagy might be an important determinant for the success of mitochondrial-targeted therapies.

Based on its role in mitochondrial protein synthesis and due to its upregulation in a wide range of tumors, TOP1MT might represent a druggable target for cancer therapy [66]. TOP1MT inhibition might present an effective strategy in the eradication of cancer stem cells, as these

cells are highly dependent on mitochondrial biogenesis [57]. In particular, tumor cells in a hypoglycemic or hypoxic microenvironment might be susceptible to TOP1MT inhibition, since cancer cell growth of TOP1MT deficient cells was significantly reduced in nutrient deprived environment compared to WT cells [22]. However, TOP1MT deficiency did not alter cell growth under standard cell culture conditions, suggesting that it becomes a limiting factor for cell proliferation under nutrient starvation *in vivo* [22]. Due to mitochondrial impairment, tumor cells might enhance glycolysis to sustain their viability. Notably, the glycolytic rate was unaltered in TOP1MT-deficient cancer cells despite the upregulation of key enzymes involved in the glycolytic pathway, suggesting that glycolysis operates at its maximum [22]. Alternatively, clearance of defective mitochondria by increased autophagy might allow for cancer cell survival [62]. The mediating role of autophagy in the observed antitumorigenic effects in TOP1MT-deficient tumor cells needs to be determined in future studies.

Given the current lack of an selective TOP1MT inhibitor [66], we propose that engineering clinically used topoisomerase I inhibitors with a mitochondrial targeting peptide [67] might be a promising approach for the development of TOP1MT targeting drugs. This strategy has been successfully employed for the targeted delivery of doxorubicin to the mitochondria [68]. Whether TOP1MT inhibition can augment additional targeted treatments to achieve a sustainable response and prevent tumor relapse needs to be explored in the future [22].

Conclusion

Similar to other topoisomerases, TOP1MT releases mtDNA topological stress generated during mitochondrial replication and transcription. Although TOP1MT is a non-essential gene and TOP1MT-deficient mice are viable [22], it becomes important under stress conditions. TOP1MT protects doxorubicin-induced cardiotoxicity [17], enables liver regeneration [36] and promotes cancer cell proliferation in metabolically challenging microenvironments [22]. We propose that TOP1MT exerts a pleiotropic function, enabling mtDNA replication and expansion, but also possesses a noncanonical role in facilitating mitochondrial translation [22]. The decrease in steady-state levels of the oxidative phosphorylation proteins in turn leads to a drop in cellular energy, metabolic intermediates, enhanced oxidative stress and the activation of ATF4 to induce the mitonuclear stress response, ultimately delaying cellular proliferation. Inhibition of mitochondrial protein synthesis has been found to be a promising treatment strategy for a variety of cancers [51-56]. Based on its role in mitochondrial protein synthesis and given its

upregulation in cancers, we propose that TOP1MT could be a potential target for intervention in combinational therapies.

Acknowledgments

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Declaration of potential conflicts of interest

No potential conflicts of interests were disclosed.

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Figure Legends

Figure 1: Role of TOP1MT in mitochondrial translation. (**A**) Transient mtDNA depletion followed by recovery in HCT-116 WT and TOP1MT KO cells: mtDNA was depleted by treatment with 50 ng/mL ethidium bromide (EB) for 8 days and its recovery was followed for 10 days after EB wash-out. Mitochondrial copy number was quantified at each timepoint as described in [69]. (**B**) Steady state levels of mtRNAs at the indicated timepoints: total RNA was isolated by standard procedures and levels of ND2 and COX2 mRNAs was assessed by Northern Blotting using P^{32} DNA labeled probes spanning regions of about 500 bp of the respective transcripts. (**C**) Delayed recovery of electron transport chain subunits in TOP1MT KO cells: Whole cell lysates from different timepoints were subject to SDS-PAGE and Western blotting as described in [69]. Antibodies were purchased from abcam (anti-NDUFB8, Ab110242; anti-MTCO2, Ab110258; anti-UQCRC2, Ab14745; anti-ATP5A, Ab14748; anti-TFAM, ab131607; anti-VCL, Ab238075).

Figure 2. Role of TOP1MT in tissue regeneration and tumorigenesis. TOP1MT couples mitochondrial functions (translation, mtDNA expansion, ATP generation and biogenesis) with cellular proliferation in response to growth factors and oncogenetic stimuli.

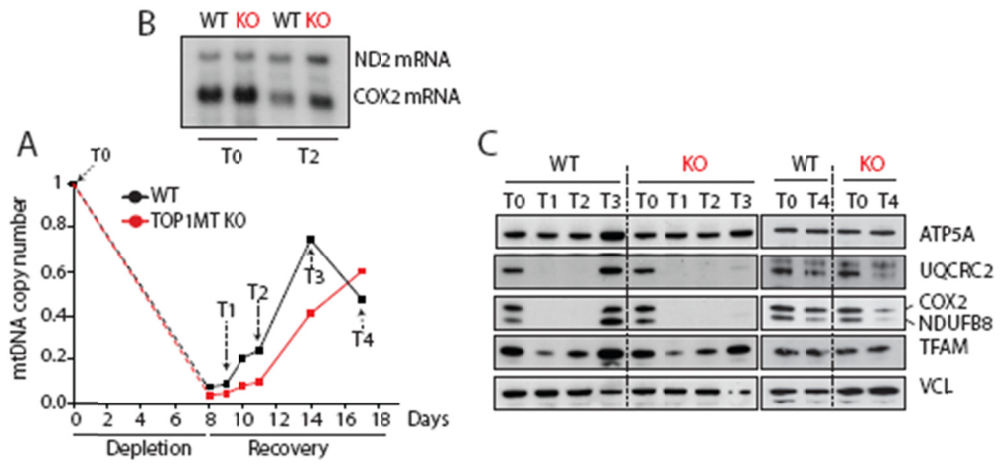
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Fig 1

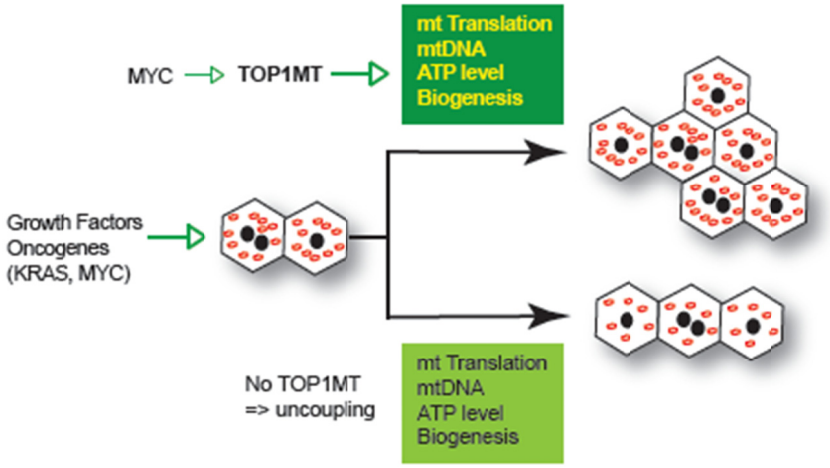
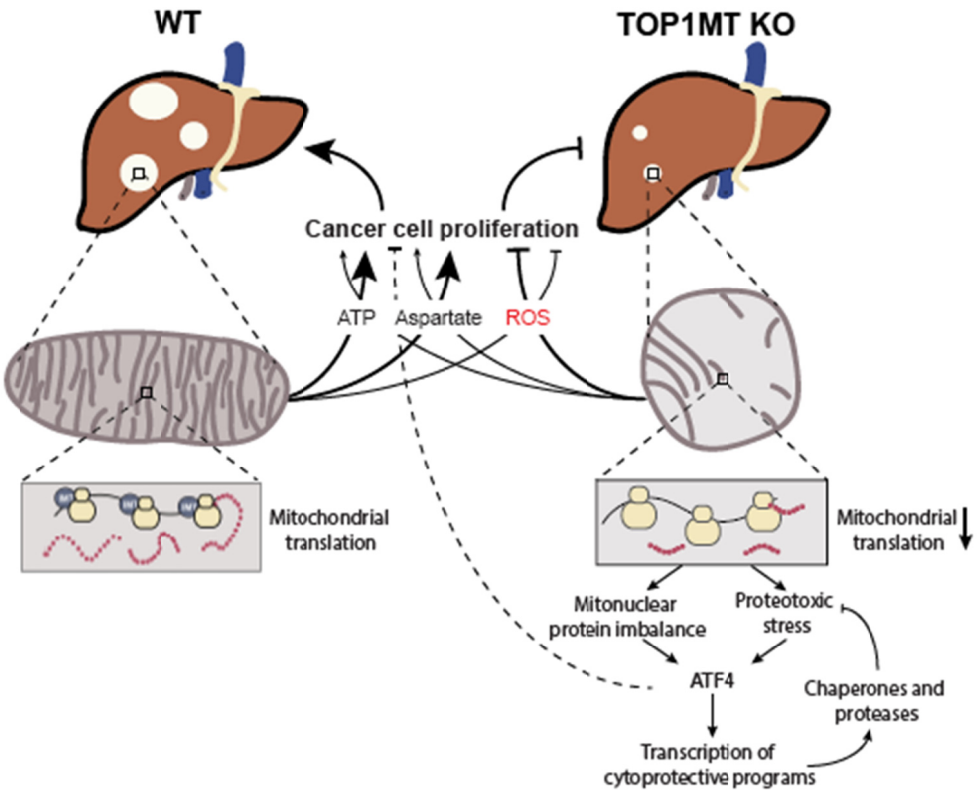

MYC
TOP1MT
mt Translation
mtDNA
ATP level
Biogenesis
Growth Factors
Oncogenes
(KRAS, MYC)
No TOP1MT
=> uncoupling
mt Translation
mtDNA
ATP level
Biogenesis

Fig 2



Graphical Abstract