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Mitochondrial topoisomerase I (Top1mt) is a novel limiting factor of doxorubicin cardiotoxicity

Salim Khiati¹, Ilaria Dalla Rosa¹, Carole Sourbier², Xuefei Ma³, V. Ashutosh Rao⁴, Leonard M Neckers², Hongliang Zhang¹, and Yves Pommier¹

¹Developmental Therapeutics Branch and Laboratory of Molecular Pharmacology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, 20892, USA

²Urologic Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, 20892, USA

³Laboratory of Molecular Cardiology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, 20892, USA

⁴Center for Drug Evaluation and Research, Food and Drug Administration, Bethesda, MD, 20892, USA

Abstract

Purpose—Doxorubicin (DOX) is one of the most effective chemotherapeutic agents. However, up to 30% of the patients treated with DOX suffer from congestive heart failure. The mechanism of DOX cardiotoxicity is likely multifactorial and most importantly, the genetic factors predisposing to DOX cardiotoxicity are unknown. Based on the fact that mtDNA lesions and mitochondrial dysfunctions have been found in human hearts exposed to DOX and that mitochondrial topoisomerase 1 (Top1mt) specifically controls mtDNA homeostasis, we hypothesized that Top1mt knockout (KO) mice might exhibit hypersensitivity to DOX.

Experimental Design—Wild type (WT) and knockout Top1mt mice were treated once a week with 4 mg/kg DOX for 8 weeks. Heart tissues were analyzed one week after the last treatment.

Results—Genetic inactivation of Top1mt in mice accentuates mtDNA copy number loss and mtDNA damage in heart tissue following DOX treatment. Top1mt knockout mice also fail to maintain respiratory chain protein production and mitochondrial cristae ultrastructure organization. These mitochondrial defects result in decreased O₂ consumption, increased ROS production and enhanced heart muscle damage in animals treated with DOX. Accordingly, Top1mt knockout mice die within 45 days after the last DOX injection under conditions whereas the wild type mice survive.

Conclusions—Our results provide evidence that mitochondrial topoisomerase I, Top1mt, which is conserved across vertebrates, is critical for cardiac tolerance to DOX and adaptive response to

Corresponding author: Dr. Yves Pommier, Developmental Therapeutics Branch and Laboratory of Molecular Pharmacology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892. Tel.: 301-496-5944; Fax: 301-402-0752; pommier@nih.gov.

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DOX cardiotoxicity. They also suggest the potential of Top1mt single nucleotide polymorphisms (SNP) testing to investigate patient susceptibility to DOX induced cardiotoxicity.

Keywords

Mitochondrial topoisomerase I; knockout mice; mitochondrial DNA; cardiotoxicity; doxorubicin

Introduction

Anthracycline antibiotics, and especially DOX are among the most widely used anticancer drugs (1). Their primary mechanism of action is by intercalation into DNA (2) and by trapping topoisomerase II-DNA cleavage complexes (Top2cc) (3, 4) as they bind at the Top2-DNA interface (5, 6). Top2cc, in turn selectively kill cancer cells by blocking replication and transcription (4, 7-9).

In spite of the efficacy of DOX in pediatric (10) and adult cancers ranging from leukemia to lymphomas and solid tumors such as breast cancers (11), the main adverse effect of DOX is cardiotoxicity, which can cause congestive heart failure in 30% of adults at high doses, and delayed heart failure after terminating treatment in children once they reach adulthood. The cardiotoxicity of DOX appears separable from its therapeutic mechanism because cardiomyocytes are generally not replicative, and Top2 α , the primary target of DOX (7, 8) is not expressed in quiescent cells and undetectable in heart tissues (12). On the other hand, Top2 α is required for cell proliferation and its gene *TOP2A* is often amplified with the HER-2 (*ERBB2*) oncogene in breast and other forms of cancers (13).

The cardiotoxicity of DOX remains difficult to predict and is often not detected until years after the completion of chemotherapy (14). Also, the genetic determinants of DOX cardiotoxicity remain unknown, at least in part because DOX cardiotoxicity is likely multifactorial and complex (15). Free radical generation is a classical mechanism by which DOX injures the myocardium (16). The chemical structure of DOX is prone to the generation of free radicals as DOX reversibly oxidizes to a semiquinone, an unstable metabolite whose futile cycling within the mitochondria releases reactive oxygen species (17). Unfortunately, free radical scavengers provide only limited heart tissue protection (18-20). The heart is selectively sensitive to reactive oxygen metabolites because of lowered antioxidant glutathione peroxidase, catalase, and superoxide dismutase levels compared to other tissue (21). An additional possibility stems from the fact that DOX not only inhibits Top2 α but also Top2 β . A recent study showed that genetically engineered mice lacking Top2 β in their heart avoid myocardial injuries after DOX treatment (22). A third possibility is the direct targeting of mitochondria by DOX (23). DOX being a cationic compound readily enters mitochondria, binds to cardiolipin and inhibits the respiratory chain. Indeed, the electron-transport chain proteins require cardiolipin to function properly, and it has been proposed that since DOX disrupts the cardiolipin-respiratory chain protein interface, more superoxide (O₂⁻) formation occurs (24-26). Finally, mtDNA could be a direct target of DOX (27), as mtDNA lesions and free radical-associated mitochondrial dysfunction have been found in the hearts of patients treated with DOX (26).

Mitochondria are the only cellular organelles containing metabolically active DNA outside the nucleus (28). DNA topoisomerases are present in mitochondria to relieve mtDNA topological stress and entanglements generated during replication and transcription. To date, three topoisomerases have been identified in vertebrate mitochondria: Top1mt (29), Top2 β (30) and Top3 α (31). Top3 α and Top2 β both function in mitochondria and the nucleus, and the only specific mitochondrial topoisomerase in vertebrates is Top1mt (29). Murine embryonic fibroblasts (MEFs) from Top1mt knockout animals show a marked increase in ROS production, calcium signaling and hyperpolarization of mitochondrial membranes (32). Top1mt activity in the regulatory region of mtDNA also suggests its importance in regulating mtDNA replication (33). However, Top1mt-deficient mice (Top1mt^{-/-}) are viable, fertile, normal in size, and do not display obvious basal physical or behavioral abnormalities, indicating compensation by other topoisomerases and metabolic reprogramming. Indeed, Top1mt deficient MEFs compensate their mitochondrial dysfunction by producing ATP through alternative metabolic pathways and increasing their antioxidant capacity (32).

Based on the fact that mtDNA lesions and radical-associated mitochondrial dysfunctions have been found in human hearts exposed to DOX (26) and that Top1mt specifically controls mtDNA homeostasis (32, 33), we hypothesized that Top1mt knockout mice might exhibit heart tissue sensitivity to DOX.

Methods

Mouse handling

Top1mt^{+/+} (WT) and Top1mt^{-/-} (Top1mt KO) mice were generated from heterozygous (Top1mt^{+/-}) and paired within the same litter. Each Top1mt KO mouse had at least one brother WT as control. Starting at 7 weeks age, mice were treated once a week with 4mg/kg intra peritoneal doxorubicin (DOX) or saline solution control for 8 weeks. Heart tissues were analyzed one week after the last treatment. For survivals studies mice were followed for up to 90 days after the last injection. Animal experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of the National Institutes of Health.

Transmission electron microscopy

Mice were euthanized and heart tissues were immediately harvested and fixed in 4% formaldehyde, 2% glutaraldehyde, 0.1M Cacodylate (pH 7.4) for 2 hour at room temperature. Small pieces of the fixed heart tissue were postfixed in 1% osmium tetroxide for 1 hour and stained in 0.5% uranyl acetate for another hour. The samples were then dehydrated in a graded series of 35%, 50%, 70%, 100% ethanol and exchanged to propylene oxide. After infiltration at 1:1 propylene oxide and epoxy resin (Poly/Bed 812, Polysciences, Warrington, PA) overnight, samples were embedded in 100% epoxy resin. Polymerization of resin was performed for 3 days at 55°C. Thin sections of 70 – 90 nm were cut with an ultramicrotome (Leica EM UC6, Leica Microsystems, Buffalo Grove, IL), stained with uranyl acetate and lead citrate, lightly carbon coated, and imaged in a Hitachi 7650 or 7600 transmission electron microscope (Hitachi High Technologies America, Gaithersburg, MD).

Images were taken with 2k × 2k AMT digital camera (Advanced Microscopy Techniques, Woburn, MA). Mitochondrial morphometric measurements were performed using ImageJ. A total of 250 mitochondria for each condition were analyzed from 10 electron micrographs taken at 3000× magnification in 6 different sections. 2 animals were used for each condition.

Mitochondria isolation

Mitochondria from hearts of mice were isolated following the protocol for rats published by Rogers et al (34). Briefly, 50 mg of heart tissue were trimmed to size of 1 mm³ and resuspended in ~10 ml mitochondria isolation buffer (225 mM mannitol, 75 mM sucrose, 10 mM HEPES, 10 mM EDTA, 1 mg/ml bovine serum albumin [BSA]). Tissues were homogenized with 40 strokes in a dounce homogenizer and centrifuged 10 min at 1000g. Supernatant was centrifuged at 12000g for 10 min and crude mitochondria pellets were washed twice with mitochondria isolation buffer without BSA. Proteins concentrations were quantified using Bio-Rad Protein Assay.

Mitochondrial membrane potential (ψ_m)

ψ_m was determined in isolated mitochondria using JC-1 according to the manufacturer's protocol. Protein concentration was used for normalization.

Reactive oxygen species (ROS) production measured by glutathione assay

ROS production was measured quantifying reduced glutathione (GSH) in heart tissue. GSH levels were assessed in 50 mg tissue lysates using the luminescence-based GSH-Glo™ Glutathione Assay (Promega) according to the manufacturer's protocol.

Mitochondrial Complex IV activity

The cytochrome C oxidase activity quantification in isolated mitochondria was performed using the absorbance-based assay Mitochondrial Complex IV (Mouse) Activity Assay Kit (Milipore) and following the manufacturer's protocol. The Complex IV is immunocaptured with the wells and its activity is determined by following the oxidation of reduced cytochrome c as an absorbance decrease at 550 nm.

Quantification of mtDNA copy number and mtDNA damage

For mtDNA quantification, total DNA was isolated from 30 mg of tissue using DNeasy Blood and tissues Kit (QIAGEN). Quantitative PCRs were performed in triplicates in 384-Wells reaction plates (Applied Biosystems). Each reaction (final volume 10 μ l) contained 25 ng DNA, 5 μ l of Power SYBR-Green PCR Master Mix (Applied Biosystems) and 0.5 μ M of each forward and reverse primer. COX1 gene was amplified and β 2 microglobulin (β 2m) was used as normalizing control.

MtDNA damage was quantified by long range PCR (35). A 10 Kb fragment and a shorter region of mtDNA were amplified. PCR reactions were limited to 18 cycles, to ensure that amplification process was still in the exponential phase. In order to compare mtDNA damage in each sample, PCR products were quantified using PicoGreen and the quantity of

the short range PCR product (Q) was normalized to amount of the long range PCR product (P) measured by analysis. The damage index is determined by the ratio of Q/P.

Primers sequences used for mtDNA analysis are:

Genes	Forward primer sequence	Reverse primer sequence
<i>B2M</i>	5'-AAATGCTGAAGAACGGGAAAA-3'	5-ATAGAAAGACCAGTCCTTGCTGAAG-3'
<i>TOP2B</i>	5'-GCTGATATGGAAAGGCATC-3'	5-GTAACCTACGCTGTCTCC-3,
Long range PCR	5'- CCATCTACCTTCTCAACCTCACC -3'	5'- GATGCTCGGATCCATAGGAATGTTG -3'
Short range PCR	5'- CAGGATTAAACTCAGATGCAG -3'	5'- CAAATAGGAAATATCATTCCGGT -3'

Western blotting

For detection of respiratory chain (RC) proteins, 50 mg of heart tissue were homogenized and lysed in RIPA buffer supplemented with 0.4 M NaCl and protease inhibitors (Roche Applied Science, Indianapolis, IN). After 1 hour at 4°C, lysates were centrifuged for 10 min at full speed and protein concentration in the supernatant was measured (Bio-Rad Protein Assay). 40 µg of protein were subjected to SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). After 1 h blocking with 5% milk in PBST (phosphate-buffered saline, Tween20 0.1%), membranes were incubated overnight with Anti-OxPhos Complex Kit antibody (#457999, Invitrogen). After three washes in PBST, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse (1:5,000 dilution) antibody (Amersham Biosciences, Piscataway, NJ) for 1 hour and then washed three times. Immunoblots were detected using enhanced chemiluminescence detection kit (Pierce).

For detection of Top1 and Top2β, 100 mg of heart tissue were trimmed to size of 1 mm³, homogenized and lysed in RIPA buffer supplemented with protease inhibitors. After 1 hour shaking at 4°C, lysates were centrifuged at full speed for 10 min at 4°C. Supernatant was discarded and the pellet was lysed a second time for 1 h in RIPA buffer supplemented with 0.4 M NaCl and protease inhibitors. After centrifugation, proteins in the supernatant were quantified and 40 µg were subjected to SDS-PAGE as described above. The primary antibodies used were: anti-Top1 (#556597, BD Pharmingen), anti-top2β (sc-25330, Santa Cruz) and anti-α-tubulin (#05-829, Millipore).

Histological analyses and immunofluorescence

Heart tissues were fixed in 10% phosphate buffered formalin, pH 7.4, at room temperature for 2 hours. Five microns sections from the paraffin-embedded hearts were stained with Hematoxylin and eosin for the analysis of nucleus hypertrophy. For Cardiomyocyte cross-dimensions analysis, heart sections were depareffined (3 times 20 min in Xylen at room temperature) and fixed with 4% formaldehyde in PBS for 1 h. After PBS washes, sections were fixed and permeabilized with pre-chilled (−20°C) 70% ethanol for 20 min and stained for 1 h with Wheat germ agglutinin coupled to Alexa Flour 488 (1:200; Wheat Germ Agglutinin, Alexa Fluor® 488 Conjugate, Invitrogen). Tissues were then washed with PBS,

and mounted using Vectashield mounting medium with DAPI (4', 6'-diamidino-2-phenylindole) to counterstain the DNA (Vector Laboratories, Burlingame, CA).

Slides after H&E staining were examined using high-resolution TV camera attached to a light microscope and the magnification was calibrated with a stage micrometer (Zeiss). Slides stained with wheat germ agglutinin were examined using a laser scanning confocal microscope (Zeiss LSM510). Images were collected and processed using the Zeiss AIM software. Nucleus size and cardiomyocyte areas were realized with ImageJ software. For each animal (n=4 for each condition) four to seven regions from sections of the right ventricular were counted and a mean value was obtained.

Results

Lack of Top1mt increases DOX-induced cardiac mitochondrial defects

To investigate the role of Top1mt in the adaptive response to DOX-induced cardiomyopathy, we treated Top1mt knockout (Top1mt^{-/-}) and wild type (Top1mt^{+/+}) mice born in similar litters from heterozygous (Top1mt^{+/-}) parents with DOX. Figure 1A shows our treatment scheme. Seven week-old mice were treated once a week with DOX at 4 mg/Kg or with saline solution (control) given by injections for 8 consecutive weeks. One week after the last injection, hearts were analyzed. Additional mice were followed for survival for up to 90 days after the last injection (see below and Figure 4).

Electron microscopy (EM) analysis of heart tissues showed no obvious difference in mitochondrial ultrastructure between WT and Top1mt KO mice treated with saline solution (Figure 1B, left panels). Accordingly, surface area analysis (Supplementary Fig. S1A) and mitochondria quantitation (Supplementary Fig. S1B) showed no significant difference between WT and Top1mt KO mice, and dense and regular cristae organizations were observed in both tissues. After DOX treatment, EM analyses showed Top1mt KO mice displaying more extensive mitochondrial damage compared to WT mice. Mitochondria were swollen (Figure 1B and supplementary Fig. S1A), and showed highly fragmented and degraded cristae (Figure 1B). In addition, compared to WT mice, the Top1mt KO mice showed an attenuated upregulation of mitochondria number in response to DOX (Supplementary Fig. S1B), indicating defective mitochondrion homeostasis in response to DOX for the Top1mt KO mice.

Top1mt is required to maintain heart mitochondrial biochemical functions and mtDNA integrity after DOX treatment

To determine whether the ultrastructural defects observed by EM were accompanied by mitochondrial dysfunction, mitochondria isolated from the heart tissue were examined biochemically. Immunoblotting showed that DOX markedly decreased the steady-state levels of complexes I, III and IV of the respiratory chain (RC) proteins in Top1mt KO mice (Figure 2A, right panel showing a representative heart muscle example, and Supplementary Fig. S2 for quantitation). Although, it is well known that complexes I and III, and especially complex IV are depleted in heart tissue after DOX treatment (36, 37), the decrease in those complexes, which are both nuclear and mitochondrial-encoded, was more dramatic in

Top1mt KO compared to WT mice (Supplementary Fig. S2B, and Figure 2A). On the other hand, proteins of complexes II and V, which are assembled even in the complete absence of mitochondrial protein synthesis, were unaffected (Figure 2A). The effect of DOX was specific for the heart muscle as the same RC proteins in skeletal muscle showed no difference after DOX treatment in both, WT and Top1mt KO mice (Supplementary Fig. S2A).

Complex IV activity was analyzed further by measuring cytochrome *C* oxidase activity in isolated heart mitochondria. Figure 2B shows that cytochrome *C* oxidase activity was decreased by 80% in Top1mt KO mice whereas it decreased only by 20% in WT mice. As the final electron acceptor in the electron transport chain is oxygen, we assessed mitochondrial respiration by measuring the rate of oxygen consumption in isolated mitochondria. Oxygen consumption was decreased by about 50% in Top1mt KO compared to WT mice treated with DOX (Figure 2C). Likewise, the membrane potential in isolated mitochondria from heart tissue decreased by 31% in Top1mt KO mice (Figure 2D). As mitochondrial dysfunction generates reactive oxygen species (ROS) (38) that are quenched by glutathione (GSH) (39), we measured reduced glutathione in Top1mt KO mice. Figure 2E shows that reduced glutathione decreased by $\approx 80\%$ in Top1mt KO mice whereas this level decreased by only $\approx 40\%$ in WT mice following DOX treatment (Figure 2E).

Each mitochondrion contains several mtDNA copies and prior observations point to the important contribution of direct and/or indirect mtDNA damage in DOX cardiotoxicity (40). Accordingly, we found that DOX decreased mtDNA copy number both in WT and Top1mt KO mice (Figure 2F). However, mtDNA depletion was significantly greater in the Top1mt KO mice (Figure 2F). Long range PCR was also performed to evaluate mtDNA damage (41). Figure 2G shows DOX-induced mtDNA damage both in WT and Top1mt KO mice. However, mtDNA damage was significantly greater in the Top1mt KO mice. The effects of DOX on the mtDNA of WT mice are consistent with previous studies (24-27). However, we show here for first time that lack of Top1mt accentuates mtDNA copy number loss and mtDNA damage.

Lack of Top1mt accentuates cardiomyocyte damage after DOX treatment

To further examine cardiomyocytes, cardiac sections were stained with fluorescein isothiocyanate-conjugated wheat germ agglutinin, which delineate cardiomyocyte dimensions by staining glycolipids and glycoproteins enveloping individual cells (42) (Figure 3A). DOX induced hypertrophy of individual cardiomyocytes in both WT (43) and Top1mt KO mice. However, the KO cardiomyocytes were significantly larger after DOX than those from WT mice (Figure 3A-B). In addition, hematoxylin and eosin (H&E) staining showed an increased cardiomyocyte nuclear size in Top1mt KO mice (Figure 3C-D). These results demonstrate that Top1mt activity prevents DOX-induced cardiomyocytes hypertrophy.

To address whether cardiomyocyte hypertrophy is accompanied by defects in cardiac muscle at the ultrastructural level, we analyzed heart tissue sections from Top1mt KO and WT mice by EM (Figure 3E). Such analysis revealed prominent defects in the hearts of Top1mt KO mice, with marked structure alterations of individual myofibrils after DOX treatment. At the

tissue level, the distance between individual cardiomyocytes was greater in Top1mt KO than in WT mice (Figure 3E, left panel, asterisks). At the intracellular level, several prominent defects in the myofibril structure could be observed. Top1mt KO mice exhibited a range of myofibril defects, including disintegrating sarcomeres with unevenly spaced filaments ‘fraying’ out of the myofibrils (Figure 3E, right panel, arrowheads).

Top1mt inactivation increases the lethality of DOX

In light of the accentuated heart abnormalities in the Top1mtKO mice, we followed the survival of seven pairs of animals (Top1mt KO vs. WT) for 90 days following the last DOX injection. None of the animals receiving saline died, whereas DOX reduced the survival of both Top1mt KO and WT mice (Figure 4). Notably, the Top1mt KO mice showed a markedly worse survival. All 7 Top1mt KO mice (100%) died within 45 days, which is in contrast to the WT mice group where only 1 of the 7 died at day 45, and 4 WT mice remained alive at day 90.

Discussion

Cumulative evidence indicates the importance of mitochondrial dysfunction as a predisposing and potentially causal factor for the cardiotoxicity of DOX. Our study adds novel evidence for this concept, which was recently proposed for Parkin in a myocardial infarction model (44). The difference is that Parkin is involved in mitochondrial recycling by mitophagy, whereas Top1mt is involved in mtDNA homeostasis (32).

The mechanism of mitochondrial toxicity of doxorubicin remains to be fully established. A recent study showing the involvement of nuclear Top2 β (22) questioned the prior notion that DOX poisons mitochondria by generating ROS. Moreover, Top2 β has been shown to present in bovine heart mitochondria (30). However, we found no evidence of Top2 β overexpression to account for the hypersensitivity of the Top1mt KO mice (Supplementary Fig. 3A-B). Figure 5 outlines our model explaining how Top2 β and Top1mt exert opposite effect on DOX-induced cardiotoxicity. While DOX traps Top2 β cleavage complexes, resulting in mitochondrial DNA damage and dysfunction (45, 46), Top1mt protects mitochondria (32) by maintaining normal mtDNA homeostasis and enabling damaged mtDNA to be replaced. Accordingly, tissue-specific mtDNA lesions, mtDNA copy loss and abnormal arrangements of cristae have been found in human heart patients exposed to DOX (26, 47). Mitochondrial protection is also supported as a cardioprotective strategy by recent evidence with mitochondrially-targeted redox active drugs in animal models (48-50).

Our study provides the first evidence that constitutive mtDNA alterations, exemplified by Top1mt deficiency, could help identify patients at risk of DOX cardiotoxicity. Notably, we found that potentially deleterious Top1mt variants exist in the normal population (Supplementary Fig. 4).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Statement of translational relevance

Doxorubicin (DOX) is one of the most widely used anticancer drugs. Yet, a significant number of patients treated with DOX develop cardiotoxicity. The exact mechanisms of DOX cardiotoxicity are likely multifactorial and complex, and identification of predicting factors for DOX toxicity remains a clinical challenge. Here, we show that the mitochondrial topoisomerase, Top1mt is critical to limit DOX cardiotoxicity. Top1mt knockout mice show hypersensitivity to DOX with significant mitochondrial dysfunction, including mtDNA and cristae ultrastructure damage and respiratory chain proteins loss. Top1mt knockout mice show heart muscle defects with increased death rate after treatment. Our study demonstrates the importance of mitochondrial DNA (mtDNA) regulation for DOX cardiotoxicity. Deleterious genomic variants for Top1mt should be tested in patients hypersensitive to DOX.

Figure 1. Increased mitochondrial damage in Top1mt KO mice after DOX treatment. **(A)** Treatment scheme: seven week old mice including paired Top1mt KO and WT mice from similar litters were treated once a week IP with DOX at 4 mg/Kg or with saline solution (controls) for 8 weeks. One week after the last treatment, heart tissues were analyzed. For survival study, animals were assessed up 90 days after the last injection. **(B)** Representative ultrastructure images of mitochondria obtained by electron microscopy from WT and Top1mt KO mice heart tissues: left panels after saline injection, and right panels after DOX treatment.

Figure 2.

Heart mitochondria and mtDNA alterations in Top1mt KO mice after DOX treatment. **(A)** Representative Western blots of respiratory chain subunits in WT and Top1mt KO mice from same litters (left: control saline injections; right after DOX). Western blot shows animals from the same litter. **(B)** Cytochrome C oxidase activity in heart tissue after saline injection or DOX treatment in WT and Top1mt KO mice ($n = 4$ for each condition). **(C)** Oxygen consumption rates of isolated mitochondria from mouse heart tissue after saline injection or DOX treatment ($n = 3$ for saline and $n = 5$ for DOX). **(D)** Mitochondrial membrane potential measured by staining isolated mitochondria from mouse heart tissue after saline injection or DOX treatment with JC-1 ($n = 3$ for saline injection and $n = 5$ for DOX treatment, *, $p < 0.05$, t test). **(E)** Drop in reduced glutathione in mouse heart tissue lysate after saline injection or DOX treatment ($n = 5$ for saline and $n = 8$ for DOX, **, $p < 0.006$, t test). **(F)** mtDNA copy number quantification in heart tissue after saline injection or DOX treatment. mtDNA copy number was expressed relative to WT after saline injection, set as 1. Normalized intensity values are on a binary log scale ($n = 6$ for saline injection and $n = 9$ for DOX treatments, **, $p < 0.006$, t test). **(G)** Left panel: representative agarose gel images of mtDNA long fragment (Long-F) and mtDNA short fragment (Short-F) PCR products of heart tissue after saline injection or DOX treatment. Top1mt KO and WT animal from the same litters were used. Right panel: ratio of Long fragment to short fragment PCR products quantified by PicoGreen. Normalized intensity values are on a

binary log scale (n = 5 for saline injection and n = 8 for DOX treatments, **, p<0.006, t test).

Figure 3.

DOX-induced cardiomyocyte hypertrophy and fiber damage in Top1mt KO mice. **(A)** Representative images of cardiac right ventricular sections stained with fluorescein isothiocyanate-conjugated wheat germ agglutinin. **(B)** Quantitation of mean mitochondrial area in Top1mt KO and WT hearts (*, $p < 0.05$, t test). **(C)** Representative H&E staining of longitudinal sections of the right ventricular heart. Black arrows indicate hypertrophic nuclei. **(D)** Quantification of nuclear sizes obtained by measuring the longer diameter after H&E staining (***, $p < 0.0001$, t test). **(e)** Representative electron microscopy images of heart muscle from WT and Top1mt KO mice after DOX treatment. Left: red asterisks indicate distances between individual cardiomyocytes. Right: red arrowheads indicate myofibril defects.

Figure 4.

Decreased survival of Top1mt-deficient mice after DOX treatment. Survival of mice receiving DOX was assessed for 90 days after last treatment. Data are plotted as Kaplan-Meier cumulative survival curves. P-value was determined using the log-Rank test. None of the control animals receiving saline died (n = 7 for each condition).

Figure 5. Schematic representation of the mechanism by which Top1mt influences DOX cardiotoxicity. Poisoning of Top2β by DOX damages mtDNA while Top1mt limit mtDNA damage.