Lack of mitochondrial topoisomerase I (*TOP1mt*) impairs liver regeneration

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The liver has an exceptional replicative capacity following partial hepatectomy or chemical injuries. Cellular proliferation requires increased production of energy and essential metabolites, which critically depend on the mitochondria. To determine whether Top1mt, the vertebrate mitochondrial topoisomerase, is involved in this process, we studied liver regeneration after carbon tetrachloride (CCl_4) administration. *TOP1mt* knockout (KO) mice showed a marked reduction in regeneration and hepatocyte proliferation. The hepatic mitochondrial DNA (mtDNA) failed to increase during recovery from CCl_4 exposure. Reduced glutathione was also depleted, indicating increased reactive oxygen species (ROS). Steady-state levels of ATP, O_2 consumption, mtDNA, and mitochondrial mass were also reduced in primary hepatocytes from CCl_4-treated KO mice. To further test whether Top1mt acted by enabling mtDNA regeneration, we tested *TOP1mt* KO fibroblasts and human colon carcinoma HCT116 cells and measured mtDNA after 3-d treatment with ethidium bromide. Both types of *TOP1mt* knockout cells showed defective mtDNA regeneration following mtDNA depletion. Our study demonstrates that Top1mt is required for normal mtDNA homeostasis and for linking mtDNA expansion with hepatocyte proliferation.

liver regeneration | mitochondrial topoisomerase I | cell proliferation | mitochondrial DNA replication | mitochondrial homeostasis

Individual cells contain hundreds to thousands of copies of mitochondrial DNA (mtDNA), which are essential for cellular metabolism because each mtDNA molecule encodes 13 indispensible genes (that complement the 153 nuclear encoded genes) involved in mitochondrial electron transport and ATP production (for review, see ref. 1). Given that mtDNA exists as small circular double-stranded DNA molecules (16,569 bp) with bidirectional replication and transcription, and is organized in nucleoids that are anchored to the mitochondrial inner membrane, topoisomerases are required for mtDNA replication and transcription.

Type IB topoisomerases are ubiquitous enzymes that dissipate the DNA topological stress generated during replication and transcription by rapidly and reversibly breaking and rejoining the phosphodiester DNA backbone (for review, see refs. 2–4). They act both in the nucleus and in mitochondria (5), but each compartment has its own enzyme: Top1mt being mitochondrial-specific (6) and topoisomerase I (Top1) nuclear-specific (6, 7). In addition to its roles in regulating mtDNA replication (8), transcription (9), and mtDNA integrity (10), Top1mt participates in mitochondrial functions. Indeed, murine embryonic fibroblasts (MEFs) generated from *TOP1mt* KO mice have mitochondrial defects, including excessive reactive oxygen species (ROS) production and altered polarization of mitochondrial membranes (11).

Regulation of mtDNA copy number is crucial to maintain mitochondrial function and ATP production during cell proliferation (12). The liver has an exceptional proliferative capacity; it can rapidly regenerate its mass both in human and animals after partial hepatectomy or viral or chemical injuries (13–15). Liver regeneration is also key to successful partial liver transplantations from living donors (small-for-size liver transplants) (for review, see ref. 14). A common and convenient model to study liver regeneration is carbon tetrachloride (CCl_4) exposure. CCl_4 is one of the most potent hepatotoxins. It is widely used to evaluate antioxidant agents, mechanisms of hepatic injury, pathogenesis of cirrhosis, and liver regeneration. CCl_4 is metabolized in the liver by cytochrome P450 2E1 (CYP2E1) to trichloromethyl radical (CCl_3*), which initiates free radical-mediated lipid peroxidation. Accumulation of lipid-derived oxidative products then causes liver injury (16). This damage triggers the activation and coordination of multiple intracellular and intercellular pathways to regenerate the liver mass and meet the metabolic needs of the organism (13, 14, 16). Notably, hepatocytes are packed with mitochondria.

To address the role of mtDNA in liver regeneration, we took advantage of the fact that mice lacking *TOP1mt* are viable (5, 11, 17). We suspected that Top1mt might play a role in liver regeneration because we recently uncovered a critical role for *TOP1mt* in maintaining mtDNA homeostasis and mitochondrial function in adaptive response to doxorubicin-induced cardiotoxicity (17). Indeed, the cardiomyocytes of *TOP1mt* KO mice exhibit pronounced mtDNA damage, and fail to maintain respiratory chain protein production and mitochondrial cristae

Significance

The liver is rich in mitochondria and has an exceptional regenerative capacity after partial hepatectomy or transplantation, viral infections, or chemical injuries; however, relatively little is known about the genetic factors for mitochondrial DNA (mtDNA) replication during liver regeneration. Here, we show that liver regeneration is markedly reduced in mice lacking mitochondrial topoisomerase I (*TOP1mt*). This defect is linked with reduced production of mtDNA and defective mitochondrial functions during acute energy demand for liver regeneration. Additionally, *TOP1mt* KO primary hepatocytes from CCl_4-treated mice showed reduced and damaged mitochondria, decreased O_2 consumption, and ATP production. Together with mtDNA depletion and regeneration experiments with ethidium bromide, these results demonstrate that Top1mt is required for mtDNA synthesis and appropriate liver regeneration.

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ultrastructure organization, resulting in decreased O_2 consumption, increased ROS production, and severe heart muscle damage (17). In the present study, we show that Top1mt is critical for mtDNA synthesis and appropriate cell proliferation to meet the massive energy demand during liver regeneration after CCl_4 injury.

Results

Top1mt Supports Cellular Replication and Liver Recovery After CCl_4 Injury. To assess the role of *TOP1mt* in liver regeneration, we compared *TOP1mt* knockout (*TOP1mt* KO) and wild-type (*TOP1mt* WT) mice born in the same litters from heterozygous (*TOP1mt*$^{+/-}$) parents. Seven-week-old mice received a single i.p. injection of CCl_4 [0.5 mL/kg body weight; 10% (vol/vol) CCl_4 in olive oil] or the vehicle, olive oil alone (control). Livers were analyzed 2 and 4 d after CCl_4 injection (Fig. 1*A*).

Following CCl_4 injection, liver regeneration is known to cause a rapid increase in liver weight (18). To evaluate the changes in liver mass, the ratio of liver to body weight was used to avoid any bias between animals having different body weights. No difference was observed between WT and *TOP1mt* KO mice after olive oil injection (Fig. 1*B*), which is consistent with the fact that *TOP1mt*-deficient mice are normal in size. By contrast, administration of CCl_4 resulted in a marked difference in liver to body weight between WT and *TOP1mt* KO mice. Two days after CCl_4 injection, liver to body weight increased by 35% in WT animals, whereas this increase was only 10% in the *TOP1mt* KO mice (Fig. 1*B*). This difference was liver-specific because body weights after CCl_4 injection were the same for the WT and *TOP1mt* KO mice (both losing approximately 6% of their original weight; Fig. S1).

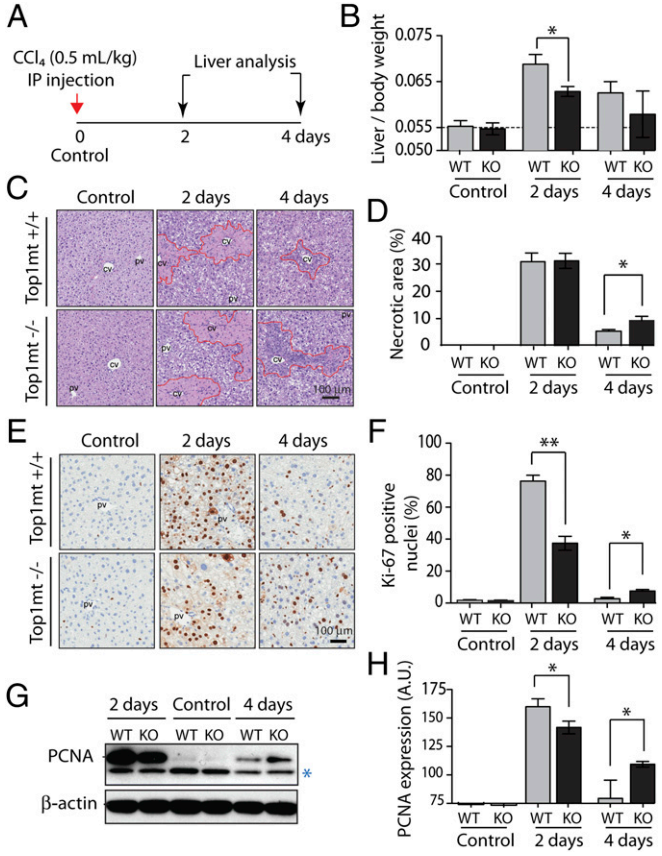

Fig. 1. Defective liver regeneration in *TOP1mt* knockout mice. (*A*) Treatment scheme: Seven-week-old paired *TOP1mt* KO and wild-type (WT) mice from same litters were treated with a single i.p. injection of CCl_4 0.5 mL/kg body weight or with olive oil as solvent control. Two and four days later, liver tissues were analyzed ($n = 5$ for control and $n = 8$ for CCl_4-treated mice). (*B*) Liver/body weight ratios. (*C*) H&E staining of liver tissue sections. CCl_4 induces necrosis in centrilobular areas around central vein (CV). (*D*) Quantification of necrotic areas around central veins (outlined with red lines). (*E*) Ki-67 IHC staining of liver tissue sections. Replication starts from the portal vein (pv) and migrates to replace the necrotic area around the CV. (*F*) Quantification of Ki-67–positive nuclei. (*G*) Western blot analysis of PCNA protein expression levels in liver tissues at days 2 and 4 of recovery. *, nonspecific band serving as loading control together with β-actin. (*H*) Quantification of PCNA expression level. *C*, *E*, and *G* show representative experiments. (*B*, *D*, *F*, and *H*) $*P < 0.05$, $**P < 0.01$, ANOVA test.

Because CCl_4 induces necrosis in the centro-lobular zones of the hepatic lobules (reviewed in ref. 14), sections of the liver tissue were analyzed after H&E staining (Fig. 1 *C* and *D*). Two days after treatment, areas of necrosis around the central vein (CV) were similar in WT and *TOP1mt* KO mice. This result was paralleled by a sharp and comparable release into blood of alanine aminotransferase (ALT), a sensitive indicator of hepatocyte integrity. Transaminases that catalyze the transfer of the amino group (−NH2) of an amino acid to a carbonyl compound are abundant in the hepatocytes and very low in the blood. Upon hepatocyte damage, the enzymes are released into the bloodstream. In both WT and KO mice, ALT levels increased ∼15-fold after 2 d of recovery and returned to the basal levels at day 4 (Fig. S2). By this time, almost all necrotic areas in WT mice were cleared and replaced by regenerating parenchyma (Fig. 1*C*). Notably, the remaining necrotic and inflamed areas still persisted at day 4 after recovery in *TOP1mt* KO mice (Fig. 1*C*).

To determine whether Top1mt affects cellular proliferation during liver regeneration, we used two classical biomarkers: Ki-67 and PCNA (proliferating cell nuclear antigen). Ki-67 staining revealed that *TOP1mt* KO mice were unable to elicit an adequate proliferative response to CCl_4-mediated liver tissue injury (Fig. 1*E*), demonstrating that the fraction of proliferating cells was significantly reduced in the *TOP1mt* KO mice. After 2 d, more than 70% of WT hepatocytes were Ki-67–positive compared with less than 40% in *TOP1mt* KO livers (Fig. 1*F*). In support of these results, CCl_4 treatment also induced a stronger PCNA expression in WT mice 2 d after CCl_4 injection (Fig. 1 *G* and *H*). Notably, both the Ki-67 staining and PCNA expression decreased progressively in WT mice 4 d after injury, reflecting a successful completion of liver regeneration. In contrast, both proliferative markers were expressed at higher levels in *TOP1mt* KO mice 4 d after injury, indicative of a delayed liver regeneration in the absence of Top1mt (Fig. 1 *C–H*). Together, these results demonstrate that genetic deletion of *TOP1mt* does not affect the extent of the initial tissue damage but leads to attenuated hepatocyte replication and delayed liver regeneration.

Top1mt Enables mtDNA Expansion During Liver Regeneration. Dividing cells have to be metabolically balanced to respond to energy demand (11, 19). Fig. 2*A* shows that 2 and 4 days after CCl_4 administration, mtDNA copy number [relative to nuclear DNA per cell normalized to the housekeeping nuclear gene β2 microglobulin (β2m)] was significantly reduced in KO mice (approximately 20% loss), whereas under the same conditions, matched WT siblings showed a slight increase in mtDNA copy number. Southern blot analysis also showed loss of liver mtDNA in KO mice (Fig. 2*B*). Mitochondrial DNA copy number is tightly regulated in a cell- and tissue-specific manner (20, 21). The decrease of mtDNA copy number observed in *TOP1mt* KO mice suggests that during acute hepatocytes proliferation, Top1mt is required for the appropriate expansion of mtDNA in the daughter cells.

Because mtDNA is packaged in nucleoids attached to the inner mitochondrial membrane near the respiratory chain proteins, the source of ROS, we tested whether mtDNA deficiency

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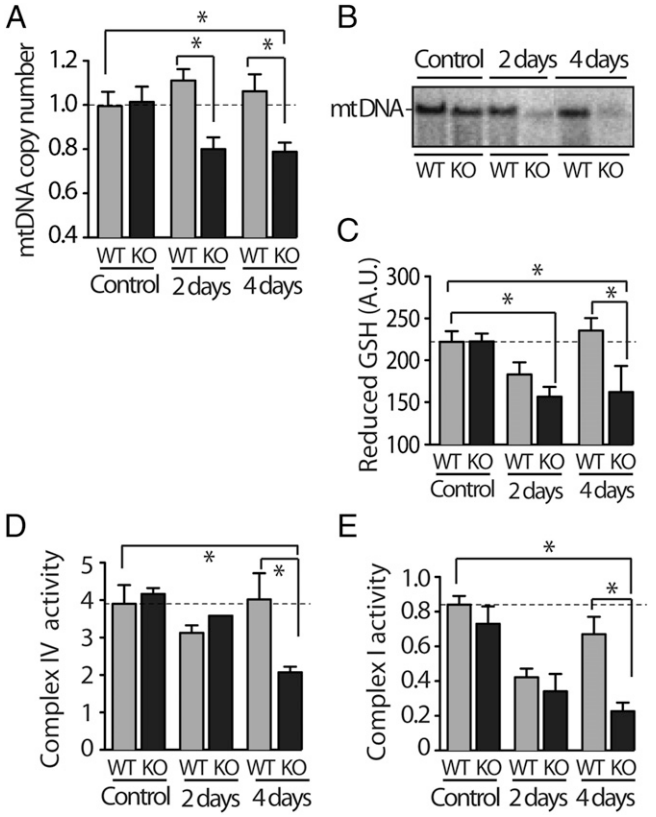

Fig. 2. Defective mtDNA and mitochondrial functions in liver tissue of *TOP1mt* knockout mice following CCl_4 administration. (*A*) MtDNA copy number was quantified relative to nuclear DNA and expressed relative to untreated (control) wild type (WT) ($n = 8$ for control and $n = 8$ for CCl_4 administration, $^*P < 0.05$, ANOVA test). (*B*) Agarose/Southern blot analysis of linearized mtDNA. (*C*) Drop in reduced glutathione in mouse liver tissue lysates from *TOP1mt* KO mice following CCl_4 exposure ($n = 5$ for control and $n = 8$ for CCl_4, $^*P < 0.05$, unpaired Student's *t* test). (*D* and *E*) Complex IV and complex I activities in isolated mitochondria from liver tissue, respectively. Data were normalized to citrate synthase activity.

in *TOP1mt* KO mice was associated with changes in reduced glutathione (GSH), the main quencher of ROS. Administration of CCl_4 significantly depleted the hepatic GSH levels (by 25%) both in WT and *TOP1mt* KO mice at day 2 after treatment (Fig. 2*C*), which is consistent with the reports that CCl_4 metabolism releases free radicals causing acute GSH depletion (22). Four days after treatment, the level of GSH in WT mice completely recovered and reached the basal level, whereas GSH remained low in *TOP1mt* KO mice (Fig. 2*C*), indicating the persistence of ROS in *TOP1mt* KO mice.

Because mtDNA alterations lead to respiratory chain defects, we assessed the enzymatic activities of the mitochondrial complexes IV and complex I in isolated mitochondria from liver tissue. All activities were normalized to the Krebs cycle enzyme citrate synthase (control for the amount of mitochondria used to measure the activity of each complex; Fig. S3). Fig. 2 *D* and *E* show that the activities of complexes IV and I were decreased by 45% and 75%, respectively, in *TOP1mt* KO mice at day 4 after CCl_4 treatment. We also tested the expression of the nuclear-encoded respiratory chain protein, COX4, which is part of the oxidative chain phosphorylation complex IV. CCl_4 markedly decreased the steady-state levels of COX4 in *TOP1mt* KO livers, consistent with the fact that respiratory chain proteins that fail to assemble in stable complexes are quickly degraded (23) (Fig. S4). However, ATP5A, a component of complex V, which is assembled even in the complete absence of mitochondrial protein synthesis (24), remained unaffected in both WT and *TOP1mt* KO mice (Fig. S4). These mitochondrial defects were accompanied by a slight increase in caspase-3 cleavage, indicative of apoptosis in *TOP1mt* KO mice at day 4 (Fig. S5*A*). Marked liver damage was also observed by increased Oil Red O staining in *TOP1mt* KO-regenerating livers 4 d after CCl_4 injection (Fig. S5*B*). Together, these results demonstrate that mtDNA as well as mitochondria and hepatocyte functions are defective in *TOP1mt* KO mice after CCl_4 treatment.

Lack of *TOP1mt* Impairs Mitochondrial Functions and Steady-State Level of ATP in Primary Hepatocytes from Mice Treated with CCl_4. Because primary hepatocytes allow direct biochemical analyses, we isolated hepatocytes by a two-step collagenase perfusion followed by Percoll purification (25). Cells were harvested 4 d after CCl_4 exposure and cultured for 24 h to allow reconstitution of cell polarity and function before analyses (26). The yield of viable *TOP1mt*-deficient hepatocytes was decreased more than twofold compared with the matched WT cells (Fig. S6), which is consistent with the reduced proliferation (Fig. 1 *E* and *F*) and more extensive hepatocyte damage (Fig. S5*B*) observed in the regenerating liver of the *TOP1mt* KO mice.

Measurement of mtDNA confirmed copy number deficiency in the primary hepatocytes from the CCl_4-treated KO mice (Fig. 3*A*; consistent with Fig. 2 *A* and *B*). Steady-state levels of intracellular ATP were also markedly reduced (threefold lower in KO than for WT hepatocytes; Fig. 3*B*). Because defective ATP production due to dysfunctions of mitochondrial oxidative phosphorylation (OXPHOS) induces a compensatory increase in aerobic glycolysis, we analyzed the oxygen consumption rate (OCR, a measure of OXPHOS) and the extracellular acidification rate (ECAR, a measure of lactate production and glycolysis) (Fig. 3 *C*–*E*). Hepatocytes from KO-treated mice showed significantly lower basal OCR (Fig. 3*C*), indicating impaired mitochondrial respiration (Fig. S7). ECAR levels were elevated in both WT and KO hepatocytes from treated mice compared with untreated mice (Fig. 3*D*). Fig. 3*E* shows similar metabolic profiles for the hepatocytes from untreated WT and KO mice. However, the metabolic profiles of the WT and KO hepatocytes from CCl_4-treated mice were different. WT hepatocytes showed robust glycolytic and respiratory activities, which is consistent with high metabolic activity to support liver regeneration, whereas the KO hepatocytes displayed a marked reduction in OCR and high ECAR, indicative of their reliance on glycolysis for energy production.

Transmission electron microscopy was performed to assess the ultrastructure and density of mitochondria in the primary hepatocyte cultures (Fig. 3 *F* and *G* and Fig. S8). There were no obvious differences in the number and ultrastructure of mitochondria in primary hepatocytes isolated from the untreated WT and *TOP1mt* KO livers. However, in the hepatocytes from CCl_4-treated mice, we observed that the KO hepatocytes displayed a significant reduction in mitochondrial density compared with the corresponding WT heptatocytes (Fig. 3*F*). In addition, the KO hepatocytes showed signs of a more extensive structural damage. The most obvious were changes in the appearance of mitochondrial cristae and disorganization and dilation of endoplasmic reticulum and frequent lipid droplets (Fig. 3*G* and Fig. S8). The accumulation of more progressive ultrastructural alterations in *TOP1mt* KO cells exposed to CCl_4 could contribute to the low yield of viable hepatocytes (Fig. S5) and diminished functional performance. These data show that loss of Top1mt reduces the fitness and adaptive responses of hepatocytes.

Top1mt Supports mtDNA Replication in MEFs and Human Cancer Cells. Top1mt sites are enriched in mtDNA replication and transcription regulatory regions (8, 27). Based on our results showing an

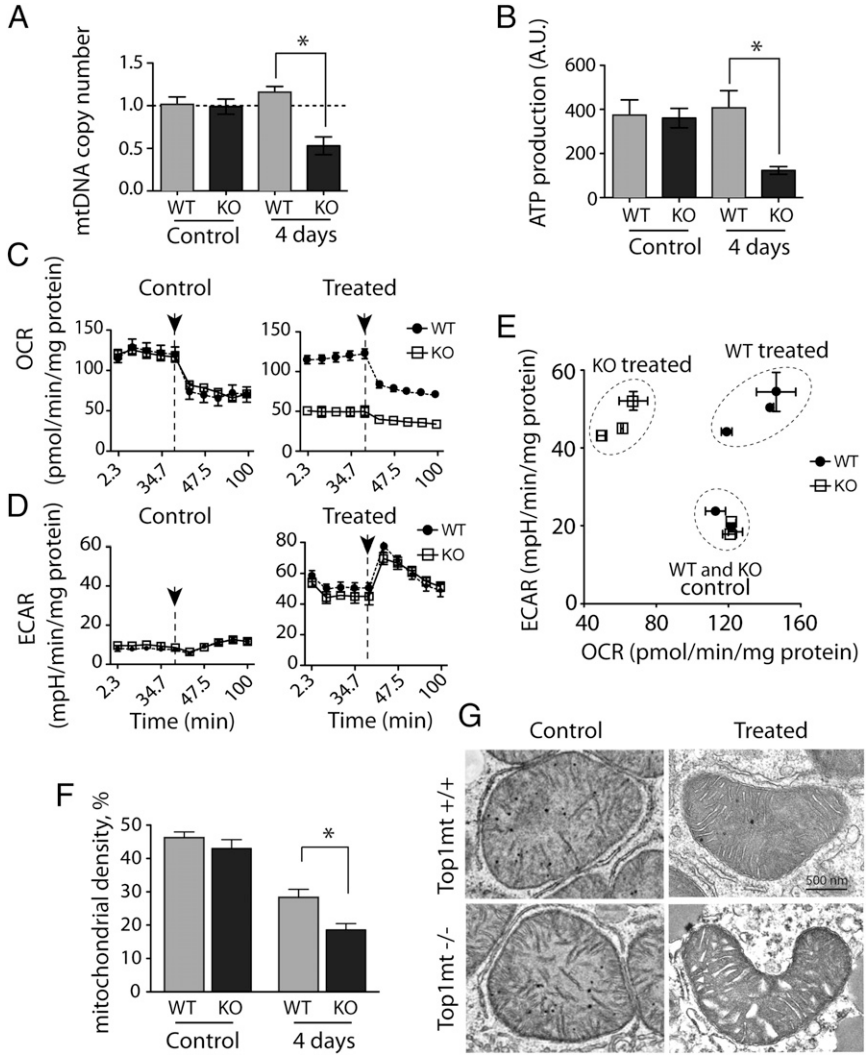

Fig. 3. Defective mitochondrial functions in primary hepatocytes from *TOP1mt* knockout mice isolated four days after CCl_4 treatment. (*A*) MtDNA copy number was quantified relative to nuclear DNA, and expressed relative to untreated (control) WT set as 1. (*B*) Intracellular ATP levels determined by the ATPlite luminescence assay. Oxygen consumption rate (OCR, a measure of OXPHOS) (*C*) and extracellular acidification rate (ECAR, a measure of lactate production and glycolysis) (*D*) measured by Seahorse XF296 Extracellular Flux Analyzer. Arrows indicate addition of oligomycin to inhibit mitochondrial respiration. (*E*) Defective metabolic profile (based on relative values of OCR and ECAR) of *TOP1mt* KO livers. Three KO and their paired WT siblings were included in each group. (*F*) Mitochondrial density assessed by electron microscopy ($n = 10$ cells for each genotype/group). *$P < 0.05$, paired t test. (*G*) Representative ultrastructure images of mitochondria from WT and *TOP1mt* KO primary hepatocytes from untreated and treated animals. (Scale bar: 500 nm.) Full-size images are shown in Fig. S8.

uncoupling of mtDNA replication and cellular proliferation (Figs. 2 and 3), we tested whether cells lacking *TOP1mt* were defective in mtDNA synthesis. We took advantage of the fact that ethidium bromide (EB) selectively depletes mtDNA and that mtDNA recovers within days following EB removal (see protocol; Fig. 4*A*) (28, 29). These results show that mtDNA depletion was similarly effective in WT and *TOP1mt* KO murine embryonic fibroblasts (MEFs) after a 3-d EB treatment. However, mtDNA copy number recovered at different rates in WT and *TOP1mt* KO cells. WT MEFs recovered almost 90% of their initial mtDNA compared with 50% in *TOP1mt* KO MEFs after 9 d of recovery, and it took 15 d to regenerate 90% of mtDNA in the KO MEFs (Fig. 4*B*).

To further substantiate the role of Top1mt in mtDNA replication, we generated human colon carcinoma HCT116 cells, in which *TOP1mt* had been inactivated by using CRISPR/Cas9 (Fig. S9). Consistent with the results obtained in the MEFs (11) and hepatocytes (Fig. 3 *C*–*E* and Fig. S7), the *TOP1mt* KO HCT116 cells also displayed a defective mitochondrial respiration (Fig. S10), and delayed mtDNA recovery after EB treatment (Fig. 4*C*). These results were reproduced in two *TOP1mt* KO clones compared with the isogenic HCT116 WT cells (Fig. 4*C*). Taken together, these experiments demonstrate the importance of Top1mt for accelerated mtDNA synthesis and mitochondrial function.

Discussion

Diverse tissues have different metabolic profiles because of their inherently different functions. In addition, energy requirements need to be adjusted to physiological or pathological conditions. Hepatocytes are rich in mitochondria, and during liver regeneration, the cellular energy demand for biosynthesis of cellular components increases considerably. Building on the fact that the capacity of the liver to regenerate is correlated with efficient oxidative phosphorylation (OXPHOS) (30), we demonstrate the critical importance of mtDNA synthesis for liver regeneration. Mice lacking the mitochondrial topoisomerase gene *TOP1mt* are deficient in liver regeneration, due to decreased mtDNA and mitochondrial functions. The molecular mechanisms underlying

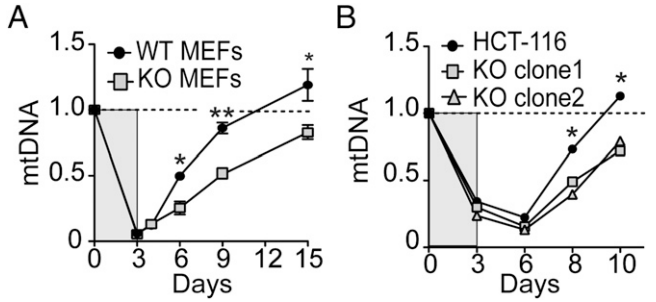

Fig. 4. Delayed mtDNA recovery after EB treatment in *TOP1mt* knockout cells. Cells were treated with 1 μg/mL EB for 3 d. After washing cell cultures free of EB, mtDNA was quantified at the indicated days. MtDNA copy number in murine embryonic fibroblasts MEFs from knockout (KO) and wild-type (WT) mice (*A*), and in human colon carcinoma HCT116 cells (WT and *TOP1mt* KO; Figs. S9 and S10) following EB treatment (*B*). MtDNA copy number is expressed relative to nontreated cells (set as 1). $n = 3$, $^*P < 0.05$, unpaired Student's *t* test.

the role of Top1mt in mtDNA adaptation are demonstrated by experiments showing that cells lacking Top1mt have impaired mtDNA synthesis. During cellular proliferation, mitochondrial mass and mtDNA must expand to provide an appropriate amount to daughter cells. Therefore, the decrease of mtDNA copy number observed in KO mice demonstrates that during acute hepatocytes proliferation, Top1mt is required to expand an appropriate number of mtDNA molecules to the daughter cells (Fig. 5).

Intact mtDNA is required for the production of the key catalytic subunits of the mitochondrial respiratory chain and, therefore, for ATP production. Here, we show that lack of Top1mt impairs mtDNA copy number expansion, delaying liver regeneration. However, lack of Top1mt was not lethal after CCl_4 exposure, which is consistent with the fact that mtDNA copy number varies among individuals, and that hepatocytes have a surplus of mtDNA. We also demonstrate that mitochondria are defective and ROS accumulate following CCl_4 treatment in the regenerating liver of *TOP1mt* KO mice. The importance of *TOP1mt* for mitochondrial regeneration and function are consistent with our recent finding that, in the heart, lack of *TOP1mt* accentuates mtDNA copy number loss, decreases respiratory chain protein expression levels, damages cristae ultrastructure organization, and increases ROS production after doxorubicin treatment (17).

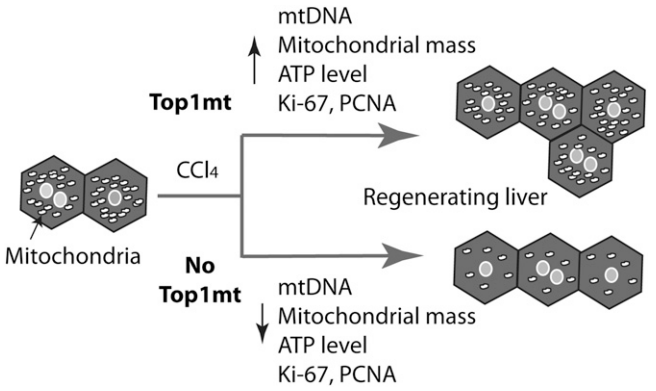

Fig. 5. Model for differential liver regeneration after CCl_4 injection in WT and *TOP1mt* KO mice. CCl_4 administration triggers hepatocytes replication. Top1mt allows mtDNA and mitochondrial mass to increase proportionally to support liver regeneration. Lack of Top1mt limits mtDNA replication, leading to decreased mtDNA in daughter cells, reduced mitochondrial mass and ATP production, and delayed liver regeneration.

Mitochondrial biogenesis occurs by division of preexisting organelles in coordination with cellular proliferation and nuclear DNA replication (12). Here, we show that Top1mt is required to expand and maintain the relative mitochondrial mass during acute hepatocytes proliferation after CCl_4 administration (Fig. 5). Mitochondrial biogenesis is also up-regulated to increase energy production during exercise (31) and to compensate for mitochondrial dysfunction and preserve cellular ATP synthesis (32). In the case of liver regeneration, many studies have shed light on the molecular pathways controlling mitochondrial biogenesis (for review, see refs. 13 and 33). However, until now, the role of *TOP1mt* in regulating mtDNA copy number was unknown (20).

Under normal conditions, *TOP1mt* KO mice do not show significant mtDNA copy number depletion in the heart (17) and liver (current study). Moreover, *TOP1mt* KO mice exhibit normal life span and reproductive cycle (5). This normal phenotype was initially unexpected (5) because knocking out the other topoisomerases genes results in severe phenotypes. The nonessentiality of *TOP1mt* is likely due to the compensation of most Top1mt functions by other topoisomerases. Topoisomerases IIα and IIβ have both recently been shown in vertebrate mitochondria (5) as well as Top3α (34, 35). Under stress conditions [doxorubicin treatment for the heart (17) or CCl_4 administration for the liver (present study)], lack of Top1mt becomes rate limiting by failing to increase mtDNA copy number. Thus, Top1mt appears to be primarily required to sustain acute mtDNA replication under stress conditions. Under such acute mtDNA replication, the compensation by other topoisomerases appears insufficient to appropriately regenerate mtDNA.

Drug-induced liver injuries and successful partial liver transplantations differ across individuals. The present study demonstrates the importance of Top1mt for mtDNA homeostasis during tissue regeneration. Because potentially deleterious genomic variants are present for *TOP1mt* in the normal population (17), further studies are warranted to determine whether deleterious genomic variant of *TOP1mt* and other mitochondrial DNA enzymes, which are all coded in the nucleus contribute to interindividual susceptibilities.

Materials and Methods

Mouse Handling. $TOP1mt^{+/+}$ (WT) and $TOP1mt^{-/-}$ (KO) mice were generated from heterozygous ($TOP1mt^{+/-}$) parents. Each KO mouse had at least one sibling WT as control. Mice were genotyped by PCR using genomic DNA from mice tail tips (5). Three primers were used as follows: *TOP1mt-A* (5′-GGTGCTAGACATTGAACTCAG-), *TOP1mt-B* (5′-CTGCAAATGGCCTCGTTAGC), and *TOP1mt-C* (5′-GTCCTGGATTCCATCTTAAGC). KO and WT gave 254-and 306-bp PCR products, respectively (5). Seven-week-old mice were treated with a single i.p. injection of CCl_4 [0.5 mL/kg body weight of CCl_4 in 100 μL of 10% (vol/vol) CCl_4 in olive oil]. Control animals received 100 μL of olive oil. Experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of the National Institutes of Health (protocol LMP-010).

Immunohistochemistry (Ki-67) and Hematoxylin/Eosin (H&E) Staining. Livers were fixed in 10% (vol/vol) neutral-buffered-formalin. Five-micrometer paraffin sections were stained with anti-Ki-67 (ab16667; Abcam). Details are provided in *SI Materials and Methods*.

Western Blotting. For detection of PCNA, actin, and respiratory chain proteins, 50 mg of liver tissue were homogenized and lysed in RIPA buffer (89900; Thermo Scientific) supplemented with 0.4 M NaCl and protease inhibitors mixture (Roche Applied Science). Further details are provided in *SI Materials and Methods*.

Quantification of mtDNA by PCR and Southern Blotting. Analyses were performed in liver tissue, primary hepatocytes, and in human colon carcinoma cells (HCT116) as described in *SI Materials and Methods*.

ROS Production Measured by Glutathione Assay. ROS production was measured by quantifying reduced glutathione (GSH) in liver tissue. GSH levels

were assessed in 50 mg of tissue lysates by using the luminescence-based GSH-Glo Glutathione Assay (Promega) according to the manufacturer's protocol.

Mitochondrial Enzymatic Activities. The activities of the complexes I and IV and the Krebs enzyme, citrate synthase, in isolated mitochondria from liver tissue were measured at 37 °C on Beckman DU-640B spectrophotometer (Beckman Coulter) by using standard methods (36). The activities of complexes I and IV were normalized to citrate synthase activity.

Primary Hepatocyte Isolation and Culture. Hepatocytes were isolated by two-step collagenase perfusion of the mouse livers followed by isodensity purification in Percoll gradient (25). After quantification of viable cells with 0.4% Trypan blue (T10282; Life Technologies), cells were seeded in 96-well plates (for Seahorse analysis and ATP production) and in six-well plates [for electron microscopy (EM) analysis] in the plating medium supplemented with 10% (vol/vol) FBS (25). After 4 h, the plating medium was replaced by serum-free medium. Cells were cultured for 24 h before analyses.

Measurements of Extracellular Acidification and Oxygen Consumption Rate. The XF96 Extracellular Flux Analyzer (Seahorse Bioscience) was used to detect rapid, real-time changes in cellular respiration and glycolysis rate as described (37). Details are provided in *SI Materials and Methods*.

Transmission Electron Microscopy. Details are provided in *SI Materials and Methods*.

Generation of Human *TOP1mt* KO Cells. *TOP1mt* KO HCT116 cells were generated by CRISPR genome editing (38) targeting exon 6 of *TOP1mt* (see details in *SI Materials and Methods*). Two independent clones (clones 1 and 2) showing undetectable Top1mt expression were used.

MtDNA Recovery After EB Depletion. WT and *TOP1mt* KO murine embryo fibroblasts (MEFs) and human colon carcinoma HCT116 cells were seeded in six-well plates at 20% confluence. After 24 h, cells were incubated with or without 0.1 μg/mL EB. After 3 d, cells were washed at least five times with 3 mL of medium without EB. For each washing time, cells were incubated with washing medium for 30 min. After washing, mtDNA was quantified after 1, 3, 6, 12, and 15 d of recovery.

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1. Bonawitz ND, Clayton DA, Shadel GS (2006) Initiation and beyond: Multiple functions of the human mitochondrial transcription machinery. *Mol Cell* 24(6):813–825.
2. Champoux JJ (2001) DNA topoisomerases: Structure, function, and mechanism. *Annu Rev Biochem* 70:369–413.
3. Pommier Y (2013) Drugging topoisomerases: Lessons and challenges. *ACS Chem Biol* 8(1):82–95.
4. Pommier Y, Leo E, Zhang H, Marchand C (2010) DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. *Chem Biol* 17(5):421–433.
5. Zhang H, et al. (2014) Increased negative supercoiling of mtDNA in TOP1mt knockout mice and presence of topoisomerases IIα and IIβ in vertebrate mitochondria. *Nucleic Acids Res* 42(11):7259–7267.
6. Zhang H, et al. (2001) Human mitochondrial topoisomerase I. *Proc Natl Acad Sci USA* 98(19):10608–10613.
7. Dalla Rosa I, et al. (2009) Adaptation of topoisomerase I paralogs to nuclear and mitochondrial DNA. *Nucleic Acids Res* 37(19):6414–6428.
8. Zhang H, Pommier Y (2008) Mitochondrial topoisomerase I sites in the regulatory D-loop region of mitochondrial DNA. *Biochemistry* 47(43):11196–11203.
9. Sobek S, et al. (2013) Negative regulation of mitochondrial transcription by mitochondrial topoisomerase I. *Nucleic Acids Res* 41(21):9848–9857.
10. Medikayala S, Piteo B, Zhao X, Edwards JG (2011) Chronically elevated glucose compromises myocardial mitochondrial DNA integrity by alteration of mitochondrial topoisomerase function. *Am J Physiol Cell Physiol* 300(2):C338–C348.
11. Douarre C, et al. (2012) Mitochondrial topoisomerase I is critical for mitochondrial integrity and cellular energy metabolism. *PLoS One* 7(7):e41094.
12. Lee S, Kim S, Sun X, Lee JH, Cho H (2007) Cell cycle-dependent mitochondrial biogenesis and dynamics in mammalian cells. *Biochem Biophys Res Commun* 357(1):111–117.
13. Böhm F, Köhler UA, Speicher T, Werner S (2010) Regulation of liver regeneration by growth factors and cytokines. *EMBO Mol Med* 2(8):294–305.
14. Mao SA, Glorioso JM, Nyberg SL (2014) Liver regeneration. *Transl Res* 163(4):352–362.
15. Durand F, et al. (2002) Auxiliary liver transplantation for fulminant hepatitis B: Results from a series of six patients with special emphasis on regeneration and recurrence of hepatitis B. *Liver Transpl* 8(8):701–707.
16. Manibusan MK, Odin M, Eastmond DA (2007) Postulated carbon tetrachloride mode of action: A review. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* 25(3):185–209.
17. Khiati S, et al. (2014) Mitochondrial topoisomerase I (top1mt) is a novel limiting factor of Doxorubicin cardiotoxicity. *Clinical Cancer Res* 20(18):4873–4881.
18. Uemitsu N, Nakayoshi H (1984) Evaluation of liver weight changes following a single oral administration of carbon tetrachloride in rats. *Toxicol Appl Pharmacol* 75(1):1–7.
19. Zoppoli G, et al. (2011) Coordinated regulation of mitochondrial topoisomerase IB with mitochondrial nuclear encoded genes and MYC. *Nucleic Acids Res* 39(15):6620–6632.
20. Moraes CT (2001) What regulates mitochondrial DNA copy number in animal cells? *Trends Genet* 17(4):199–205.
21. Shadel GS, Clayton DA (1997) Mitochondrial DNA maintenance in vertebrates. *Annu Rev Biochem* 66:409–435.
22. Shimuzu M, Morita S, Yamano T, Yamada A (1989) Relationship between hepatic glutathione content and carbon tetrachloride-induced hepatotoxicity in vivo. *Toxicol Lett* 47(1):95–102.
23. Park CB, et al. (2007) MTERF3 is a negative regulator of mammalian mtDNA transcription. *Cell* 130(2):273–285.
24. Buchet K, Godinot C (1998) Functional F1-ATPase essential in maintaining growth and membrane potential of human mitochondrial DNA-depleted rho degrees cells. *J Biol Chem* 273(36):22983–22989.
25. Kao CY, Factor VM, Thorgeirsson SS (1996) Reduced growth capacity of hepatocytes from c-myc and c-myc/TGF-alpha transgenic mice in primary culture. *Biochem Biophys Res Commun* 222(1):64–70.
26. Wanson JC, Drochmans P, Mosselmans R, Ronveaux MF (1977) Adult rat hepatocytes in primary monolayer culture. Ultrastructural characteristics of intercellular contacts and cell membrane differentiations. *J Cell Biol* 74(3):858–877.
27. Dalla Rosa I, et al. (2014) Mapping topoisomerase sites in mitochondrial DNA with a poisonous mitochondrial topoisomerase I (Top1mt). *J Biol Chem* 289(26):18595–18602.
28. Meyer RR, Simpson MV (1969) DNA biosynthesis in mitochondria. Differential inhibition of mitochondrial and nuclear DNA polymerases by the mutagenic dyes ethidium bromide and acriflavin. *Biochem Biophys Res Commun* 34(2):238–244.
29. Leibowitz RD (1971) The effect of ethidium bromide on mitochondrial DNA synthesis and mitochondrial DNA structure in HeLa cells. *J Cell Biol* 51(1):116–122.
30. Guerrieri F, et al. (1995) Correlation between rat liver regeneration and mitochondrial energy metabolism. *Biochim Biophys Acta* 1272(2):95–100.
31. Russell AP, Foletta VC, Snow RJ, Wadley GD (2014) Skeletal muscle mitochondria: A major player in exercise, health and disease. *Biochim Biophys Acta* 1840(4):1276–1284.
32. Gonzalvez F, et al. (2013) Barth syndrome: Cellular compensation of mitochondrial dysfunction and apoptosis inhibition due to changes in cardiolipin remodeling linked to tafazzin (TAZ) gene mutation. *Biochim Biophys Acta* 1832(8):1194–1206.
33. Diaz F, Moraes CT (2008) Mitochondrial biogenesis and turnover. *Cell Calcium* 44(1):24–35.
34. Wang Y, Lyu YL, Wang JC (2002) Dual localization of human DNA topoisomerase IIIalpha to mitochondria and nucleus. *Proc Natl Acad Sci USA* 99(19):12114–12119.
35. Wu J, Feng L, Hsieh TS (2010) Drosophila topo IIIalpha is required for the maintenance of mitochondrial genome and male germ-line stem cells. *Proc Natl Acad Sci USA* 107(14):6228–6233.
36. Medja F, et al. (2009) Development and implementation of standardized respiratory chain spectrophotometric assays for clinical diagnosis. *Mitochondrion* 9(5):331–339.
37. Yang Y, et al. (2010) UOK 262 cell line, fumarate hydratase deficient (FH-/FH-) hereditary leiomyomatosis renal cell carcinoma: in Vitro and in vivo model of an aberrant energy metabolic pathway in human cancer. *Cancer Genet Cytogenet* 196(1):45–55.
38. Cong L, et al. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* 339(6121):819–823.
39. Loiseau D, et al. (2007) Mitochondrial coupling defect in Charcot-Marie-Tooth type 2A disease. *Ann Neurol* 61(4):315–323.