Mitoribosomal defects aggravate liver cancer via aberrant glycolytic flux and T cell exhaustion

Supplemental Materials and Methods

Isolation of hepatic tumoral mononuclear cells in humans

Liver mononuclear cells were isolated as previously described.[1] Briefly, liver tissue from humans or mice was cut into small pieces and incubated for 30 min at 37°C with pre-warmed medium that included dissociation enzymes (Miltenyi Biotec Inc., Auburn, CA, USA). After enzymatic digestion, the hepatic cell suspensions were quickly homogenized in C-Tubes using a GentleMACS Dissociator (Miltenyi Biotec) and dissociated with the h_tumor_01. After debris removal, the cells were resuspended in phosphate-buffered saline and centrifuged at 1,000 × g for 5 min for hepatocyte elimination. The supernatants were removed by mechanical suction and passed through a cell strainer with a 70 μm nylon filter (BD Falcon, Millville, NJ). Hepatic mononuclear cells were isolated by centrifugation at 1,200 × g for 10 min at 4°C and resuspended in RPMI-1640 medium (Welgene, Daegu, South Korea). Isolated hepatic mononuclear cells were subjected to real-time PCR and FACS analysis.

Isolation of lymph node lymphocytes for flow cytometry

First, 40-week-old diethylnitrosamine (DEN)-injured mice were euthanized with isoflurane and the draining lymph nodes (portal, coeliac, and mesenteric lymph nodes) were harvested, as previously described.[2] Lymph nodes were minced through a cell strainer (70 μm nylon filter;

BD Falcon, Millville, NJ), and washed with PBS. The cells were isolated by centrifugation at 1,200 × g for 10 min at 4°C and resuspended in RPMI-1640 medium (Welgene). Isolated lymphocytes were stained with the antibodies listed in Supplemental Table 3. Stained cells were analyzed using a BD LSRFortessa flow cytometer (BD Biosciences, San Jose, CA, USA), and the data were analyzed using FlowJo software (FlowJo, LLC, Ashland, OR, USA).

Serum chemistry and ELISA

Blood was collected from the retro-orbital sinus, incubated at room temperature for 1 h, and then centrifuged at 600 × g for 5 min to obtain serum. Biochemical measurement of alanine transaminase (ALT), aspartate transaminase (AST), triglycerides, and total cholesterol was conducted using a Fuji DRI-CHEM 7000i instrument (Fujifilm, Tokyo, Japan). Supernatants from CD4+ or CD8+ T cells activated with anti-CD3/CD28 antibodies were removed and IFN-γ levels were measured using a standard sandwich ELISA (BD Biosciences).

Western blots

Mouse tissues in lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA, pH 8.0; 0.1% Triton X-100) containing a protease inhibitor cocktail (#11836145001, Roche, Basel, Switzerland) and phosphatase inhibitors (04906837001, Roche) were homogenized on ice for 30 min using a TissueLyser II. After centrifugation at 16,000 × g for 15 min, the protein concentrations of the supernatants were measured in a BCA protein assay (#23227, Thermo Fisher Scientific, Waltham, MA). Fifty micrograms of protein per sample were loaded onto 8–12% polyacrylamide gels and electrophoresis was performed. The separated proteins were electrotransferred to 0.45 μm PVDF membranes (#IPVH00010, Millipore) at 200 mA for 2 h.

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The membranes were blocked for 1 h with 5% skimmed milk (#T145.2, Roth) in TBS/T buffer (20 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.6) and then incubated overnight at 4°C with appropriate primary antibodies. After washing three times with TBS/T, the membranes were incubated for 1 h at room temperature with secondary antibodies and visualized using ECL solution (#34580, Thermo Fisher Scientific). Target protein levels were normalized to those of glyceraldehyde 3-phosphate. The antibodies used are listed in Supplemental Table 4.

Blue-native PAGE

For mitochondrial isolation, 30 mg of liver tissue was homogenized in isolation buffer (210 nM mannitol, 70 mM sucrose, 1 mM EGTA, 5 mM HEPES, pH 7.2) using WiseStir (HS-30E; Daihan, Wonju, South Korea). The samples were then centrifuged at 600 × g for 10 min at 4°C and the supernatants were re-centrifuged at 17,000 × g for 10 min at 4°C. The pelleted mitochondria were resuspended and the constituent proteins were separated using a Native PAGE Novex Bis-Tris Gel system (Invitrogen, Carlsbad, CA, USA). To quantify the mitochondrial OxPhos complexes, 30 μg of each mitochondrial fraction in Native PAGE sample buffer (Invitrogen) and 10% n-dodecyl-β-D-maltoside were loaded onto Native PAGE 3–12% Bis-Tris gels. After electrophoresis, the separated proteins were transferred to PVDF membranes using iBlot gel transfer stacks (Invitrogen) and fixed in 8% (v/v) acetic acid. After overnight drying, membranes were incubated for 90 min with anti-OxPhos complex cocktail (#457999, Invitrogen; sc-58347, Santa Cruz) and visualized using a Western Breeze Chromogenic Western Blot Immuno-detection kit (Invitrogen). The antibodies used are listed in Supplemental Table 4.

Data selection and gene expression analysis

RNA-seq gene expression data from 368 subjects with hepatocellular carcinoma were acquired from the NIH's Genome Data Commons as raw count-mapped reads. Samples belonging to control or normal types were excluded. RNA-seq data were also generated for control and LivKO mice, and their gene counts were analyzed with DESeq R package (data are available under accession number GSE149553).[3]

Gene set enrichment analyses

To explore potential molecular signatures underlying the constructed prognostic gene signatures, GSEA (Gene Set Enrichment Analyses) was performed to identify enriched terms predicted to correlate with the following terms: Hallmark in C1; the Kyoto Encyclopedia of Genes and Genomes pathway in C2; and a gene set that contain genes annotated by the same gene ontology term in C5. A P value of < 0.01 and a FDR (false discovery rate) of $q < 0.05$ were considered statistically significant.

Reporter metabolite analysis

Dysregulated metabolites were identified by reporter metabolite analysis based on changes in expression of genes encoding metabolic enzymes catalyzing production of the given metabolites.[4] Reporter metabolite analysis was performed using a high-quality generic genome-scale metabolic model of humans,[5] and differential gene expression statistics (i.e., p-values and fold changes) from the LivKO and control groups of mice, and the tumor and non-tumor groups, of patients with HCC.

Metabolite enrichment measurement

Tracer labeled metabolites of glycolysis, serine biosynthesis, and the TCA cycle were analyzed using GC-MS (Models 7890A/5975C; Agilent Technologies, Santa Clara, CA). To extract metabolites from liver samples, ~30 mg of frozen liver tissue were ground using a mortar, mixed with 70% ethanol, heated at 70°C, and vortexed for 30 seconds. After this, samples were incubated at 95°C for 5 min, as previously described.[6] After centrifugation at 14,000 × g for 5 min, supernatants were collected and desiccated under a Speed Vac (Savant Instruments, Farmingdale, NY). The labeled metabolites of glycolysis, serine biosynthesis, and the TCA cycle were determined following two sequential derivatizations to yield a methoxylamine derivative and a tert-butyldimethylsilyl derivative (Sigma-Aldrich, St. Louis, MO). To quantify glycolysis metabolites, ions with a mass to charge ratio (m/z) of 484 (M+0) to 487 (M+3) for dihydroxyacetone phosphate (DHAP), 571 (M+0) to 574 (M+3) for glycerol-3-phosphate (G3P), 585 (M+0) to 588 (M+3) for 3-phosphoglyceric acid (3PG), and 453 (M+0) to 456 (M+3) for phosphoenolpyruvate (PEP), were monitored. To quantify serine biosynthesis pathway metabolites, ions with an m/z of 390 (M+0) to 393 (M+3) for serine and 246 (M+0) to 248 (M+2) for glycine were monitored. To quantify TCA cycle metabolites, ions with an m/z of 174 (M+0) to 177 (M+3) for pyruvate, 261 (M+0) to 264 (M+3) for lactate, 260 (M+0) to 263 (M+3) for alanine, 459 (M+0) to 464 (M+5) for citrate, 346 (M+0) to 349 (M+3) for α-ketoglutarate, 432 (M+0) to 436 (M+4) for glutamate, 431 (M+0) to 434 (M+3) for glutamine, 289 (M+0) to 293 (M+4) for succinate, 287 (M+0) to 291 (M+4) for fumarate, 419 (M+0) to 423 (M+4) for malate, and 302 (M+0) to 306 (M+4) for aspartate were monitored. Methionine and creatine enrichment was analyzed using the Agilent 1200 series high performance liquid chromatograph (Agilent Technologies) coupled to a 4000 QTRAP (AB Sciex, Foster City, CA).

As previously described (Hui S et al., Nature, 2017), a solution of methanol and water (80:20) at -80°C was added to plasma to extract the metabolites. Following incubation at 4°C for 10 min, samples were centrifuged at 16,000 × g for 10 min. To extract metabolites from liver samples, ~20 mg of frozen tissue was weighed and ground using a mortar; mixed with a solution of methanol, acetonitrile, and water (40:40:20) at -20°C; vortexed for 10 sec; and incubated for 10 min at 4°C. The samples were then centrifuged at 16,000 × g for 10 min at 4°C. Supernatants from liver and plasma samples were transferred to autosampler vials for LC-MS analysis. Labeling of metabolites was analyzed in a quadrupole mass spectrometer using positive ion mode coupled with electrospray ionization. An XBridge BEH Amide column (2.1 mm × 150 mm, 2.5 μm particle size, 130 Å pore size; Waters, Milford, MA) was utilized for LC separation via a gradient of solvent A (20 mM ammonium acetate + 20 mM ammonium hydroxide in 95:5 water:acetonitrile, pH 9.45) and solvent B (acetonitrile). Multiple reaction monitoring mode was utilized to detect the transition from precursor to product ions. The transitions m/z 149.8 → 104.1 and 153.9 → 108.1, for creatine and methionine, respectively, were monitored. Data were analyzed using Analyst software (AB Sciex, Foster City, CA). Glucose and lactate enrichment was analyzed using GC-MS. To extract metabolites from plasma samples, 70% acetonitrile was added to plasma, the mixture was centrifuged at 10,000 × g for 10 min at 4°C, and the supernatant was desiccated under a SpeedVac. The dried sample was derivatized as described above. Ions with an m/z of 319 (M+0) and 323 (M+4) for glucose and 219 (M+0) and 222 (M+3) for lactate were monitored.

Labeling of metabolites from [U-$^{13}C_6$]-glucose

To calculate the relative metabolic flux originating from glycolysis, labeling (%) of

metabolite mass isotopomers was determined based on incorporation of carbon isotopes from [U-$^{13}C_6$]-glucose, followed by correction for the natural abundance of mass isotopomers[7], using the following equation:

Labeling (%) = TTR / (TTR +1) × 100

where TTR is tracer to tracee ratio, expressed as M+i / M+0 (i = mass number of mass isotopomer above the most abundant lowest mass, M+0).

Calculation of metabolic kinetics

Glucose flux, i.e., the appearance rate (R_a same as R_d in fasting state, nmol/g/min) and the metabolic clearance rate (MCR, ml/g/min), was calculated at steady state:[8]

$$R_a \text{ glucose} = F / E_p$$

$$MCR = R_d / [(C1 + C2)/2]$$

where F represents the isotope tracer infusion rate (nmol/g BW/min); E_p represents plateau isotopic enrichment (%); and C_1 and C_2 are the concentrations of glucose (mmol/L) at sampling times t_1 and t_2, respectively.

Hematoxylin and eosin (H&E) and Ki-67 staining

Liver samples were obtained from DEN-injured control and LivKO mice that had been fed a chow diet for 40 weeks. Tissue was fixed overnight in 10% neutral formalin (BBC Biochemical, Mt. Vernon, WA, USA) and then embedded in paraffin wax. After sectioning (4 μm thick), tissue samples were deparaffinized by three immersions in xylene for 4 min each and washed with a graded ethanol series for 2 min each. After washing with distilled water for 4 min, the tissue sections were stained with hematoxylin (S2-5, YD Diagnostics, Yongin, South

Korea) for 4 min and eosin (#HT1101128, Sigma-Aldrich) for 3 min. The sections were then dehydrated in a graded ethanol series (for 2 min each) and immersed three times in xylene (for 4 min each) before mounting and examination under an upright microscope (#BX53, Olympus, Tokyo, Japan). For Ki-67 immunohistochemistry, slides were incubated with a primary antibody specific for Ki-67 (DAKO, IR626), followed by incubation with an HRP-labeled secondary antibody. The KI-67-positive cellular index was defined as the simple presence of nuclear staining. All stained nuclei were scored as positive regardless of the intensity of staining. Cell counts in five randomly selected fields were made at ×100 magnification under a conventional light microscope. The percentage of cells expressing KI-67 was determined by counting 1,000 cells per slide.

Measurement of the oxygen consumption rate and extracellular acidification rate

The mitochondrial oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using a Seahorse XF-96 Extracellular Flux Analyzer (Seahorse Bioscience Inc., North Billerica, MA, USA). To obtain tumor-infiltrating lymphocytes, tumor tissue from DEN-injured mice was cut into small fragments and treated for 30 min at 37°C with pre-warmed medium that included dissociation enzymes (Miltenyi Biotec). After enzymatic digestion, the hepatic cell suspensions were quickly homogenized in C-Tubes using the GentleMACS Dissociator (Miltenyi Biotec) and the m_impTumor_04 program. After debris removal, the cells were resuspended in Hank's balanced salt solution and centrifuged at 1,000 × g for 5 min. The supernatants were removed by mechanical suction and filtered through a cell strainer (70 μm strainer; BD Falcon, Millville, NJ). Lymphocytes were purified by 40% Percoll-gradient centrifugation at 1,200 × g for 10 min at 4°C and resuspended in RPMI-1640

medium (Welgene) containing 10% FBS. Tumor CD4+ or CD8+ T cells were sorted using a magnetic CD4+ or CD8+ T cell Isolation Kit (#130-104-454, #130-104-075; Miltenyi Biotec). Tumor CD4+ or CD8+ T cells (300,000 cells per well) were cultured and activated by anti-CD3 (2 μg/mL)/CD28 (5 μg/mL) in Seahorse XF-96 plates. Next, the cells were incubated for 1 h with RPMI-1640 medium lacking sodium bicarbonate at 37°C in a non-CO_2-containing incubator. The calibration plate was then loaded into the Seahorse XF-96 analyzer and calibration was performed for 30 min at 37°C. The medium containing unbuffered RPMI supplemented with 1% BSA and 25 mM glucose, 1 mM pyruvate, 2 mM glutamine, and mitochondrial OxPhos inhibitors was adjusted to pH 7.4 on the day of the OCR assay. Measurement of the OCR and ECAR was made under basal conditions and after addition of 1 μM oligomycin (Sigma-Aldrich), 0.5 μM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (Sigma-Aldrich), 0.5 μM rotenone (Sigma-Aldrich), glucose (10 mM, Sigma-Aldrich), and 2-DG (50 mM, Sigma-Aldrich). The measurements were programmed for four cycles of 2 min of mixing, 2 min of waiting, and 3 min of measurement at both baseline and following the addition of each inhibitor. OCR and ECAR were calculated and recorded by a sensor cartridge and Seahorse XF-96 software.

Isolation of primary hepatocytes

As previously described[1], *in situ* perfusion of control and LivKO male mice was conducted for 30 min using EGTA solution (0.5 mM EGTA, 25 mM Tricine, 5.4 mM KCl, 0.44 mM KH2PO4, 140 mM NaCl, 0.34 mM Na2HPO4, pH 7.2) and collagenase solution (0.8 mg/ml collagenase type I in HBSS; Worthington, Freehold, NJ, USA) to isolate primary hepatocytes. Cells in the suspension were filtered using 70 μm cell strainers (BD Falcon) and then

centrifuged at 1,000 × g for 5 min. Pelleted cells were resuspended and isolated on 50% Percoll solution (GE Healthcare, Buckingham, UK) by centrifugation at 1,200 × g for 10 min at 4℃. Isolated primary hepatocytes (2 × 10^5 per well) were seeded in low-glucose DMEM (Welgene) containing 10% fetal bovine serum (Thermo Fisher Scientific) and 1% PS (penicillin and streptomycin; Welgene).

Exposure of HepG2 cell lines to hypoxia

HepG2 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in low-glucose DMEM (Welgene) containing 10% fetal bovine serum (Thermo Fisher Scientific) and 1% PS (penicillin and streptomycin; Welgene). Cell cultures were authenticated and checked routinely to ensure that they were mycoplasma-free. HepG2 cells were seeded onto each well of 12-well plates and exposed to 20% and 1% O_2 for 24 h. Transcription of *Hspd1*, *Clpp*, and *Lonp1* was measured by qRT-PCR.

Measurement of global translation

Primary hepatocytes from WT and LivKO mice were treated, or not, for 12 h with 1 or 2 μM gamitrinib (LegoChem Biosciences Inc., Daejeon, Republic of Korea). Puromycin (1 μg/mL) was added during the last 30 min of incubation. Cells were washed with 1× PBS and collected for protein analysis.

ROS measurements

Primary hepatocytes (2 × 10^5 cells) were washed with PBS, and the cells were then incubated at 37°C for 15 min with the ROS detection reagent 6-chloromethyl-2′,7′-

dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCF-DA from Invitrogen). Oxidation of the CM-H2DCF-DA probe was analyzed by monitoring excitation at 492–495 nm and fluorescence emission at 517–527 nm using a LSRFortessa flow cytometer (BD Biosciences). A histogram of relative fluorescence intensities was used to compare levels of intracellular ROS generation by primary hepatocytes.

Lactate measurement

Lactate measurement was performed at room temperature using the EnzyChrom L-Lactate Assay Kit (BioAssay Systems, Hayward, CA, USA). Briefly, tumor tissue was weighed and homogenized in PBS. The volume of PBS was adjusted to yield the same concentration of tissue in each sample. Tissue samples and sera from control and LivKO mice were then incubated with the working solution of the assay. The optical density of the samples was read at 560 nm (in triplicate) and compared with a standard curve at both time zero and following a 20 min incubation. The lactate concentration in the initial tissue was calculated using the recorded weight of the tumor tissue and the volume of PBS that was added to the tissue sample.

***In vitro* analysis of CD8+ T cells**

CD8+ T cells were isolated from the spleens of C57BL/6J mice by magnetic bead separation using the CD8α+ T cell Isolation Kit (Miltenyi Biotec). Purified CD8+ T cells (1 × 10^5) were cultured in RPMI medium containing 10% FCS, 100 µg/ml penicillin/streptomycin, 2 mM L-glutamine, 20 mg/ml NEAA (Sigma-Aldrich), 5 µl/ml β-mercaptoethanol (Gibco; Thermo Fisher Scientific), and recombinant mouse IL-2 protein (20 ng/ml; R&D Systems, Minneapolis, MN, USA). CD8+ T cells were incubated with or without 5 mM or 15 mM L-

lactic acid (Sigma-Aldrich) for 24 h. For cytokine and *Ifng* mRNA expression analysis, cells were stimulated for 5 h with ionomycin (500 ng/ml), PMA (5 ng/ml), and Golgi plug (1 μg/ml). Then, IFN-γ levels from the culture supernatants were measured. Surface marker expression by CD8+ T cells was determined by flow cytometry analysis.

Real-time PCR analysis

Real-time PCR analysis was performed using specific primers. Total RNA was extracted from liver and adipose tissue using TRIzol Reagent (Invitrogen, Eugene, OR, USA). Complementary DNA (cDNA) was synthesized from the same quantity of RNA using M-MLV reverse transcriptase and oligo-dT primers (Invitrogen). Quantitative real-time PCR was performed using cDNA, 2×SYBR Green PCR Mix (Applied Biosystems) and analyzed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The comparative Ct method was used to quantitate transcript levels, the expression of which was normalized to that of 18s RNA. The results were analyzed using the ΔΔCt method and the values expressed as fold differences relative to the control.

Single-cell analysis

Gene-cell matrix of single-cell transcriptomics was download from the GEO (GSE151530)[9] and checked to exclude poor-quality cells (cells expressing a low number of genes). After normalization for scaling the sequencing depth and batch corrections, dimension reduction analysis was performed using Seurat package to cluster cell types based on single-cell gene expression. To this end, highly variable genes across single cells were identified based on mean expression and dispersion, and principal component analysis based on highly variable

genes was performed, followed by selection of significant principal components (PCs) by the jack-straw function in the Seurat package. Lastly, selected PCs were used as inputs for t-Distributed Stochastic Neighbor Embedding (tSNE), thereby identifying cell clusters from tSNE maps. Pre-defined cell clusters from the original papers were overlaid onto the tSNE maps to identify cell-specific gene expression. Trajectory analysis of subclusters of given cell types were performed and visualized using R *monocle* package.[10]

Transmission electron microscopy

Liver tissues from 8-week-old control and LivKO mice were fixed at 4°C in 1% (wt/vol) glutaraldehyde and then washed in 0.1 M cacodylate buffer (pH 7.2, 4°C). Washed liver tissues were fixed for 1 h at 4°C with 1% (wt/vol) osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2, containing 0.1% (wt/vol) calcium chloride. Liver samples were dehydrated in a graded series of ethanol solutions (50%, 75%, 90%, 95%, and 100%), treated with propylene oxide, and then embedded in Embed-812 (Electron Microscopy Sciences, Hatfield, PA, USA). The resin blocks were then polymerized at 60°C for 48 h. Tissues were sectioned with an EM UC6 ultramicrotome (Leica Microsystems, Vienna, Austria) and post-stained with 4% (wt/vol) uranyl acetate and citrate. Specimens were observed under a JEM ARM 1300S high-voltage electron microscope (JEOL, Japan). We also performed trajectory analysis using the R monocle package.

Statistical analysis

Statistical analysis was performed using R software v4.0.2. Qualitative variables were analyzed using Pearson's chi-squared (χ^2) test or Fisher's exact test. Quantitative variables

were analyzed using a t-test for paired samples or a non-parametric approach. Differentially-expressed genes were detected using the negative-binomial exact test, with a FDR (Benjamini-Hochberg) < 0.05, and these were visualized in a heat map. Kaplan–Meier survival analysis was performed using GraphPad Prism software 8.0, and the statistical p-values were generated using the Cox-Mantel log-rank test or Gehen-Breslow-Wilcoxon test. Cox proportional hazards regression was used to analyze the effect of clinical variables on patient overall survival and recurrence-free survival. For all analysis, $P < 0.05$ was considered statistically significant.

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Supplemental figure legends

Supplemental Fig. 1. Plots showing expression of genes encoding mitoribosomal proteins (according to the cell type identified from single-cell RNA-seq). Among the six cell types (B cells, cancer-associated fibroblasts (CAFs), malignant cells, T cells, tumor-associated macrophage (TAMs), and tumor endothelial cells (TECs)), expression of A) *MRPS25*, B) *MRPL2*, C) *MRPS12*, D) *MRPL13*, E) *MRPS15*, F) *MRPL51*, G) *MRPS26*, H) *MRPS21*, and I) *MRPL55* was identified.

Supplemental Fig. 2. Plots showing expression of genes encoding mitoribosomal proteins according to cell type identified from single-cell RNA-seq. Among the six cell types (B cells, cancer-associated fibroblasts (CAFs), malignant cells, T cells, tumor-associated macrophage (TAMs), and tumor endothelial cells (TECs)), expression of A) *AURKAIP1*, B) *MRPS18A*, C) *DAP3*, D) *MRPL40*, E) *MRPL27*, F) *MRPL21*, G) *MRPS23*, H) *MRPL34*, and I) *MRPS33* was identified.

Supplemental Fig. 3. Plots showing expression of genes encoding mitoribosomal proteins according to cell type identified from single-cell RNA-seq. Among six cell types (B cells, cancer-associated fibroblasts (CAFs), malignant cells, T cells, tumor-associated macrophage (TAMs), and tumor endothelial cells (TECs)), expression of A) *MRPS18B*, B) *MRPS35*, C) *CHCHD1*, D) *MRPS28*, E) *MRPL16*, F) *MRPS5*, G) *MRPL11*, H) *MRPL22*, and I) *MRPL46* was identified.

Supplemental Fig. 4. Plots showing expression of genes encoding mitoribosomal proteins according to cell type identified from single-cell RNA-seq. Among six cell types (B cells, cancer-associated fibroblasts (CAFs), malignant cells, T cells, tumor-associated macrophage

(TAMs), and tumor endothelial cells (TECs)), expression of A) *MRPL57*, B) *MRPS34*, C) *MRPS30*, D) *MRPS10*, E) *MRPL4*, F) *MRPL35*, G) *MRPL9*, H) *MRPS9*, and I) *MRPS22* was identified.

Supplemental Fig. 5. Plots showing expression of genes encoding mitoribosomal proteins according to cell type identified from single-cell RNA-seq. Among six cell types (B cells, cancer-associated fibroblasts (CAFs), malignant cells, T cells, tumor-associated macrophage (TAMs), and tumor endothelial cells (TECs)), expression of (A) *MRPS2*, (B) *MRPS18C*, (C) *MRPL19*, (D) *MRPL3*, (E) *MRPL20*, (F) *MRPL47*, (G) *MRPL50*, and (H) *GADD45GIP1* (same as *MRPL59*, *CRIF1*) was identified.

Supplemental Fig. 6. Single-cell landscape of lactate dehydrogenase A (LDHA) expression in HCC samples. A) Expression of the gene encoding *LDHA* was detected in B cells, cancer-associated fibroblasts (CAFs), malignant cells, T cells, tumor-associated macrophage (TAMs), and tumor endothelial cells (TECs). B) Violin plots showing expression of the *LDHA* gene by six different cell types. Highest *LDHA* gene expression was observed in malignant cell populations.

Supplemental Fig. 7. Expression of mitochondrial stress response-related genes in HepG2 cells under normoxic and hypoxic conditions. RT-qPCR to detect expression of *Hspd1*, *Clpp*, and *Lonp1* mRNA in HepG2 cells exposed to 20% and 1% O_2 for 24 h. Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01 (two-tailed t-test).

Supplemental Fig. 8. Trajectory analysis of malignant cells. A) We selected a subcluster of malignant cells (red circle) and B) performed trajectory analysis of cellular differentiation. We observed increasing expression of (C) MRP and (D) mitochondrial stress-related proteins upon

differentiations of malignant cells.

Supplemental Fig. 9. Trajectory analysis of tumor-associated macrophages. A) We selected a subcluster of tumor-associated macrophages (TAMs) (red circle) and B) performed trajectory analysis of cellular differentiation. We observed increasing expression of (C) MRP and (D) mitochondrial stress-related proteins as TAMs differentiated.

Supplemental Fig. 10. Expression of MRPs, mitochondrial stress proteins, and T cell exhaustion markers in different patients. (A) MRP and (B) mitochondrial stress-related proteins were expressed by malignant cells, as were (C) T cell exhaustion markers. The x-axis shows patients ordered according to expression of MRP in malignant cells.

Supplemental Fig. 11. Expression of OxPhos complex and mitochondrial stress response-related genes by non-tumor and tumor tissue from humans and mice. (A) Representative western blot and quantification of band density show expression of OxPhos complex subunits by non-tumor and tumor tissue from patients with hepatocellular carcinoma. (B) BN-PAGE analysis of the assembled OxPhos complex in tumor and non-tumor tissues from patients with hepatocellular carcinoma. (C) Expression of mitochondrial stress response-related genes in non-tumor and tumor-bearing DEN-injured mice (40 weeks old). Data are expressed as the mean ± SEM. $^{**}P < 0.01$ ((A,C): two-tailed t-test).

Supplemental Fig. 12. Differences in overall survival of HCC patients in the TCGA dataset according to expression of genes encoding MRPs and mitochondrial stress markers such as LONP1, CLPP, and HSPD1. Kaplan–Meier curves comparing overall survival according to the expression of (A) MRPs, (B) LONP1, (C) CLPP, and (D) HSPD1 among patients with HCC. ($p < 0.05$; log-rank test).

Supplemental Fig. 13. Liver-specific mitoribosomal defects promote tumor progression in a mouse model of chemically-induced liver cancer. (A) Strategy for hepatocyte-specific deletion of *Crif1* using Alb:cre and Crif1:floxed mice. (B) Bar graphs show band density of OxPhos complexes in the liver (normalized to β-actin) of 10-week-old control and LivKO mice. (C) OCR and (D) ECAR were measured in primary hepatocytes from 10-week-old control and LivKO male mice. (E) Primary hepatocytes from 8-week-old control and LivKO mice were treated with puromycin (1 μg/mL) for 30 min. Protein was extracted from mitochondria and cytosol isolated from cells, and then analyzed by western blotting with an anti-puromycin antibody to detect basal levels of mitochondrial and cytosolic translation. (F, G) Hepatic expression of *Hspd1* and *Lonp1* in 10-, 20-, and 40-week-old control and LivKO mice. (H) Representative western blot and quantification of band density showing expression of OPA1 and Parkin in the liver of control and LivKO mice. Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01 (B–D; F–H: two-tailed t-test).

Supplemental Fig. 14. Differences in mitochondrial morphology and T cell exhaustion marker expression in mice harboring liver-specific mitoribosomal defects. (A) Mitochondrial mass and liver morphology in 10-week-old control and LivKO mice were observed by confocal microscopy. (B) Representative transmission electron microscopic images of livers from 10-week-old control and LivKO mice. (C–D) Percentages of hepatic T cells with high PD-1 expression in tumor-free 20- or 40-week-old control mice and LivKO mice. Data are expressed as the mean ± SEM. **P < 0.01 (C,D: two-tailed t-tests).

Supplemental Fig. 15. Relationship between MRP expression and tumor progression in HCC patients from the TCGA dataset. (A) Kaplan–Meier curves comparing disease-free survival of patients with HCC (TCGA dataset) according to expression of genes encoding MRPs. (B)

Tumor stage and (C) liver disease severity (Child-Pugh score) were significantly different between the high- and low-MRP patient subgroups (Chi-square test; $p < 0.01$).

Supplemental Fig. 16. Appearance and characteristics of tumors from LivKO mice after DEN injection. (A) Appearance of gross tumors in the liver of control and LivKO mouse (aged 20 weeks) given DEN injections at 2-weeks-of-age. (B) Liver weight, (C) number of tumor nodules, and (D) maximal size of tumors in 20-week-old DEN-injured control and LivKO mice. Data are expressed as the mean ± SEM. *$P < 0.05$, **$P < 0.01$ (B–D: two-tailed t-test).

Supplemental Fig. 17. Lipid profiles and hepatic histologic findings from 40-week-old DEN-injured control and LivKO mice. Male and female DEN-injured control and LivKO mice (aged 40 weeks) were compared with respect to (A) maximal size of tumor and serum levels of (B) total cholesterol and (C) triglyceride. Typical HCC features were confirmed by (D) H&E and (E) Ki67 staining. Scale bars: 200 µm. Ki67 staining ($n = 5$ mice per condition per time point; five random fields of view of the tumor area) was quantitated and plotted. Data are expressed as the mean ± SEM. **$P < 0.01$ (A–C, E: two-tailed t-tests).

Supplemental Fig. 18. [U-$^{13}C_6$]-labeled glucose flux in non-cancer and cancer liver tissues from control and LivKO mice. Atom tracing of glycolytic intermediates and TCA cycle metabolites from [U-$^{13}C_6$]-labeled glucose (red; shown in Figure 3A). Mass isotopomer distributions were determined by GC-MS. Incorporation of ^{13}C atoms from [U-$^{13}C_6$]-labeled glucose into (A) alanine, (B) DHAP, (C) 3PG, (D) PEP, (E) G3P, (F) glutamine, and (G) glutamate were denoted as M+n, where n is the number of ^{13}C atoms. Data are expressed as the mean ± SEM. *$P < 0.05$, **$P < 0.01$ (A–G: one-way ANOVA).

Supplemental Fig. 19. *Crif1* deficiency-mediated mitoribosomal defects increase expression

of mitochondrial pyruvate carrier 1 and 2 in the liver. (A) Representative blots showing mitochondrial pyruvate carrier 1 and 2 in liver tissue of control and LivKO mice aged 10 weeks. (B) Relative band density in representative blots of liver tissue from control and LivKO mice. Data are expressed as the mean ± SEM. *P < 0.05 (B: two-tailed t-test).

Supplemental Fig. 20. Gating strategy used for analysis of CD4+ and CD8+ T cells, regulatory T cells, NK cells, NKT cells, B cells, monocytes, neutrophils, and eosinophils in the liver of DEN-injured mice aged 40 weeks.

Supplemental Fig. 21. Comparison of PD-1 expression in draining lymph nodes (portal, coeliac and mesenteric lymph nodes) and tumors from DEN-injured mice aged 40 weeks. (A and B) Representative flow cytometry plots and PD-1 mean fluorescence intensity of CD4+ and CD8+ T cells from the draining lymph nodes (black line) and hepatic tumors (red line), respectively. (C) Expression of genes related to T cell exhaustion in the draining lymph nodes and hepatic tumors in 40-week-old DEN-injured mice. Data are expressed as the mean ± SEM. **P < 0.01 (A–C: two-tailed t-test). The shaded areas depict the isotype control.

Supplemental Fig. 22. Immunophenotype of tumor-infiltrating immune cells in 40-week-old DEN-injured control and LivKO mice. Hepatic tumor tissue from DEN-injured control and LivKO mice (aged 40 weeks) was compared. (A–D) Representative flow cytometry plots showing the percentages of NK cells, NKT cells, and T cells. (E–G) Representative flow cytometry plots showing the percentages of CD4+ and CD8+ T cells. (H–J) Representative flow cytometry plots of tumor-infiltrating monocytes, neutrophils, and Tregs. (K–N) Representative flow cytometry plots showing the percentages of B cells and eosinophils. Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01 (two-tailed t-test).

Supplemental Fig. 23. Hepatic mitoribosomal defects increase the number of PD-1+ immune cells in the liver tumors of DEN-injured mice. Hepatic tumor tissues from DEN-treated control and LivKO mice (aged 40 weeks) were compared. Numbers of (A) CD3+PD-1+ T cells, (B and C) CD4+PD-1+ and CD8+PD-1+ T cells, and (D–F) PD-1+NKT, PD-1+NK, and PD-1+B in a tumor measuring 1 mm. Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01 (A–F: two-tailed t-test).

Supplemental Fig. 24. PD-1+ immune cells are more abundant in tumor tissue than in non-tumor tissue from DEN-injured mice. Hepatic tumor tissues from DEN-treated control and LivKO mice (aged 40 weeks) were compared. Percentage of (A) CD3+PD-1+ T cells, (B and C) CD4+PD-1+ and CD8+PD-1+ T cells, and (D) PD-1+NKT cells. Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01 (A–D: two-tailed t-test).

Supplemental Fig. 25. Hepatic mitoribosomal defects increase expression of exhaustion markers and apoptosis-related genes in tumor-infiltrating CD4+ and CD8+ T cells in 40-week-old DEN-injured mice. (A–C) qRT-PCR analysis data showing expression levels of *Tim3*, *Lag3*, and *Casp3* in tumoral CD4+ and CD8+ T cells from the liver of 40-week-old DEN-injured mice. Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01 (A–C: two-tailed t-test).

Supplemental Fig. 26. Hepatic ROS production depends on mitoribosomal function. (A) Black and red violin plots represent expression of the ROS gene set in patients showing the lowest 25% (n = 92) and highest 25% (n = 92) percentiles of MRP expression. (B) Normalized MFI values for DCFDA-positive cultured primary hepatocytes from 10-week-old control and LivKO mice. Data are expressed as the mean ± SEM. **P < 0.01, ***P < 0.01 (two-tailed t-test).

Supplemental Fig. 27. Hepatic mitoribosomal defects in mice are associated with lower levels of whole-body endogenous glucose production. (A,B) Labeled serum glucose levels at 130, 140, and 150 min after initiation of tracer infusion (for determination of plateau tracer enrichment) in control and LivKO mice. (C) Serum levels of glucose in control and LivKO mice at 130, 140, and 150 min after initiation of tracer infusion. (D) Whole-body glucose turnover rate in control and LivKO mice aged 8 weeks. Data are expressed as the mean ± SEM. **P < 0.01, ***P < 0.01 (B–D: two-tailed t-test).