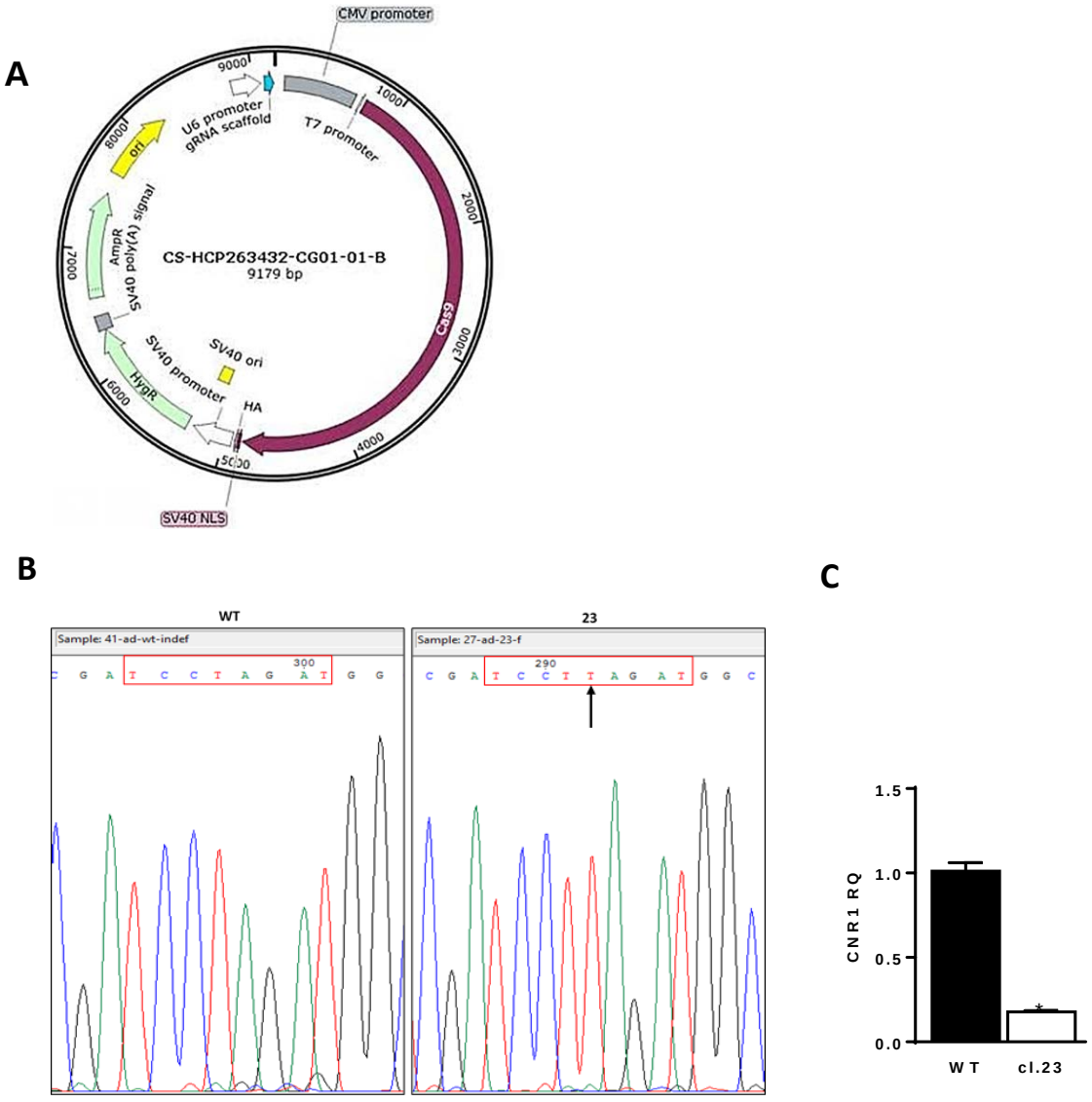
Cannabinoid-1 Receptor Regulates Mitochondrial Dynamics and Function in Renal Proximal Tubular Cells

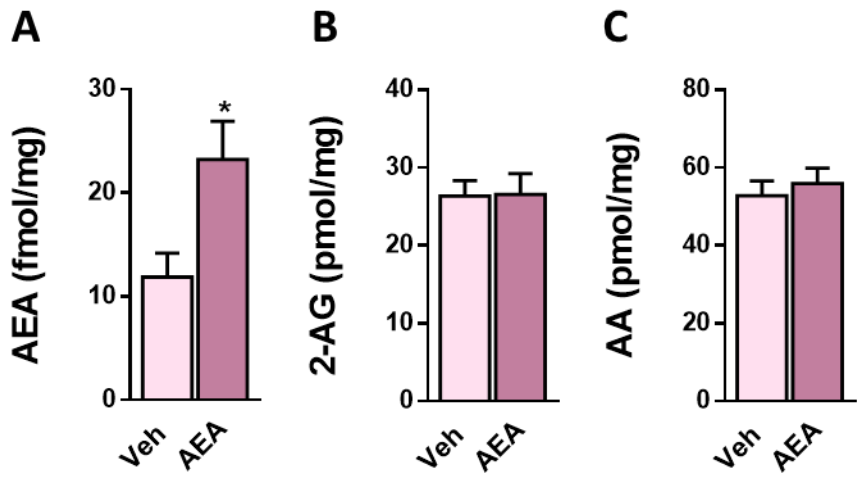
Adi Drori, Anna Permyakova, Rivka Hadar, Shiran Udi, Alina Nemirovski, and Joseph Tam

Inventory of Supplemental Information:

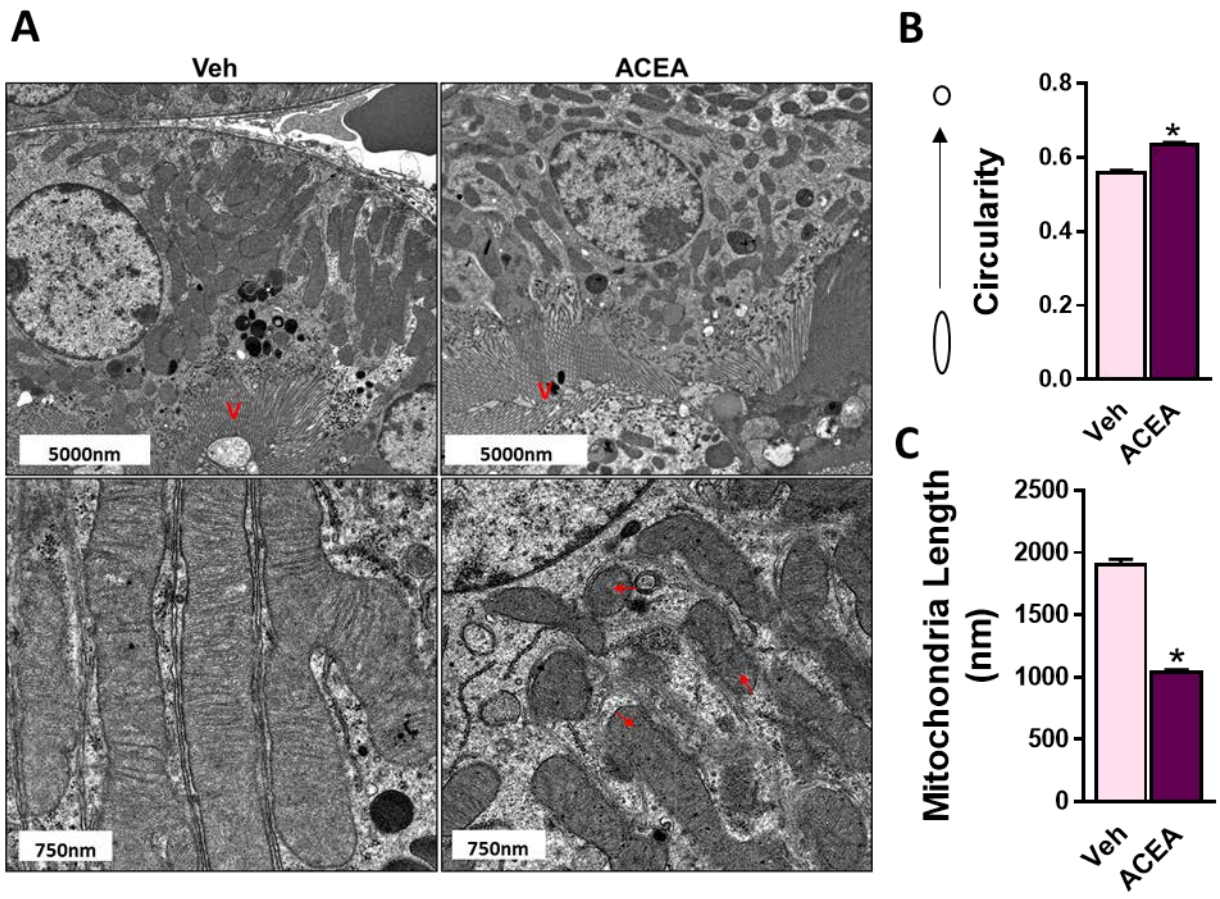
The following Supplemental Table, Figures and Methods provide additional information supporting the role of proximal tubule CB_1R in regulating mitochondrial fragmentation.



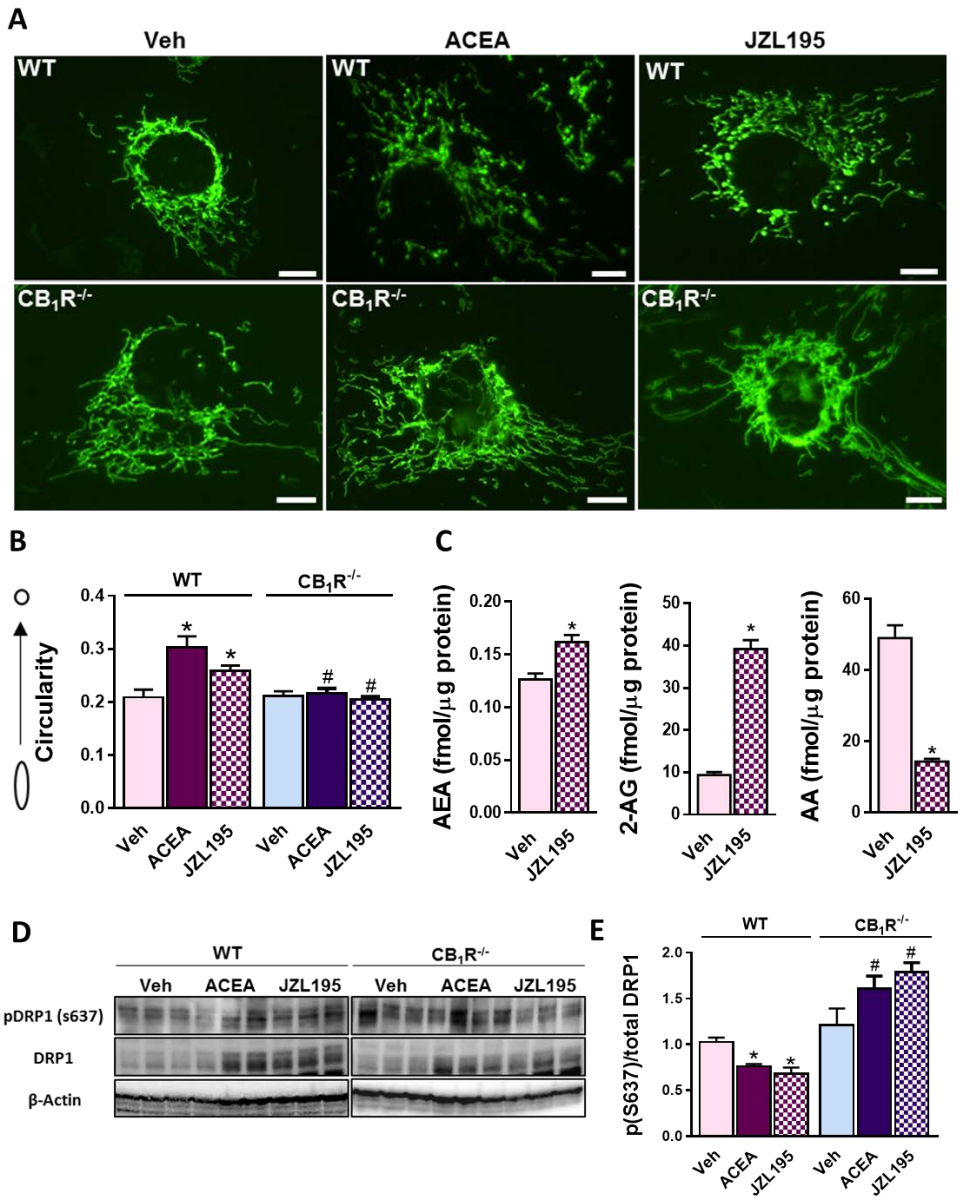
Supplementary Figure 1. ***Generation of $CB_1R^{-/-}$-HK-2 cells.*** (**A**) Cas9-expressing vector containing gRNA sequence to target all *hCNR1* isoforms was designed and provided by GeneCopoeia™. Wild-type HK-2 were transfected with the vector, and selected for Hygromycin resistance. Resistant cells were serially diluted, seeded in single-cell density and expanded. (**B**) DNA from different clones was isolated and sequenced. Clone no.23 had a T insertion mutation within the gRNA target sequence and low CB_1R mRNA expression (**C**). It was designated as $CB_1R^{-/-}$-HK-2 in the manuscript.



Supplementary Figure 2. ***Renal endocannabinoid levels following AEA administration.*** Male, C57Bl6/J mice were administered an intraperitoneal injection of either vehicle (Veh) or 10 mg/kg AEA. Six hours following the administration, AEA (**A**), 2-AG (**B**), and AA (**C**) were quantified by LC/MS-MS. Data represent the mean ± SEM from 5 mice in each group. **P*<0.05 relative to Veh-treated animals.



Supplementary Figure 3. ***CB_1R activation by ACEA induces in vivo mitochondrial fission in RPTCs.*** Male, C57Bl6/J mice were administered an intraperitoneal injection of either vehicle (Veh) or 10 mg/kg ACEA. Mice were euthanized 6 hours after drug administration and their kidneys were processed for electron microscopy. (**A**) Note the disrupted mitochondrial morphology induced by ACEA treatment in RPTCs. A scale bar is indicated for each picture. Legend: V, Brush border villi; red arrows, obstructed cristae. (**B, C**) Mitochondrial circularity and length were measured with Adobe Photoshop C3S software. Data represent mean ± SEM of 650-850 mitochondria from 11-12 RPTCs (from 2 (Veh) or 3 (ACEA) mice per group). **P*<0.05 relative to Veh-treated animals.



Supplementary Figure 4. ***In vitro mitochondrial fission induced by ACEA or increased endocannabinoid 'tone' is CB1R mediated***. WT-HK-2 or $CB_1R^{-/-}$-HK-2 cells were stably transfected with MitoGFP and treated with either vehicle (Veh), 5 µM ACEA, or 250 nM JZL195 for 6 hours. (**A**) Live cells were examined under a fluorescent microscope to visualize the mitochondrial morphology. (**B**) Mitochondrial circularity was measured using a publically available version of ImageJ macro for mitochondrial morphology. Data represent the mean ± SEM from 20-45 cells in each group. Scale bar, 10 µm. *P<0.05

relative to Veh-treated HK-2 of the same cell line. $^{\#}P<0.05$ relative to the same treated group in WT-HK-2 cells. (**C**) Increased endocannabinoid 'tone' after JZL195 administration in HK-2 cells. Measurements of AEA, 2-AG, and AA were done using LC-MS/MS, and normalized to cellular protein content. Data represent the mean ± SEM from 12 replicates in each group. *P<0.05 relative to Veh-treated HK-2 cells. (**D, E**) Reduced ratio between the phosphorylated (S637) and total forms of DRP1 was measured in ACEA (5 μM), and JZL195 (250 nM)-treated WT-HK-2 cells. Data represent the mean ± SEM of 3-6 replicates from 2 independent experiments. *P<0.05 relative to Veh-treated HK-2 of the same cell line. $^{\#}P<0.05$ relative to the same treated group in WT-HK-2 cells.

Supplementary Table 1. Primer List

Gene (*homo sapiens)*	Forward	Reverse
MitoDNA	CACTTTCCACACAGACATCA	TGGTTAGGCTGGTGTTAGGG
β2M	TGTTCCTGCTGGGTAGCTCT	CCTCCATGATGCTGCTTACA
DRP1	TCACGAGACAAGTTAATTCAGGA	GCCTTTGGCACACTGTCTTG
RPLP0	CTTCCTTAAGATCATCCAACTA	ACATGCGGATCTGCTGCA

Complete Materials and Methods

Animals and Experimental Protocol. All animal studies were approved by the Institutional Animal Care and Use Committee of the Hebrew University of Jerusalem (AAALAC accreditation #1285). Male, 8-10-week-old RPTC-$CB_1R^{-/-}$ mice[1,2] and their wild-type (WT) littermate controls were used for the *in-vivo* experiments. To activate CB_1R, mice were administered a single intraperitoneal injection of 10 mg/kg AEA or arachidonyl-2'-chloroethylamide (ACEA) (90050 or 91054, respectively; Cayman Chemicals, Ann Arbor, MI). To test the effect of fatty acid flux-induced CB_1R activation on the kidney, mice were fed with a STD (14% fat, 24% protein, 62% carbohydrates; NIH-31 rodent diet) or a HFD (60% fat, 20% protein and 20% carbohydrates; Research Diet, D12492) for 7 days. Mice were euthanized by a cervical dislocation under anesthesia, and blood and kidneys were harvested for further analyses.

Electron Microscopy. 3 mm kidney slices were fixed overnight in 2% paraformaldehyde and 2.5% Glutaraldeyde in 0.1 M Cacodylate buffer (pH 7.4) at room temperature, then washed 4 times in cacodylate buffer. Tissue slices were stained with 1% osmium tetroxide, 1.5% potassium ferricyanide in 0.1 M cacodylate buffer for 1 hour, washed 4 times in cacodylate buffer and dehydrated. Following dehydration, slices were infiltrated with increasing concentrations of Agar 100 resin in propylene oxide, consisting of 25, 50, 75, and 100% resin for 16 hours each, and then embedded in fresh resin and let to polymerize at 60°C for 48 hours. Embedded tissues in blocks were sectioned with a diamond knife on a Leica Reichert Ultracut S microtome, and ultrathin sections (80 nm) were collected onto 200 Mesh, carbon-formvar-coated copper grids. The sections on grids were sequentially

stained with uranyl acetate and lead citrate for 10 minutes each, and viewed with Tecnai 12 TEM 100kV (Phillips, Eindhoven, the Netherlands) equipped with a MegaView II CCD camera and Analysis® version 3.0 software (SoftImaging System GmbH, Münstar, Germany).

Cell Culture. WT or $CB_1R^{-/-}$-HK-2 cells (Human immortalized RPTCs) were maintained in a low glucose DMEM (01-050-1A; Biological Industries, Israel) supplemented with 5% FCS, 100 mM Glutamine, 100 mM Na-Pyruvate, and Pen/Strep. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2/95% air. To test the effect of CB_1R activation, cells were seeded in 6-well plates ($25x10^4$ cells/well) for 24 hours. Then, growth medium was replaced with a serum-free medium (SFM) for an additional 12 hours. At the morning of the experiment the medium was replaced with fresh SFM containing either vehicle (EtOH), 5 µM AEA, 5 µM ACEA, or 250 nM JZL195 for 6 hours. To mimic fatty conditions, cells were overloaded with a mixture of fatty acids containing 0.1 mM sodium Oleate (O7501; Sigma Aldrich) and sodium Palmitate (P9767; Sigma Aldrich) in a ratio of 2:1 (O:P, respectively), dissolved in 11% free-fatty acid BSA solution (A7030; Sigma Aldrich). At indicated time points, cells were harvested for further analyses as described below.

Stable Transfection of RPTCs for Mitochondrial Overexpression of GFP. HK-2 cells were transfected with pLYS1-FLAG-MitoGFP-HA (#50057; Addgene, Cambridge, MA), using Lipofectamin3000 (L3000-001; Invitrogen) according to the manufacturer's protocol. Transfected cells were selected with Puromycin (1 µg/µL; P8833, Sigma

Aldrich). Antibiotic-resistant cells were then sorted for high GFP expression using FACSAria II (Becton Dickinson, NJ), were plated, and MitoGFP expression was validated using a IX-73 fluorescent microscope (OLYMPUS).

Genetic Deletion of CB_1R in RPTCs. HK-2-MitoGFP cells were transfected with CRISPR-CAS9 vector containing a sgRNA sequence to target all human isoforms of *CNR1* (Genecopeia™ CS-HCP263432-CG01-01-B) using Lipofectamine3000 (L3000-001, Invitrogen), and selected with Hygromycin (200 μM; H3274, Sigma Aldrich). Antibiotic-resistant cells were then seeded in single-cell density, and allowed to grow. Single-cell clones were analyzed by DNA sequencing and mRNA analysis to confirm the deletion of CB_1R (**Supplementary Figure 1**).

Fat accumulation in RPTCs. RPTCs were seeded in 96-microwell black plates (Nunc, Denmark) at 10,000 cells per well, and left to adhere. At 36 hours after seeding, medium was replaced with fresh serum-free DMEM, containing either vehicle, AEA, or O:P for the indicated time points. Cells were then washed in 1x PBS and stained with Nile-Red for 15 min at room temperature. At the end of the incubation, cells were washed again, and fluorescence was measured with the Cytation™ 3 Cell Imaging Multi-Mode Reader (BioTek™; ex. 488, em. 550).

Extracellular Flux Analysis. The cellular oxygen consumption rate (OCR) was measured using a Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Agilent technologies). Briefly, wild-type-MitoGFP and $CB_1R^{-/-}$-MitoGFP HK-2 cells were seeded

in an XF24 cell culture plate ($25x10^3$ cells/well), and incubated overnight in a 37°C humidified incubator with 5% CO_2. Then, vehicle, AEA, or O:P was added to the cells in fresh SFM for the indicated times. Basal OCR was measured at the end of the incubation time. Data were calculated from 3 consecutive measurements, post instrument calibration.

Multi-parameter Metabolic Assessment. The mice were metabolically assessed by using the Promethion High-Definition Behavioral Phenotyping System (Sable Instruments, Inc., Las Vegas, NV, USA) as described previously.[2]

Real-time PCR. For total mRNA isolation, HK-2 cells were washed in PBS and harvested using Bio-Tri RNA lysis buffer (Bio-Lab, Israel). Extracted RNA was treated with DNase I (Thermo Scientific, IL), and reverse transcribed using the Iscript cDNA kit (Bio-Rad Laboratories, CA). Quantitative PCR reactions for dynamin-related protein 1 (*DRP1*) were performed using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, CA), and the CFX connect ST system (Bio-Rad Laboratories, CA). RQ values of all tested genes were normalized to RPLP0. Primers are listed in **Supplementary Table 1**.

Western blot Analysis. Adherent cells were washed in cold PBS x1, and harvested in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS), vortexed and incubated for 30 min at 4°C, then centrifuged for 10 min at 14,000 rpm. Protein concentrations were determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, IL). Cleared lysates were added with protein sample buffer,

resolved by SDS-PAGE (4-15% acrylamide, 150V) and transferred to PVDF membranes using the Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories, CA). Membranes were incubated for 1 hour in 5% milk (in TBS-T) to block unspecific binding, washed briefly, and incubated overnight at 4°C with primary antibodies for either phosphorylated DRP1(S637) (ab193216, Abcam), DRP1 (ab5788, Abcam), phosphorylated $ERK_{1/2}$ (ab201015, Abcam), total $ERK_{1/2}$ (ab184699, Abcam), GFP (#598, MBL International), Hsp60 (kindly provided by O. Pines, Hebrew University of Jerusalem), or β-Actin (ab49900, Abcam). Anti-rabbit (ab97085, Abcam) or mouse (ab98799, Abcam) horseradish peroxidase (HRP)-conjugated secondary antibodies were used for 1 hour at room temperature, followed by chemiluminescence detection using Clarity™ Western ECL Blotting Substrate (Bio-Rad Laboratories, CA). Densitometry was quantified using ImageLab software and calculated as the ratio between phosphorylated and non-phosphorylated signals, unless stated otherwise.

Immunofluorescence. Wild-type-MitoGFP and $CB_1R^{-/-}$-MitoGFP HK-2 cells were seeded in an 8-chambered cover-glass system (C8-1.5H-N, In Vitro Scientific). At the end of the experiment, cells were fixed for 10 min in 2% paraformaldehyde, washed 3 times with PBS x1, and permeabilized using 0.25% Triton X-100 for 7 min. Then, cells were washed 3 times and blocked in x1 PBS containing 2% BSA for an additional 2 hours at room temperature. Following blocking, cells were incubated overnight with either anti-DRP1 (S616) (3455, Cell Signaling) or anti-HSP60 and anti-DRP1 (ab5788, Abcam) at 4°C. APC-conjugated donkey anti-rabbit IgG (711-136-152, Jackson) and Cy3-conjugated goat anti-mouse IgG (ab97035, Abcam) were used as secondary antibodies for 1 hour. Stained

cells were added with Hoechst 33342 (H3570, Sigma Aldrich), washed in PBS and analyzed. Images were taken with an OLYMPUS IX-73 fluorescent microscope, and processed with CellSens Dimension software or a NIKON A1R confocal microscope followed by analysis using NIS elements software.

Cellular Fractionation. Mitochondria were isolated from cells using the Mitochondria Isolation Kit for Cultured Cells (89874, Thermo Scientific), following the manufacturer's protocol with modifications. Briefly, $8x10^6$ HK-2 MitoGFP cells, treated with either vehicle or 5 µM AEA, were used per sample. Cells were lysed in Buffer A, using a dounce homogenizer. Lysates were centrifuged in 1200 x g for 5 min to pellet nuclei. Supernatants were transferred to clean tubes and centrifuged at 3000 x g for 15 min to pellet mitochondria. Pellets were washed twice in Buffer C and re-suspended in a RIPA buffer for Western Blot analysis. The supernatants, containing the cytoplasmic proteins, were also collected for the analysis.

Determination of the NAD^+/NADH Ratio. An NAD^+/NADH Assay kit (ab65348, Abcam) was used to determine the ratio between NAD^+ and NADH according to the manufacturer's instructions. Absorbance was measured with a Spectra Max Plus plate reader (Molecular Devices, CA) at a wavelength of 450 nm.

Lactate Measurements. Cellular lactate levels were measured with a Picoprobe L-Lactate Assay Kit (ab169557, Abcam) following the manufacturer's protocol. For intra-cellular

lactate, 20 µL of lysate prepared from $1x10^6$ cells were used. Fluorescence was measured with a SYNERGY HT microplate reader (ex. 530/25, em. 590/20).

ATP Quantification. ATP content in wild-type-MitoGFP and $CB_1R^{-/-}$-MitoGFP HK-2 cells was quantified using an ATP assay kit (ab83355, Abcam), following the manufacturer's protocol. Briefly, $2.5x10^6$ cells/sample were harvested, washed, and re-suspended in 100 µL ATP assay buffer. Sample de-proteinization was carried out using a TCA precipitation kit (ab204708, Abcam), and 50 µL of each sample were used for the assay. The colorimetric signal was measured with a Spectra Max Plus plate reader (Molecular Devices, CA) at a wavelength of 570 nm.

Reactive Oxygen Species (ROS) Measurements. Cellular ROS levels were measured using Cellular Reactive Oxygen Species Detection Assay Kit (Deep Red Fluorescence) (ab186029, Abcam) according to manufacturer's protocol. Briefly, wild-type-MitoGFP and $CB_1R^{-/-}$-MitoGFP HK-2 cells were seeded in a 96-well plate format ($15x10^4$ cells/well) and treated with either vehicle or AEA for 6h. 30 min before the end of the experiment, ROS probe was added to cells and incubated for 30 min in 37°C. Fluorescence was then measured with a SYNERGY HT microplate reader (ex. 650, em. 675).

Total DNA Extraction and mtDNA/nDNA Measurement. Cells were harvested using trypsin, and DNA was extracted with a DNeasy Blood & Tissue Kit (69056, QIAGEN), according to the manufacturer's protocol. Isolated DNA served as a template for a qPCR

reaction using primer sets for β2-microglobulin (β2m) to amplify nuclear DNA (nDNA), and a D-loop region to amplify mitochondrial DNA (mtDNA). Primers are listed in **Supplementary Table 1**.

Cell Viability and Proliferation. To evaluate cell viability, HK-2 cells treated with vehicle or 5 μM AEA for 6 and 24 hours were stained using an Annexin V-FITC Apoptosis Detection Kit (ab14085, Abcam), according to the manufacturer's protocol. Stained cells were analyzed by LSR II flow cytometer (Becton Dickinson, NJ), and collected data were analyzed by FCS Express V3 (De Novo Software, CA, USA). Cell proliferation was assessed using CFSE staining according to a standard protocol. Briefly, untreated HK-2 cells were loaded with CellTrace™ CFSE (C34554, Invitrogen) for 30 min at room temperature, washed twice in complete DMEM, seeded, and left to adhere. Culture cells were then incubated with vehicle or 5 μM AEA for 24 hours. At the end of the experiment, cells were analyzed by flow cytometry, as described above.

Quantification of Mitochondrial Morphology. Fluorescence images in .vsi format were processed in ImageJ software using the OlympusViewer Plugin, then subjected to analysis with the publically available ImageJ macro for mitochondrial morphology, designed by Ruben K. Dagda and named Mito-Morphology.[3] The macro returns the circularity value for each mitochondrion in the cell and the average circularity value of each measured cell. The average value ± SEM was plotted. Number of replicates is indicated in each figure legend. The perimeter of each mitochondria, and the total mitochondrial area were also

measured using the same macro. Interconnectivity was calculated as AVG area/perimeter ratio.

Electron microscope images in .tiff format were analyzed in Adobe Photoshop C3S software. Images were analyzed in RGB mode, and the measurement scale was set according to image scale bar. Each mitochondrion was marked using the magnetic laso tool, and measurements of area and perimeter were recorded. Circularity was calculated by the software as $4 * л(\frac{area}{perimeter})\text{^}2$, and value of 1 represents a perfect circle. The length of mitochondria was measured by manually drawing a line along mitochondrial major axis, using the ruler tool, and recording the value.

Endocannabinoid measurements by LC-MS/MS. eCBs were extracted, purified, and quantified in kidney and cells, as described previously.[2] LC-MS/MS was analyzed on an AB Sciex (Framingham, MA, USA) Triple Quad™ 5500 mass spectrometer coupled with a Shimadzu (Kyoto, Japan) UHPLC System. eCBs were detected in a positive ion mode using electron spray ionization (ESI) and the multiple reaction monitoring (MRM) mode of acquisition.

The levels of each compound were analyzed by monitoring multiple reactions. The molecular ion and fragment for each compound were measured as follows: m/z 348.3→62.1 (quantifier) and 91.1 (qualifier) for AEA, m/z 379.3→287.3 (quantifier) and 91.1(qualifier) for 2-AG, m/z 305.2 →91.1 (quantifier) and 77.1 (qualifier) for AA, and m/z 352.3→66.1 (quantifier) and 91.1 (qualifier) for [$^2H_4$] AEA. The levels of AEA, 2-AG, and AA in the cell and kidney samples were measured against standard curves.

Statistical Analysis. Data are presented as mean ± SEM. Unpaired two-tailed Student's t-test was used to determine variations between groups (GraphPad Prism v6 for Windows). Statistical significance was set at $P < 0.05$.

References

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