Supplementary Methods

Intraperitoneal Glucose Tolerance Test (IPGTT)

All the mice were fasted overnight and then received an intraperitoneal injection of glucose solution (2 g glucose/kg body weight). The blood glucose was monitored at various time points (0, 30, 60, 90, 120, and 180 min) by cutting the tail and gently massaging blood onto a glucose test strip.

Indirect Calorimetry

The oxygen consumption (Volume O_2), carbon dioxide generation (Volume CO_2), HEAT, and respiratory exchange ratio (RER) of mice were measured using the Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments). The above metabolic parameters were normalized according to body weight.

Computed Tomography (CT)

The fat mass of subcutaneous and epididymal adipose tissue (eWAT, sWAT) in WT mice were determined using a Micro-PET-CT scanner (PINGSENG Healthcare Inc., China). The mice were anesthetized with 3% isoflurane and positioned with all legs fully extended. The mass of sWAT and eWAT were quantified in the area between proximal end of lumbar vertebra L1 and the distal end of L6.

Immunofluorescence staining

The sWAT and eWAT were fixed in 4% 10% formalin and paraffin sections were prepared for immunofluorescence staining. The paraffin sections were incubated with primary antibody overnight, including Cx43 (Cell Signaling Technology, 3512S, 1: 400), CD206 (Abcam, ab64693, 1:400), CCR2 (Abcam, ab203128, 1:400), CD11c (Abcam, ab219799, 1:400), F4/80 (Abcam, ab6640, 1:400), and UCP-1 (Abcam, ab10983, 1:400), followed by incubation with fluorophore-conjugated secondary antibody, including goat anti-rat (Abcam, ab150167, 1:400) or goat anti-rabbit (Invitrogen, A32738, 1:400), for 1 h. The Immunofluorescence images were obtained using a confocal microscopy (A1HD25, Nikon, Tokyo, Japan).

Transmission electron microscopy

For transmission electron microscopy assay (TEM), RAW264.7 cells were incubated with 2% osmium tetroxide and 0.1 mM sodium cacodylate (pH 7.4) for 1 h at 4 °C. The sections were stained with uranyl acetate and lead citrate and examined under a Philips CM10 electron microscope (Eindhoven, the Netherlands).

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Netherlands).

Evaluation of ROS

The ROS generation were measured using a dihydroethidium (DHE) fluorescent probe and MitoSOX Red (Thermo Fisher Scientific), for cytosolic and mitochondrial ROS, respectively. Briefly, the stimulated cells were incubated with DHE or MitoSOX (5 μM) at 37°C for 30min the ROS generation were quantified using a Fluoroskan Ascent Fluorometer (Thermo Fisher Scientific) and visualized by an inverted fluorescence microscope (TE2000; Nikon).

High-Resolution Respirometry

The mitochondrial respiratory function was analyzed using Oxygraph-2k (O2k, Oroboros Instruments). RAW264.7 cells were harvested and transferred to oxygraphy chambers. The routine respiration was monitored and the plasma membrane was permeabilized with digitonin. Glutamate (G, 10mM), malate (M, 0.5mM), and ADP (5mM) were added successively to evaluate Complex I–dependent oxidative phosphorylation (CI OXPHOS). Subsequently, succinate (S, 10mM) was added to induce the maximal OXPHOS capacity of CI + Complex II (CII) OXPHOS. Next, CI+II supported maximal convergent respiratory capacity of the CI + II electron transfer system (ETS) was measured via addition of 2-([4-(trifluoromethoxy) phenyl hydrazinylidene) propanedinitrile (FCCP, 0.05uM steps). CI + Complex II (CII)

OXPHOS was evaluated after addition of succinate (S). Subsequently, CII ETS was measured after addition of rotenone (Rot, 0.5μM). Residual oxygen consumption (ROC) was evaluated after the inhibition of CIII with antimycin A (Ama, 2.5μM).

Respiratory Chain Enzyme Activity Assay

The respiratory chain enzyme activities (Complex I, II, III, and IV) were measured using Mitochondrial Complexes Activity Assay Kit (Solarbio, Beijing, China).

Mitochondrial and ER Ca^{2+} Measurement

The mitochondrial and ER Ca^{2+} fluorescence signals were measured using Rhod-2 AM and MagFura-2 AM (Thermo Fisher Scientific), respectively. RAW264.7 cells were incubated with Rhod-2 AM (5 μM) or MagFura-2 AM (5 μM), as well as 0.025% F-127, at 37 ℃ for 40min and washed 3 times. Then, cells were permeabilized with digitonin and washed 3 times. For mitochondrial Ca^{2+} fluorescence signal, the fluorescence was measured at an emission wavelength of 581 nm and an excitation wavelength of 552 nm at baseline and after treatment. As for ER Ca^{2+} fluorescence signal, an emission wavelength of 510 nm and excitation wavelength of 340 nm and 380 nm. Varioskan Flash microplate reader (Thermo Electron Corporation) was used to measure the fluorescent signal.

MTT measurement

The SDH activities were assayed according to manufactures' instructions (Beyotime,

China).

Mitochondrial membrane potential

The mitochondrial membrane potential was determined using Mitochondrial membrane potential assay kit with JC-1 (Solarbio, Beijing, China). All the analyses were performed according to the manufacturer's instructions and the fluorescence signals were recorded with Varioskan Flash (Thermo Scientific, MA, USA).

Real-time PCR

The RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufactures' instructions and then reverse-transcribed into cDNA using a Transcriptor First-Strand cDNA Synthesis Kit (Roche). The SYBR Green (Roche) was used for the amplification reactions using the three-step protocol described by the manufacturer (LightCycler 96; Roche). Data were normalized to housekeeping genes (GAPDH). The fluorescence curves were analyzed using LightCycler 96 software (version 1.1). The primers are listed in Supplementary Table.

Isolation of mitochondria, MAM, and inner mitochondrial membrane

The isolation of mitochondrion and MAM were conducted according to previous reports [1]. Briefly, RAW264.7 cells were harvested and homogenized followed by gradient centrifugation to acquire the crude mitochondria. Subsequently, the crude mitochondria were resuspended in mitochondrial resuspending buffer (MRB)

containing 30% percoll medium and then, ultracentrifuged at 95,000g for 30min at 4 °C in a Beckman Coulter Optima L-100 XP Ultracentrifuge (SW40 rotor, Beckman, Fullerton, CA, USA) to obtain purified mitochondria and crude MAM. The crude MAM was further purified at 100,000g for 1 h (70-Ti rotor, Beckman) at 4 °C.

The isolation of inner mitochondrial membrane (IMM) was performed according to previous studies[2] with minor modifications. RAW264.7 cells were harvested and broken in cold mannitol buffer (MB) (210 mM mannitol, 70 mM sucrose, 10 mM HEPES-KOH pH 7.4, 1 mM EDTA) with protease inhibitors (Roche) and centrifuged gradient to obtain mitochondrial-rich fraction. The pellet was further washed and centrifuged to get sub-mitochondrial fractionation. The mitoplasts were resuspended in cold 10 HEPES buffer containing protease inhibitors (Roche) and further incubated for 20 min on ice. After incubation, the mitoplasts were centrifuged for 15 min at 15,000g at 4 °C and sonicated using a water bath with 30-s pulses for 15 cycles at 4 °C. The samples were finally ultra-centrifuged for 30 min at 45,000g at 4 °C, to obtain a pellet corresponding to the IMM-rich fraction.

Western blot

The RAW264.7 cells, MAM, IMM, and mitochondria were prepared using RIPA lysis buffer (1% Triton-X, 0.1% SDS, 0.5% deoxycholate, 50 mM Tris pH 7.5, 150 mM NaCl) with protease inhibitor cocktail tablets (Roche). The concentrations were measured using bicinchoninic acid assay (BCA) method. Equal amounts of protein (20 mg/lane) were separated by SDS-PAGE and transferred onto polyvinylidene difluoride

membranes (Millipore). After blocking, the filters were incubated with the following primary antibodies: Cx43 (Cell Signaling Technology, 3512S), Cx43 (Abcam, ab235585), IL-10 (Abcam, ab189392), Arg (Cell Signaling Technology, 93668S), iNOS (Cell Signaling Technology, 20609S), TNF-α (Abcam, ab92324), IL-1β (Abcam, ab234437), HIF-1α (Cell Signaling Technology, 36169S), GLUT1 (Abcam, ab115730), HKII (Abcam, ab209847), LDHa (Abcam, ab52488), SDHB (Abcam, ab175225,), COX IV(Cell Signaling Technology, 11967S), IP3R (Abcam, ab264281), MCU (Cell Signaling Technology, 14997S), Tom20 (Cell Signaling Technology, 42406S), VDAC (Abcam, ab14734). After washes and incubation with the appropriate horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnology), the immune complexes were visualized using a chemiluminescence horseradish peroxidase substrate (WBKLS0100; Millipore).

Immunoprecipitation

The Immunoprecipitation (IP) was conducted to explore the relationship between Cx43 and MAM markers according to a previous protocol [1]. Briefly, the purified MAM was lysed with RIPA buffer (1% Triton-X, 0.1% SDS, 0.5% deoxycholate, 50 mM Tris, 150 mM NaCl, Protease inhibitor). The lysate was incubated with Cx43/SDHB antibody overnight at 4 °C and equal amount of IgG was used as the control, followed by 3 h in the presence of Dynabeads Protein A/G (Invitrogen). Dynabeads were washed 5-6 times with IP buffer. The protein was eluted with loading buffer at 100 °C for 8 min and was further analyzed by Western blot for Tom20, IP3R, VDAC, MCU, and Cx43.

1. Wieckowski MR, Giorgi C, Lebiedzinska M, et al. Isolation of mitochondria-associated membranes and mitochondria from animal tissues and cells. *Nat Protoc*. 2009, 4:1582-1590.

2. Rey T, Zaganelli S: Mitochondrial RNA granules are fluid condensates positioned by membrane dynamics. *Nat Cell Biol*.2020, 22:1180-1186.

Supplementary Figures legends

Supplementary Figure 1. Inhibiting Cx43 alleviates HFD-induced adipose tissue inflammation.

(A) Oxygen consumption (Volume O_2), carbon dioxide production (Volume CO_2), RER, and Heat of WT mice fed with ND or HFD, detected by CLAMS (n=5). (**B and C**) Representative images of IF staining **(B)** and quantitative results **(C)** of CD11c (Green), CD206 (Green), CCR2 (Green), Cx43 (Green), and F4/80 (Red) in sWAT of mice fed with ND or HFD, respectively. Scales bars, 50 μm (n=5). **(D-F)** The mRNA levels of indicated genes in RAW264.7 cells treated with BSA or PA **(D,** n=3**)**, saline or LPS **(E,** n=3**)**, LPS or LPS+18α-GA **(F,** n=3**).** The data are presented as the mean ± SEM. $^{*}P<$ 0.05, $^{**}P<$ 0.01, $^{***}P<$ 0.001, $^{****}P<$ 0.0001 compared with ND **(A-C)**, BSA **(D)**, saline **(E)**, or LPS **(F)**.

Supplementary Figure 2. Inhibition of Cx43 alleviates PA-induced M1 macrophages polarization.

(A) The protocol used to measure mitochondrial respiration. G, glutamate (10mM); M, malate (0.5mM); S, succinate (10mM); FCCP, 2-([4-(trifluoromethoxy) phenyl hydrazinylidene) propanedinitrile (0.05uM steps); Rot, rotenone (0.5μM); AMA, antimycin A (2.5μM). **(B)** Representative images of mitochondrial ROS measured by MitoSOX fluorescent staining and quantitative results of RAW264.7 cells treated with or without 18α-GA (n=3). **(C and D) T**he mRNA levels of indicated genes in

RAW264.7 cells treated with si-con+PA or si-Cx43+PA **(C,** n=3**)**, LV-con+PA or LV-Cx43+PA **(D, n=**3**)**. The data are presented as the mean ± SEM. $^{*}P< 0.05$, $^{**}P< 0.01$, $^{***}P< 0.001$, $^{****}P< 0.0001$ compared with con **(B)**, si-con+PA **(C)**, or LV-con+PA **(D)**.

Supplementary Figure 3. Inhibiting SDH alleviates PA-induced mitochondrial dysfunction and pro-inflammatory M1 macrophages polarization.

(A-F) The representative images of cytosolic ROS measured by DHE and mitochondrial ROS measured by MitoSOX fluorescent staining and the individual quantitative results **(A, D,** n=3**)**, the mitochondrial membrane potential that assayed by JC1 **(B, E,** n=3**)**, and the mitochondrial respiration indexes **(C, E,** n=2**)** in RAW264.7 cells treated with PA or DMM+PA **(A-C)**, PA or Suc+PA **(D-F)**. **(G and H)** The mRNA levels of indicated genes in RAW264.7 cells treated with PA or DMM+PA **(G, n=3)**, PA or Suc+PA **(H, n=3)**. The data are presented as the mean ± SEM. $^{*}P< 0.05$, $^{**}P< 0.01$, $^{***}P< 0.001$, $^{****}P< 0.0001$ compared with PA.

Supplementary Figure 4. Inhibition of Cx43 improves MCU-mediated mitochondrial Ca^{2+} overload in macrophages.

(A) Representative immunoprecipitation of Cx43 in isolated MAM of RAW264.7 cells treated with BSA or PA and immunoblotted by IP3R (n=3). The immunoblot results of lysate input are showed below. **(B-I and K-N)** The DHE and mitoSOX fluorescent

staining and the individual quantitative results **(B, F, K,** n=3**)**, the mitochondrial respiration indexes **(C, H, L,** n=2**)**, the mitochondrial membrane potential that assayed by JCI **(D, G, M,** n=3), and mRNA levels of indicated genes **(E, I, N,** n=3**)** in RAW264.7 cells treated with LV-Cx43+PA or LV-Cx43+si-MCU+PA **(B-E)**, si-Cx43+PA or si-Cx43+LV-MCU+PA **(F-I)**, LV-Cx43+PA or LV-Cx43+LV-MCU+PA **(K-N). (J)** The changes of mitochondrial Ca^{2+}, labeled with Rhod-2 AM, in digitonin-permeabilized RAW264.7 cells treated with LV-Cx43+PA or LV-Cx43+LV-MCU+PA (n=3). The data are presented as the mean ± SEM. $^{*}P< 0.05$, $^{**}P< 0.01$, $^{***}P< 0.001$, $^{****}P< 0.0001$ compared with LV-Cx43+PA **(B-E and J-N)**, si-Cx43+PA **(F-I)**.

Supplementary Table. Primers Used for qRT-PCR in This Study.

mouse GAPDH-reverse	CATGGCCTTCCGTGTTCCTA
mouse GAPDH-reverse	CCTGCTTCACCACCTTCTTGAT
mouse Arg-forward	GGGCAACCTGTGTCCTTTCTCC
mouse Arg-reverse	GGTCTACGTCTCGCAAGCCAAT
mouse IL-10-forward	GGGTTGCCAAGCCTTATCGGAA
mouse IL-10-reverse	CTTCACCTGCTCCACTGCCTTG
mouse TNF-α-forward	GGAACTGGCAGAAGAGGCACTC
mouse TNF-α-reverse	GTAGACAGAAGAGCGTGGTGGC
mouse iNOS-forward	CCCTCCTCGTTCAGCTCACCTT
mouse iNOS-reverse	CCGCTCTCATCCAGAACCTCCA
mouse IL-1β-forward	CCTGTGTCTTTCCCGTGGACCT
mouse IL-1β-reverse	TCGGAGCCTGTAGTGCAGTTGT
mouse GLUT1-forward	CTTCTCTGTGGGCCTTTTCGT
mouse GLUT1-reverse	CAACAAAGGACTTGCCCAGTTTCG
mouse LDHa-forward	ATATCTTGACCTACGTGGCTT
mouse LDHa-reverse	CTCTCCCATCAGGTAACGGAA
mouse HIF-1α-forward	CTCAAAGTCGGACAGCCTCA
mouse HIF-1α-reverse	CCCTGCAGTAGGTTTCTGCT
mouse HKII-forward	ATCTACGCCATTCCCGAGGACA
mouse HKII-reverse	ATCTCTGCCTTCCACGCCACT
mouse MCU-forward	TCTCTGACTCAGTCGGCGTGTT
mouse MCU-reverse	GTCATCGAGGAGCAGGAGGTCT

mouse SDHB-forward	TACTGGTGGAACGGAGAC
mouse SDHB-reverse	TACTGGTGGAACGGAGAC