Supplementary Data

AMPK-induced mitochondrial biogenesis decelerates retinal pigment epithelial cell degeneration under nutrient starvation

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MATERIALS AND METHODS

Reagents and antibodies

2′,7′-dichlorofluorescin diacetate (DCF-DA; D6883), compound C (Comp C; 171260), chloramphenicol (CAP; C0378), 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR; A9978), and metformin (Met; D150959) were purchased from Sigma-Aldrich (Burlington, MA, USA). 4′,6-diamidino-2-phenylindole (DAPI; D1306), MitoTracker Red CMX/Ros (M7512), MitoTracker Green FM (M7514), ProLong Gold antifade reagent (P36934), and Lipofectamine 3000 (L3000015) were supplied by Invitrogen (Waltham, MA, USA). Protease & phosphatase inhibitor cocktail (78,442) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12 (DMEM/F12; 11330-032), Hank's buffered salt solution (HBSS; 24020117) and Opti-MEM (31985070) were purchased from GIBCO (Waltham, MA, USA). Fetal bovine serum (FBS) and penicillin-streptomycin solutions (LS202-02) were supplied by Welgene (Deagu, Korea). 10X Phosphate-buffered saline (PBS; L615-500) was supplied by Biowest (Riverside, MO, USA).

The following antibodies were used: anti-AMPKα (2532S) and phospho-AMPKα (2535S) antibodies were purchased from Cell Signaling (Danvers, MA, USA). Anti-GAPDH (ab181602) and PGC1-α (ab54481) were supplied by Abcam (Cambridge, UK). Horse radish peroxidase conjugated goat anti-rabbit immunoglobulin (sc-2004) and Horse radish peroxidase conjugated goat anti-mouse immunoglobulin (sc-2031) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin (A-11008), Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin (A-11001), and Alexa Fluor 555-conjugated goat anti-rabbit immunoglobulin (A-21428) were purchased from Invitrogen.

Cell culture and siRNA transfection

Retinal pigment epithelial cells (ARPE-19; CRL-2302; American Type Culture Collection, ATCC) were maintained in DMEM/F12 medium supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Cells were kept in an incubator, humidified at 37 ˚C with 5% CO_2. Cells were starved with HBSS minimal medium. To knockdown TWIST1, ARPE-19 cells were incubated in Opti-MEM medium and transfected with anti-TWIST1 siRNA (sc-38604; Santa Cruz Biotechnology) using Lipofectamine 3000.

Reactive oxygen species detection

ARPE-19 cells were seeded on glass coverslips (Φ12 mm) placed in a 24-well plate and starved with HBSS minimal medium under various conditions at 37 ˚C. After incubation, cells were treated with DCF-DA (10 μM) in the dark at 37 ˚C for 10 min. Cells were washed with PBS and examined under a fluorescence microscope (AMF7000; Invitrogen).

Western blot analysis

RIPA buffer (50 mM Tris-HCl, pH 8, 150 nM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1.0% Tergitol®, 10 mM 2-mercaptoethanol, 1 mM phenylmethanesulfonylfluoride) including a protease & phosphatase inhibitor cocktail was used to lyse the cells. Bradford assay (5000006; Bio-Rad; Hercules, CA, USA) was used to determine the protein concentration. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins (15-20 μg per lane) and then proteins were transferred to an Immobilon-P PVDF membrane (IPVH00010; Millipore; Burlington, MA, USA). Membranes were blocked with 3% bovine serum albumin (BSA100; LPS solution; Daejeon, Korea) dissolved in tris buffered saline tween-20 (TBST) buffer (25 mM Tris, 150 mM NaCl, 2 mM KCl, 0.1% Tween 20, pH 7.4) for 1 h at room temperature, followed by incubation overnight with primary antibodies (1:1000 to 1:80,000) at 4 ˚C. After washing with TBST buffer, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit and anti-mouse immunoglobulin for 2 h at room temperature. After 2 h, membranes were revealed using an enhanced chemiluminescence system (WBKLS0500; Millipore) and signals were detected using a G:BOX Chemi XL (Syngene; Frederick, MD, USA).

Analysis of mitochondrial morphology

Cells were seeded on glass coverslips (Φ12 mm) placed in a 24-well plate and starved with HBSS minimal medium under various conditions at 37 ˚C. After incubation, cells were washed with PBS and incubated with 100 nM of MitoTracker Red CMX/Ros (diluted in Opti-MEM) at 37 ˚C for 10 min in the dark. Cells were subsequently washed thrice with pre-warmed PBS and fixed with 4% paraformaldehyde for 1 h. After washing, the nuclei were stained with 300 nM of DAPI (diluted in PBS) in the dark at room temperature. Cells were washed with PBS for 10 min three times after each step. Coverslips were mounted with ProLong Gold antifade reagent on a glass slide. Red fluorescence resulting from the mitochondria was observed using a confocal fluorescence microscope (LSM800; Carl Zeiss, Jena, Germany).

MitoTimer fluorescence assay for mitochondrial turnover

Cells were seeded on glass coverslips (Φ12 mm) placed in a 24-well plate and transfected with plasmids encoding MitoTimer (52659; Addgene, Watertown, MA, USA) using Lipofectamine 3000. After transfection, cells were washed with PBS and the medium was replaced with 10% DMEM/F12 medium. Cells were starved with HBSS minimal medium under various conditions at 37 ˚C. After incubation, cells were fixed with 4% paraformaldehyde for 1 h. Nuclei were stained with 300 nM of DAPI (diluted in PBS). Cells were washed with PBS for 10 min three times after each step. Cells were observed under a confocal fluorescence microscope (LSM800; Carl Zeiss). MitoTimer fluorescence was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Flow cytometric analysis

Cells were seeded in 6-well plates and starved with HBSS minimal medium under various conditions at 37 ˚C. The cells were washed with pre-warmed PBS and stained with MitoTracker Red CMX/Ros (100 nM) and MitoTracker Green FM (50 nM) at 37 ˚C for 10 min in the dark. Cells were collected and resuspended in PBS. Cells were analyzed using a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA).

Quantitative real-time PCR analysis

Cells were seeded in 6-well plates and starved with HBSS minimal medium under various conditions at 37 ˚C. After collecting the cells, total RNA was isolated using TransZol-Up reagent (ET111-01; TransGen Biotech, Beijing, China) according to the manufacturer's instructions. cDNAs were synthesized from 1 μg of total RNA using ReverTra Ace™ qPCR RT Master Mix (FSQ-201; TOYOBO, Osaka, Japan) according to the manufacturer's instructions. cDNAs were amplified by quantitative real-time PCR using the QuantiNova SYBR Green PCR Kit (208054; QIAGEN, Hilden, Germany). Primer sequences used are listed in Table S1. The fluorescence signal was detected using Rotor-Gene Q (QIAGEN). The following cycle conditions were applied: 95 ˚C for 5 min, followed by 40 cycles at 95 ˚C for 10 s, and 53 ˚C for 45 s, and melting step from 58 ˚C to 95 ˚C.

Immunocytochemistry

Cells were seeded on glass coverslips (Φ12 mm) placed in a 24-well plate and starved with HBSS minimal medium under various conditions at 37 ˚C. After incubation, cells were fixed with 4% paraformaldehyde for 1 h. Cells were then permeabilized with 0.2% Triton X-100 for 15 min at room temperature, followed by

blocking with 3% bovine serum albumin for 1 h at room temperature. Primary antibodies were incubated overnight at 4 ˚C, and fluorescence-conjugated secondary antibodies were added for 2 h at room temperature. Nuclei were stained with 300 nM of DAPI (diluted in PBS). Cells were washed with PBS for 10 min three times after each step. Cells were mounted with ProLong Gold antifade reagent on a slide glass and observed under a confocal fluorescence microscope (LSM800; Carl Zeiss).

Dual-luciferase reporter assay

Cells were seeded in a 24-well plate and co-transfected with luciferase-expressing NF-κB plasmids (Promega; Madison, WI, USA) and luciferase-expressing Renilla plasmids (Promega) using Lipofectamine 3000. After transfection, cells were washed with PBS and the medium was replaced with 10% DMEM/F12 medium. Cells were starved with HBSS minimal medium under Met pretreatment (1 mM for 2 h) at 37 ˚C for 12 and 24 h. After incubation, the cells were lysed using a passive lysis buffer (Promega). Luciferase assay was performed using the Dual-Luciferase Reporter Assay System (E1910; Promega) according to the manufacturer's instructions.

Transmission electron microscopy

Cells were seeded in a 100-mm dish and starved with HBSS minimal medium under Met pretreatment (1 mM for 2 h) at 37 ˚C for 36 h. After incubation, cells were fixed with Karnovsky's fixative (2% glutaraldehyde and 2% paraformaldehyde in 0.05 M sodium cacodylate buffer) at 4 ˚C overnight. After three washes with 0.05 M sodium cacodylate buffer, cells were post-fixed with 1% osmium tetroxide diluted in 0.1 M cacodylate buffer at 4 ˚C for 1 h. Cells were washed three times with distilled water and en bloc stained in 0.5% uranyl acetate for 30 min at 4 ˚C. Cells were dehydrated with various concentrations (30% to 100%) of ethanol and transitioned into 100% propylene oxide. Dehydrated cells were embedded with Spurr's resin in propylene oxide and polymerized in Spurr's resin at 70 ˚C overnight. After ultramicrotome sectioning (PowerTome PC; RMC Boeckeler, Tucson, AZ, USA), sections were sequentially stained with 2% uranyl acetate and Reynolds lead citrate. Samples were observed using a transmission electron microscope (LIBRA 120; Carl Zeiss).

Statistical analysis

Replicate data were expressed as the mean ± standard error of the mean (SEM). Paired data were evaluated using Student's *t*-test, in which a value of $P < 0.05$ was considered statistically significant.

Supplementary Table and Figures

Table S1. Primer sequences for PCR

Gene Name		Sequence
TFAM	Forward Primer	5' AGC TCA GAA CCC AGA TGC 3'
	Reverse Primer	5' CCA CTC CGC CCT ATA AGC 3'
TWIST1	Forward Primer	5' TTC TCG GTC TGG AGG ATG GA 3'
	Reverse Primer	5' CCC ACG CCC TGT TTC TTT GAA 3'
SNAI1	Forward Primer	5' GAA AGG CCT TCA ACT GCA AA 3'
	Reverse Primer	5' TGA CAT CTG AGT GGG TCT GG 3'
GAPDH	Forward Primer	5' ACC ACA GTC CAT GCC ATC AC 3'
	Reverse Primer	5' TCC ACC ACC CTG TTG CTG TA 3'

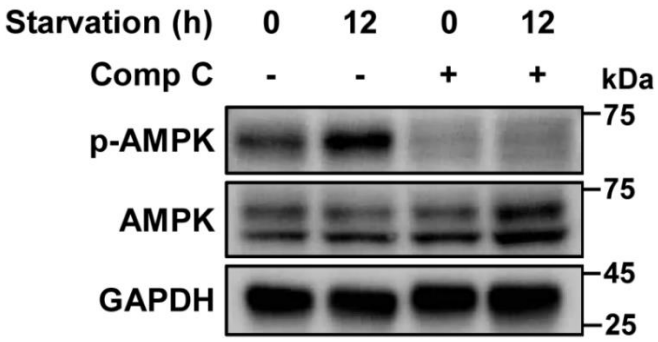

Fig S1. Comp C treatment inhibits AMPK activation under nutrient starvation. ARPE-19 cells were starved with HBSS media for 12 h in the presence or absence of Comp C (5 μM). Western blot analysis of AMPK and p-AMPK under nutrient starvation.

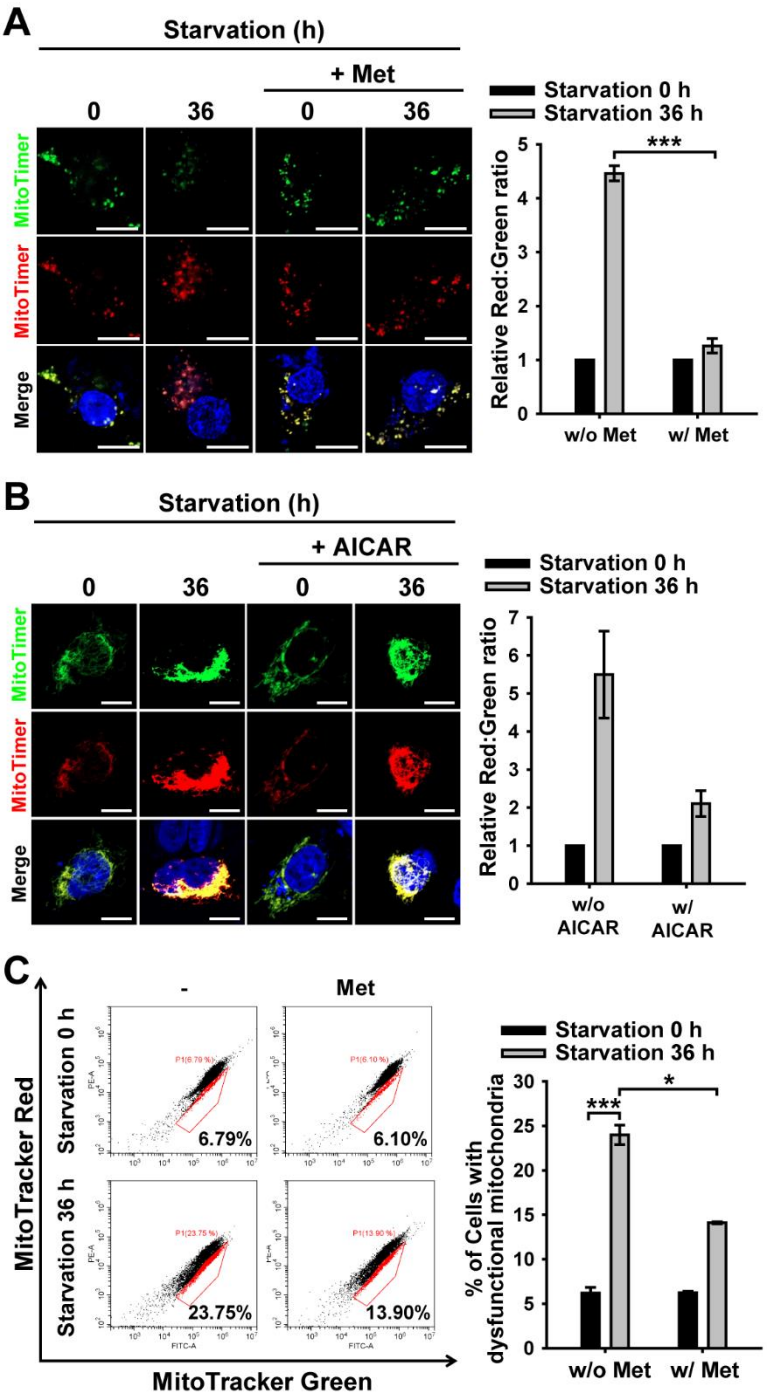

Fig. S2. AMPK activation promotes mitochondrial quality control under nutrient starvation. (A) Confocal fluorescence microscopy images of MitoTimer-expressing cells under nutrient starvation for 36 h in the presence or absence of Met (1 mM; pre-treated for 2 h). Scale bar: 10 μm. Bar graph represents the red-to-green fluorescence ratio of MitoTimer. Data are presented as the mean ± SEM, n = 3. $^{***}P$ 〈 0.005. **(B)** Confocal fluorescence microscopy images of MitoTimer-expressing cells under nutrient starvation for 36 h in the presence or absence of AICAR (2 mM;

pre-treated for 1 h). Scale bar: 10 μm. Bar graph represents the red-to-green fluorescence ratio of MitoTimer. **(C)** Flow cytometric assay for mitochondrial dysfunction in ARPE-19 cells under nutrient starvation for 36 h in the presence or absence of Met (1 mM; pre-treated for 2 h). The percentage population of cells with dysfunctional mitochondria is represented in each graph. Bar graphs indicate the percentage of cells with dysfunctional mitochondria. Data are presented as the mean ± SEM, n = 3. $^{*}P < 0.05$ and $^{***}P < 0.005$.

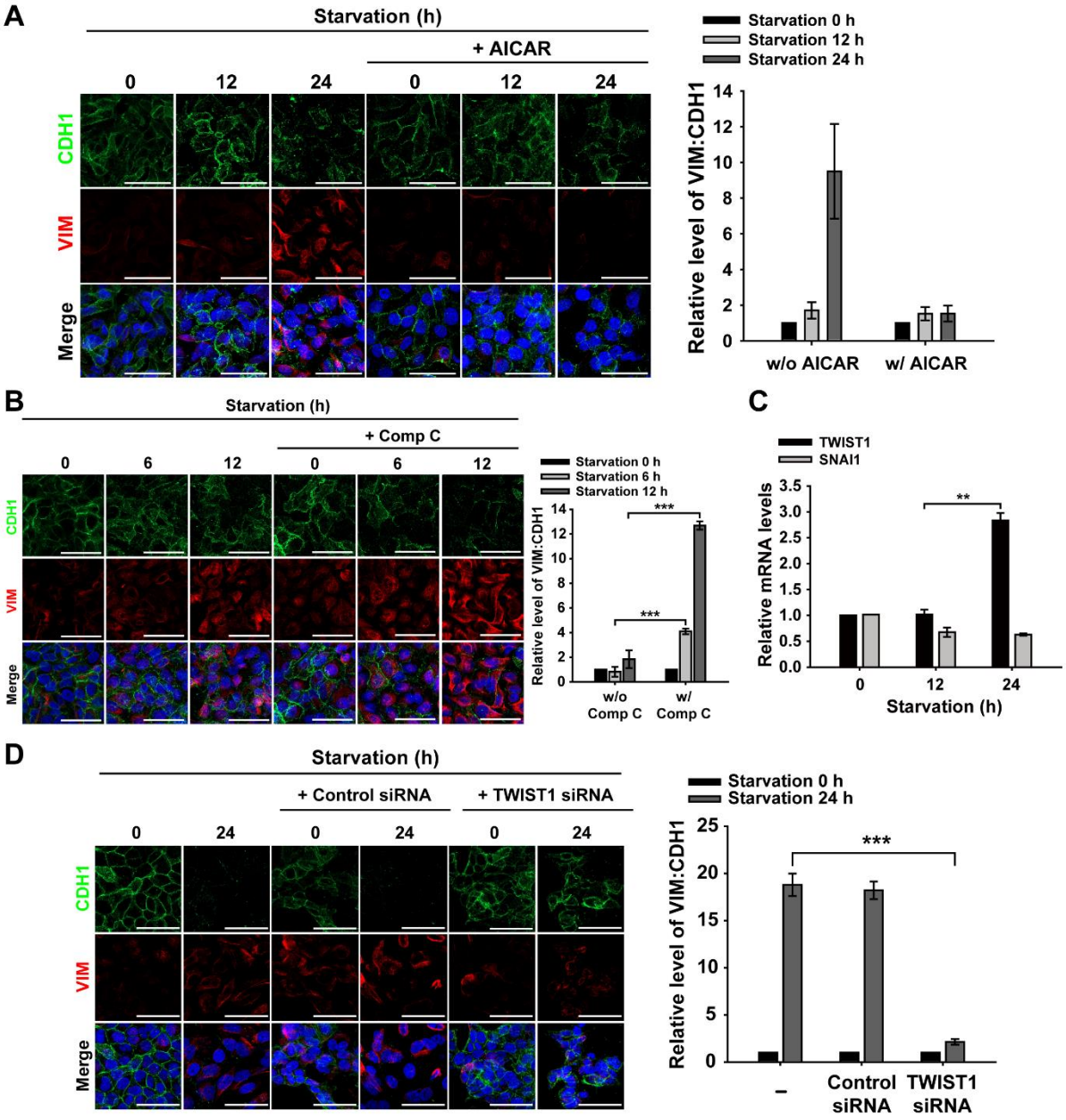

Fig. S3. AMPK decelerates EMT by down-regulating TWIST1 under prolonged starvation. (A) Confocal fluorescence microscopy images of CDH1 and VIM-immunostained (green and red, respectively) ARPE-19 cells under nutrient starvation for 0 - 24 h in the presence or absence of AICAR (2 mM; pre-treated for 1 h). Nuclei were stained with DAPI (blue). Scale bar: 50 μm. The graph represents the VIM-to-CDH1 fluorescence ratio. **(B)** Confocal fluorescence microscopy images of CDH1 and VIM-immunostained (green and red, respectively) ARPE-19 cells under nutrient starvation for 0 - 12 h in the presence or absence of Comp C (5 μM). Nuclei were stained with DAPI (blue). Scale bar: 50 μm. The graph represents the

VIM-to-CDH1 fluorescence ratio. Data are presented as the mean ± SEM, n = 3. $^{***}P < 0.005$. **(C)** Quantitative real-time PCR for ARPE-19 cells under nutrient starvation for 0 - 24 h. Bar graph indicates the relative mRNA level of TWIST1 and SNAI1 which were normalized to starvation 0 h. Data are presented as the mean ± SEM, n = 3. $^{**}P < 0.01$. **(D)** Confocal fluorescence microscopy images of CDH1 and VIM-immunostained (green and red, respectively) ARPE-19 cells under nutrient starvation for 0 - 24 h after control and TWIST1 siRNA transfection. Nuclei were stained with DAPI (blue). Scale bar: 50 μm. Bar graph represents the VIM-to-CDH1 fluorescence ratio. Data are presented as the mean ± SEM, n = 3. $^{***}P < 0.005$.