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Supplemental Information

Fatty Acid Synthesis Is Indispensable for Survival of Human Pluripotent Stem Cells

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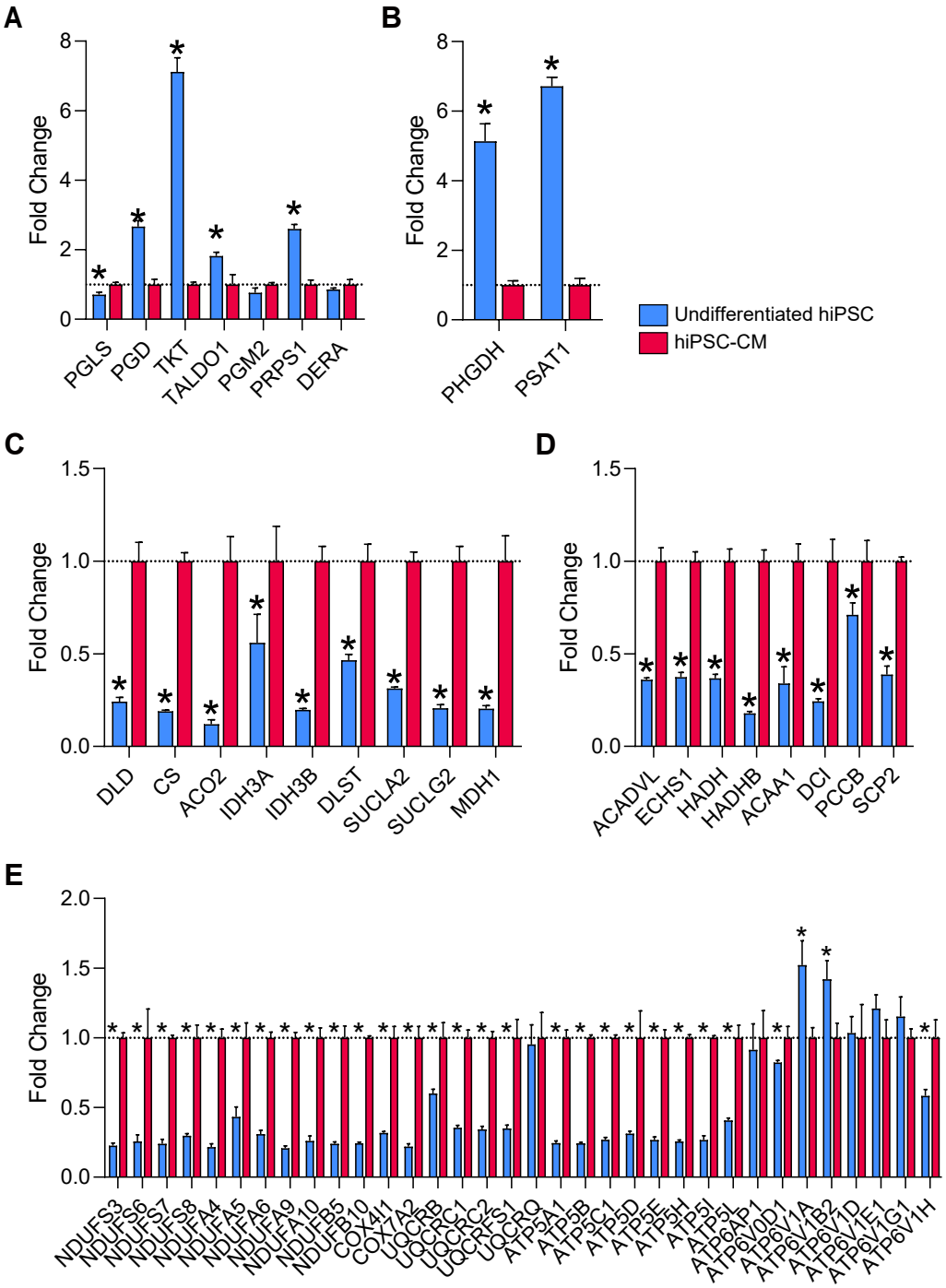
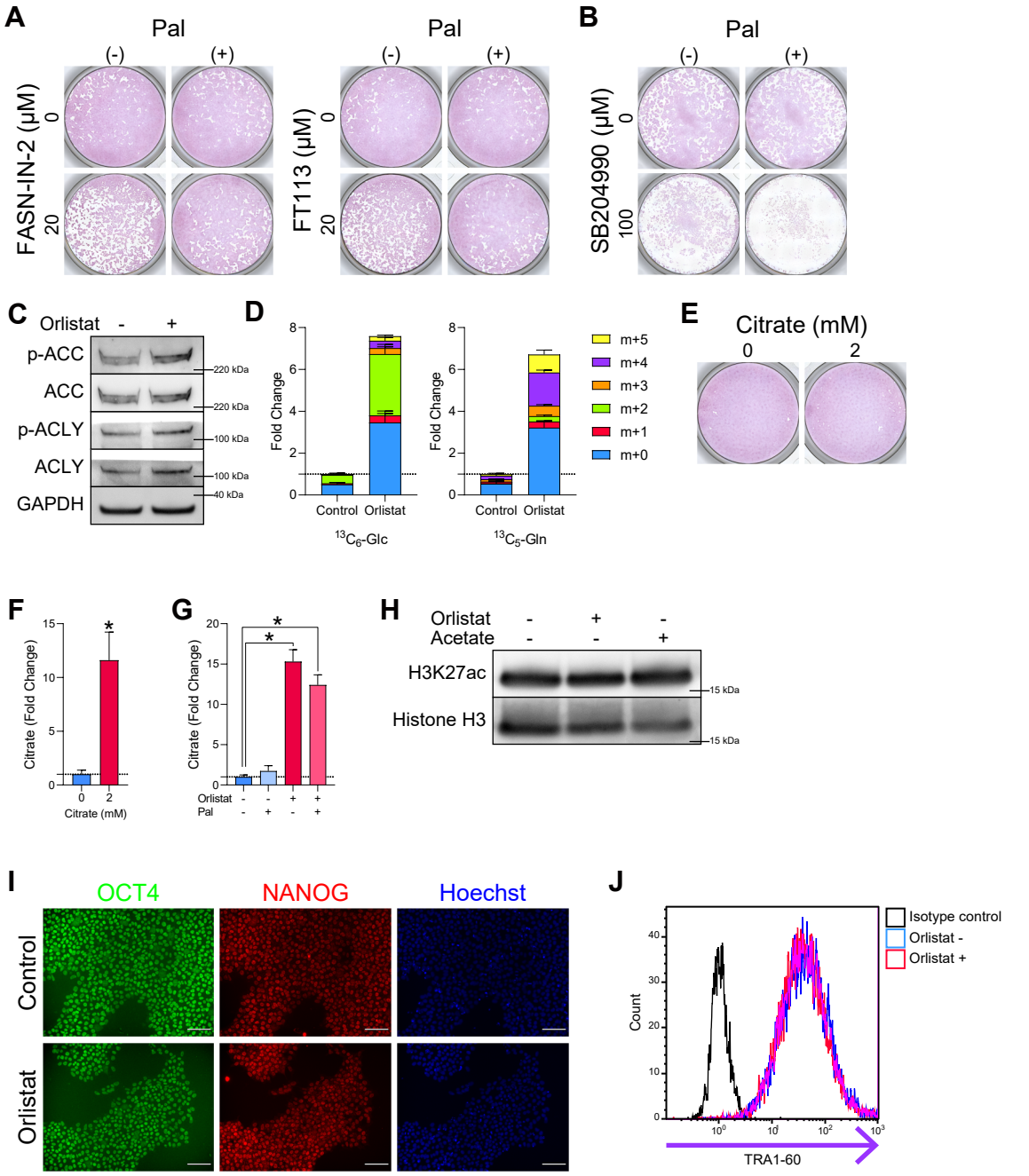

A
B
C
D
E
Fold Change
PGLS
PGD
TKT
TALDO1
PGM2
PRPS1
DERA
PHGDH
PSAT1
Undifferentiated hiPSC
hiPSC-CM
DLD
CS
ACO2
IDH3A
IDH3B
DLST
SUCLA2
SUCLG2
MDH1
ACADVL
ECHS1
HADH
HADHB
ACAA1
DCI
PCCB
SCP2

Figure S1


A
Pal
(-)
(+)
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0
20
FT113 (μM)
B
SB204990 (μM)
100
C
Orlistat
p-ACC
ACC
p-ACLY
ACLY
GAPDH
220 kDa
100 kDa
40 kDa
D
Fold Change
Control
Orlistat
$^{13}C_6$-Glc
$^{13}C_5$-Gln
m+5
m+4
m+3
m+2
m+1
m+0
E
Citrate (mM)
F
Citrate (Fold Change)
G
H
Acetate
H3K27ac
Histone H3
15 kDa
I
OCT4
NANOG
Hoechst
J
Count
TRA1-60
Isotype control
Orlistat -
Orlistat +

Figure S2

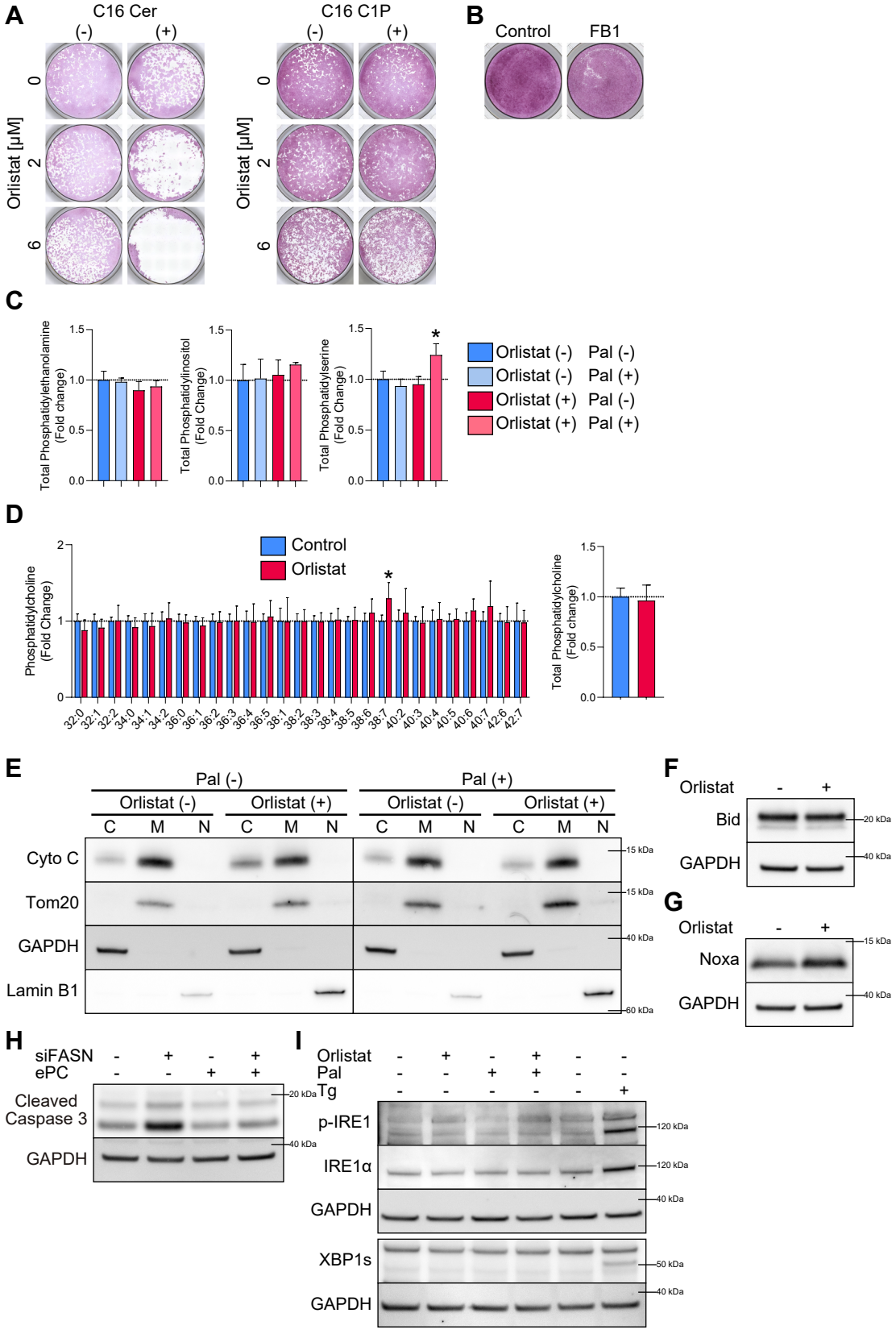
A
C16 Cer
(-)
(+)
C16 C1P
(-)
(+)
Orlistat [μM]
0
2
6
B
Control
FB1
C
Total Phosphatidylethanolamine (Fold change)
Total Phosphatidylinositol (Fold Change)
Total Phosphatidylserine (Fold Change)
Orlistat (-) Pal (-)
Orlistat (-) Pal (+)
Orlistat (+) Pal (-)
Orlistat (+) Pal (+)
D
Control
Orlistat
Phosphatidylcholine (Fold Change)
Total Phosphatidylcholine (Fold change)
32:0
32:1
32:2
34:0
34:1
34:2
36:0
36:1
36:2
36:3
36:4
36:5
38:1
38:2
38:3
38:4
38:5
38:6
38:7
40:2
40:3
40:4
40:5
40:6
40:7
42:6
42:7
E
Pal (-)
Pal (+)
Orlistat (-)
Orlistat (+)
C
M
N
Cyto C
Tom20
GAPDH
Lamin B1
15 kDa
40 kDa
60 kDa
F
Orlistat
Bid
GAPDH
20 kDa
40 kDa
G
Orlistat
Noxa
GAPDH
15 kDa
40 kDa
H
siFASN
ePC
Cleaved Caspase 3
GAPDH
20 kDa
40 kDa
I
Orlistat
Pal
Tg
p-IRE1
IRE1α
GAPDH
XBP1s
GAPDH
120 kDa
120 kDa
40 kDa
50 kDa
40 kDa
J
Control
Orlistat
p53 Hoechst
p53 Hoechst

Figure S3

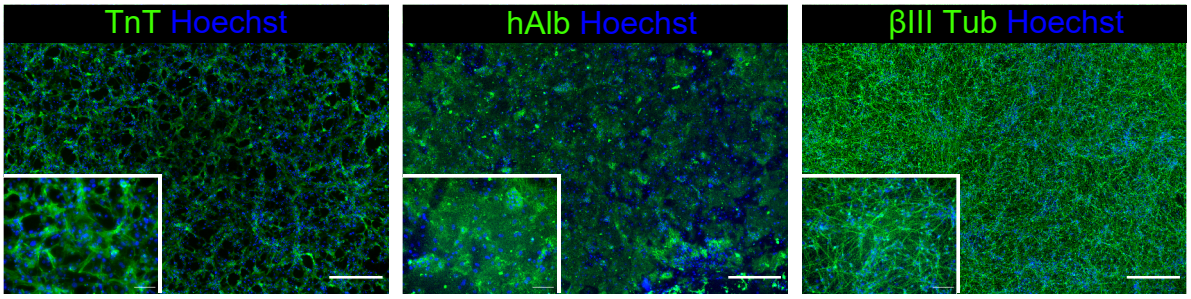

Figure S4

Supplemental Figure Legends

Figure S1 Detailed Proteomics Data, Related to Figure 1

(A-E) Relative abundance of enzymes related to pentose phosphate pathway (A), serine synthesis pathway (B), TCA cycle (C), FA oxidation (D) and oxidative phosphorylation (E) in hiPSCs (253G4) (blue) and hiPSC (253G4)-CMs (red). Student's *t*-test was performed for each protein (n = 3). Data are presented as means ± S.D. *p < 0.05.

Figure S2 Palmitic Acid Is Essential for the Survival of Undifferentiated hPSCs, Related to Figure 2

(A) Representative AP staining image of hiPSCs (253G4) treated with FASN-IN-2 (left) or FT113 (right) and Pal (50 μM) for 24 h.
(B) Representative AP staining image of hiPSCs (253G4) treated with SB204990 and Pal (50 μM) for 24 h.
(C) Representative western blot image showing phosphorylation of ACC (p-ACC) and ACLY (p-ACLY) of hiPSCs (253G4) after 3 h of orlistat treatment.
(D) Stacked bar graphs showing relative change in total amount of citrate, and the percentage of each labeled form (n = 3).
(E) Representative AP staining image of hiPSCs (253G4) treated with 2 mM citrate for 24 h.
(F) CE-MS of citrate in hiPSCs (253G4) after 3 h of 2 mM citrate treatment. Student's *t*-test, n = 3.
(G) CE-MS of citrate in hiPSCs (253G4) after 3 h of 6 μM orlistat and 50 μM Pal treatment. The same concentration of BSA (8.3 μM) was used as a control for Pal. One-way ANOVA with Dunnett's test was performed with orlistat (-) and Pal (-) as a control, n = 3.
(H) Western blotting image of lysine 27 acetylation on histone H3 (H3K27ac) in hiPSCs (253G4) after 12 h of 6 μM orlistat treatment, or 24 h of 10 mM acetate treatment.
(I) Representative image of immunocytochemistry for OCT4 and NANOG in hiPSCs (253G4) after 12 h of 6 μM orlistat treatment. Scale bar: 100 μm.
(J) Flow cytometry analysis of TRA1-60 in hiPSCs (253G4) after 12 h of 6 μM orlistat treatment.

Data are presented as means ± S.D. *p < 0.05.

Figure S3 Lipidomic Analysis, Exogenous Supplementation of Lipids, and Mechanism of Cell Death, Related to Figure 3

(A) AP staining of hiPSCs (253G4) treated with orlistat and C16 Cer or C16 C1P for 24 h.

(B) AP staining of hiPSCs (253G4) after 24 h of FB1 treatment.

(C) LC-MS of phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine of hiPSCs (253G4) after 3 h of 6 μM orlistat treatment, with or without 50 μM Pal. Data are normalized to orlistat (-) Pal (-) as a control. One-way ANOVA with Dunnett's test was performed with orlistat (-) Pal (-) as a control (n = 3).

(D) LC-MS of PC in hiPSC (253G4)-CMs after 3 h of 6 μM orlistat treatment. Data are normalized to controls. Numbers indicate carbon chain lengths followed by degree of desaturation. Student's *t*-test, n = 3.

(E) Representative western blotting image of cytochrome c subcellular localization in hiPSCs (253G4) treated with orlistat and Pal..

(F) Western blotting image of Bid in hiPSCs (253G4) treated with orlistat.

(G) Western blotting image of Noxa in hiPSCs (253G4) treated with orlistat.

(H) Representative western blotting image of cleaved caspase 3 in hiPSCs (253G4) treated with orlistat and chicken egg PC.

(I) Representative western blotting quantification of ER stress response proteins in hiPSCs (253G4) treated with orlistat. The positive control was comprised of 12 h, 3 μM thapsigargin (Tg) treatment. XBP1s, spliced XBP1.

(J) Representative immunocytochemistry images of p53 in undifferentiated hiPSCs (253G4) after 12 h of 6 μM orlistat treatment. Scale bar: 200 μm.

Data are presented as means ± S.D. *$p < 0.05$.

Figure S4 Immunofluorescent Staining of hiPSC-Derived Cells, Related to Figure 4

Representative immunocytochemistry images of hiPSC (253G4)-CMs, hepatocytes, and neurons. Scale bar: 500 μm and 100 μm.

Transparent Methods

Ethics declarations

The Animal Care and Use Committee of Keio University approved all of the experimental procedures and protocols. This work was conducted in line with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Mouse teratoma model

Male NOG mice (NOD/Shi-scid,IL-2RγKO Jic) aged 6 weeks were purchased from In-Vivo Science. Three days prior to transplantation, $5x10^5$ human dermal fibroblasts(neonatal) (Thermo Fisher Scientific, C0045C) and $0.1x10^5$ undifferentiated hiPSCs (253G4) were plated on 10-cm dishes with StemFit medium, AS103C (Ajinomoto), with or without 6 μM orlistat (Sigma, O4139-25MG). Upon transplantation, cells were rinsed with D-PBS (FUJIFILM Wako Pure Chemical, 045-29795) and incubated with Accutase (Thermo Fisher Scientific, A1110501) for 5 min at 37 °C. Cells were collected with AS103C supplemented with Y-27632 (FUJIFILM Wako Pure Chemical, 034-24024) and centrifuged for 4 min at 300 rcf. Cells were resuspended in diluted Matrigel (Corning, 354230). Supplemented with Y-27632. Orlistat-treated and control cells were transplanted to left and right legs, respectively, of isoflurane-anesthetized mice. Mice were euthanized 8 weeks after transplantation. Tumors were resected and fixed in formaldehyde, followed by hematoxylin-eosin stating. Tumors were observed microscopically by BZ-X710 (Keyence).

Cell lines

All cells were cultured in a humidified 5% CO_2 incubator at 37 °C and routinely tested for mycoplasma contamination.

hPSCs

hiPSC lines (253G4, 201B7 and FfI14) were provided by the Center for iPS Cell research and Application, Kyoto University. hESCs line (H9) was provided by WiCELL. The use of hESCs complied with the Guidelines on the Distribution and Utilization of Human Embryonic Stem cells, Ministry of Education, Culture, Sports, Science and Technology, Japan. The hPSCs were maintained with modified StemFit medium, AS103C (Ajinomoto) (Tohyama et al., 2017). Cells were passaged every 4-7 d. Upon passage, cells were washed with D-PBS and incubated for 3 min with TrypLE Select (Thermo Fisher Scientific, 12563-011) at 37 °C. Cells were collected with AS103C supplemented with

10 μM Y-27632 and pelleted for 4 min at 300 rcf. Cells were resuspended and were counted using Vi-CELL (Beckman Coulter). Cells were plated in dishes and plates coated with Matrigel.

hPSC-derived hepatocytes

hiPSC-derived hepatocytes (ReproCELL, RCDH001N) were purchased from ReproCELL and cultured according to the manufacturer's instructions. Briefly, 48-well plates were coated with Matrigel. Hepatocytes were thawed at 37 °C and resuspended in ReproHepato Culture Medium (ReproCELL, RCDH101), then pelleted for 5 min at 350 rcf. Cells were resuspended in ReproHepato Culture Medium and replated at 1.8 x 10^5/well. Medium was changed on days 1, 3, and 5. On day 6, cells were treated with 6 μM orlistat or vehicle control (0.05% dimethyl sulfoxide (DMSO) (Sigma, D250-100ML)) in AS103C.

hPSC-derived neurons

hiPSC-derived neurons (ReproCELL, RCDN001N) were purchased from ReproCELL and cultured according to the manufacturer's instructions. Briefly, 48-well plates were coated with poly-L-lysine solution (Sigma, P4832) followed by ReproNeuro Coat (ReproCELL, RCDN201). Neurons were thawed in 37 °C water bath and resuspended in ReproNeuro Culture Medium (ReproCELL, RCDN101), and pelleted for 5 min at 350 rcf. Cells were resuspended in ReproNeuro Culture Medium and replated at 5.6x10^4/well. Half of the medium was changed on day 3, 7, and 14. On day 14, cells were treated with 6 μM orlistat or vehicle control (0.05% DMSO) in AS103C medium.

Human dermal fibroblasts

Human dermal fibroblasts (neonatal) (Thermo Fisher Scientific, C0045C) were purchased from Thermo Fisher Scientific and were maintained with DMEM (Thermo Fisher Scientific, 11885076) supplemented with 10% FBS (Biowest, S1560-500). Cells were passaged every 4-7 d. Upon passage, cells were washed with D-PBS and incubated for 5 min with trypsin-EDTA solution (NACALAI TESQUE, 35554-64) at 37 °C. Cells were collected with DMEM supplemented with 10% FBS and pelleted for 4 min at 300 rcf. Cells were resuspended and counted using Vi-CELL. Cells were plated in dishes coated with gelatin (Sigma, G9382).

Cardiomyocyte differentiation from hiPSCs

The hiPSCs were differentiated to cardiomyocytes as described in previous reports (Burridge et al., 2014; Lian et al., 2013). Briefly, on day 0, cells were rinsed with D-PBS and incubated with RPMI-

1640 (FUJIFILM Wako Pure Chemical, 189-02025) supplemented with 2% B27 supplement without insulin (Thermo Fisher Scientific, A1895601) and 6 μM CHIR99021 (FUJIFILM Wako Pure Chemical, 034-23103) for 1 day. On day 1, cells were rinsed with D-PBS and incubated with RPMI-1640 supplemented with B27 supplement without insulin. On day 3, cells were rinsed with D-PBS and incubated with RPMI-1640 supplemented with B27 supplement without insulin and 5 μM IWR-1 (Sigma, I0161-25MG). On day 6, cells were rinsed with D-PBS and incubated with RPMI-1640 supplemented with B27 supplement without insulin. On day 7, cells were incubated with MEM-α (Thermo Fisher Scientific, 12571-048) supplemented with 5% FBS (Biowest, S1560-500) and 2 mM sodium pyruvate (Sigma, S8636-100ML). On day 12-14, cells were rinsed with D-PBS and incubated for 5 min with trypsin-EDTA solution. Cells were collected with MEM-α supplemented as previously described and were pelleted for 4 min at 300 rcf. Cells were resuspended and counted using Vi-CELL (Beckman Coulter). Cells were plated in dishes coated with collagen type I (AGC TECHNO GLASS, 4020-010). After 2 d, culture medium was replaced to glucose and glutamine-free medium supplemented with 4 mM lactate, StemFit medium AS501 (Ajinomoto) (Tohyama et al., 2016). After 4-7 d, culture medium was replaced by MEM-α, supplemented as previously described. Day 21 or more cardiomyocytes were used for experiments.

Neural differentiation from hiPSCs using synthetic mRNAs

hiPSCs (201B7) were differentiated into neurons under feeder-free conditions in StemFit medium, AK02N (Ajinomoto) on laminin-511 (Nippi, 892011)-coated dishes. Neuronal induction was performed by transfection with synthetic mRNA encoding NEUROGENIN2 as previously described (Goparaju et al., 2017). In this experiment, the cells were cultured without a replating step so that undifferentiated cells were remained on the dishes and coexisted with neural cells. The differentiating cells were treated with orlistat for 4 days from day 3 post neuronal induction. For immunostaining, the cells were fixed at day 7.

Proteomics

For undifferentiated hiPSCs, cells were rinsed with D-PBS and incubated for 3 min with TrypLE Select in 37 °C. Cells were collected with ice-cold StemFit medium, AS103C, and centrifuged for 4 min at 300 rcf. Supernatant was aspirated and cells were resuspended in ice-cold D-PBS and centrifuged for 4 min at 300 rcf. This procedure was repeated one more time. Following removal of supernatants, cells were resuspended in D-PBS and centrifuged for 5 min at 500 rcf. Supernatant was aspirated and cell pellets were stored at -80 °C and used for proteomic analysis.

For hiPSC-CMs, cell were incubated with prewarmed D-PBS for 3 min at 37 °C. D-PBS was aspirated and cells were incubated with trypsin-EDTA for 5 min at 37 °C. Cells were collected with ice-cold MEMα with 5% FBS and centrifuged for 4 min at 300 rcf. Following procedures were done in the same way as that for undifferentiated hiPSCs and used for proteomic analysis.

Proteomic analysis was performed as previously reported (Matsumoto et al., 2017). Briefly, cell pellets were lysed with 150 µL of 7 M urea (GE Healthcare, 17-1319-01), 2% SDS (Nippon Gene, 311-90271), and 100 mM Tris-HCl, pH 8.8 (Nippon Gene, 311-90391) with sonication by a Bioruptor II (Cosmobio, BR2006A). An equal amount of ultrapure water was added, and sonication was repeated. After centrifugation, the supernatant was used for further analysis. Protein concentrations were measured by a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, 23225) according to the manufacturer's instructions. Two hundred-µg protein samples were used. Five µL 200 mM Tris-(2-Carboxyethyl)phosphine hydrochloride (Thermo Fisher Scientific, T2556) solution and 5 µL 400 mM 2-iodoacetamide (Sigma, 8.04744) solution were sequentially added to the samples. Proteins were purified by acetone precipitation and precipitates were resuspended in digestion solution (0.5 M triethylammonium bicarbonate buffer (Sigma, T7408-100ML)). Lysyl endopeptidase (FUJIFILM Wako Pure Chemical, 125-05061) solution was added and samples were incubated for 3 h at 37 °C. Four µg trypsin (Thermo Fisher Scientific, 20233) was added and samples were incubated for 3 h at 37 °C. Four µg trypsin was added again and samples were incubated overnight at 37 °C. Protein concentration was measured by Pierce BCA Protein Assay Kit and samples were lyophilized. Proteins were labeled with mTRAQ reagent Δ0 (AB Sciex, 4440015). Samples were lyophilized and stored at -80 °C. Samples were reconstituted with 0.1% trifluoroacetic acid (Merck, 4.80112.2500) to obtain 1 µg/µL solution. Twenty µL of the solution, 4 µL RTM solution (1 µL MRMplus Retention Time Marker (Funakoshi, FMR-002) and 199 µL 0.1% trifluoroacetic acid), 4 µL internal standard peptide solution which contains synthetic peptides (Funakoshi and Genscript) labeled with mTRAQ reagent Δ4 (AB Sciex, 4427698) after reductive alkylation, and 12 µL 0.1% trifluoroacetic acid (Merck, 4.80112.2500) were mixed. The samples were subjected to reversed-phase liquid chromatography (Waters, ACQUITY UPLC H-Class) followed by multiple reaction monitoring analysis (AB Sciex, QTRAP500). The acquired data were analyzed with iMPAQT quant (Kyushu University) and R (R Foundation).

Immunocytochemistry

Cells were rinsed with D-PBS and fixed in 4% paraformaldehyde (Muto Pure Chemicals, 33111) for 15-20 min. Cells were treated with 0.1% Triton X-100 (Sigma, T9284) diluted in D-PBS for 5-15 min and rinsed with D-PBS. Cells were treated with blocking solution of either ImmunoBlock (KAC,

CTKN001) or D-PBS with 2% BSA. Cells were treated with primary antibodies diluted in blocking solution at dilutions recommended by manufacturers' instructions at 4 °C overnight. Cells were rinsed with D-BPS twice and were treated with secondary antibodies diluted in blocking solution at dilutions recommended by manufacturer's instructions at room temperature for 2 h. Cells were treated with 5 μg/mL Hoechst 33342 (Thermo Fisher Scientific, H3570) at room temperature for 1 hour or DAPI (Dojindo, D523) at room temperature for 5 min. Cells were imaged using Axio Oberver D1 (Zeiss), BZ-X710 (Keyence), or IX73 (Olympus) microscopes. The mouse monoclonal antibody against OCT4 was purchased from Santa Cruz Biotechnology. The rabbit polyclonal antibody against human NANOG was purchased from ReproCELL. The rabbit polyclonal antibody against FASN was purchased from abcam. The mouse monoclonal antibody against TnT was purchased from Thermo Fisher Scientific. The rabbit monoclonal antibody against p53 and the rabbit monoclonal antibody against βIII Tub were purchased from Cell Signaling. The goat polyclonal antibody against hAlb was purchased from Bethyl Laboratories. The mouse monoclonal antibody against βIII Tub was purchased from Promega. Alexa Fluor 488-labeled anti-mouse IgG, Alexa Fluor 546-labeled anti-mouse IgG, Alexa Fluor 488-labeled anti-rabbit IgG, Alexa Fluor 546-labeled anti-rabbit IgG, and Alexa Fluor 488-labeled anti-goat IgG were purchased from Thermo Fisher Scientific.

Western blotting

Cells were treated with indicated reagents or siRNA prior to preparation of samples, then lysed in cell lysate solution (25% NuPAGE™ LDS Sample Buffer (4X) (Thermo Fisher Scientific, NP0007), 10% NuPAGE™ Sample Reducing Agent (10X) (Thermo Fisher Scientific, NP0009), 65% ultrapure water, 1 tablet of PhoSTOP EASYpack (Roche, 4906837001), and 1 tablet of cOmplete™ ULTRA Tablets, Mini, EASYpack Protease Inhibitor Cocktail (Roche, 5892970001)). Lysates were sonicated by an ultrasonic disruptor (TOMY, UR-21P). Protein concentrations were measured by Qubit Protein Assay Kit (Thermo Fisher Scientific, Q33211) and Qubit 3 fluorometer according to the manufacturer's instructions. Electrophoresis and transfer were done according to the manufacturer's instructions. Briefly, proteins were denatured for 10 min at 70 °C. Twenty μg of protein was loaded into each well of a NuPAGE 4-12% Bis-Tris gel (Thermo Fisher Scientific, NP0321BOX). Subsequently, electrophoresis was done by 200V for 35 min in NuPAGE MES SDS running buffer (Thermo Fisher Scientific, NP0002). Transfer was done with iBlot2 PVDF mini Stacks (Thermo Fisher Scientific, IB24002) and an iBlot™ 2 Gel Transfer Device (Thermo Fisher Scientific, IB21001) according to the manufacturer's instructions. Following transfer, membranes were stained with Ponceau-S (Beacle, BCL-PSS-01) for 5 min and rinsed with 1% acetate (FUJIFILM Wako Pure Chemical, 017-00256) for

1-2 min. Membranes were imaged using an iBright FL1000 imaging system (Thermo Fisher Scientific). Membranes were rinsed with TBS-T (TAKAR BIO, T9142) for 3 times 5 min each. Membranes were blocked with Blocking One (NACALAI TESQUE, 03953) for 30 min and rinsed with ultrapure water. Membranes were probed with primary antibodies diluted in TBS-T or TBS-T supplemented with 5% BSA (FUJIFILM Wako Pure Chemical, 019-27051) overnight at 4 °C. Membranes were washed with TBS-T for 3 times 5 min each and probed with secondary antibodies. For chemiluminescence, membranes were probed with horse radish peroxidase-conjugated antibodies at 1:25,000 for 30 min, then washed with TBS-T followed by ultrapure water. Proteins were imaged using ChemiLumi One (NACALAI TESQUE, 07880-70), Pierce™ ECL Plus Western Blotting Substrate (Thermo Fisher Scientific, 32132), or SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, 34095) with LAS-3000 (FUJIFILM) or iBright FL1000. For fluorescence, membranes were probed with fluorescent secondary antibodies at 1:10,000 for 30 min; membranes were washed three times with TBS-T and imaged with iBright FL1000. The mouse monoclonal antibody against GAPDH was purchased from Thermo Fisher Scientific. The rabbit monoclonal antibodies against p-ACC, ACC, cleaved Caspase 3, and IRE1α and the rabbit polyclonal antibodies against p-ACLY, ACLY, and Bid were purchased from Cell Signaling. The rabbit polyclonal antibodies against H3K27ac, Histone H3, and XBP1; the rabbit monoclonal antibodies against Cyto C and p-IRE1; and the mouse monoclonal antibody against Noxa were purchased from abcam. The mouse monoclonal antibody against Tom20 was purchased from Santa Cruz Biotechnology. The mouse monoclonal antibody against Lamin B1 was purchased from Proteintech. HRP-labeled anti-mouse IgG, HRP-labeled anti-rabbit IgG, and Alexa Fluor 680-labeled anti-mouse IgG were purchased from Thermo Fisher Scientific.

FASN knockdown in hPSCs

Undifferentiated hiPSCs were transfected with 10 nM Ambion Silencer Negative Control siRNA (Thermo Fisher Scientific, AM4611) or Ambion *FASN* siRNA (Thermo Fisher Scientific, s5030) using Lipofectamine™ RNAi MAX Transfection Reagent (Thermo Fisher Scientific, 13778030) and Opti-MEM (Thermo Fisher Scientific, 31985070) according to the manufacturer's instructions. The medium was changed 24 h after transfection and cells were incubated for up to 48 h. Proliferation was determined by measuring confluence by IncuCyte ZOOM (Essen Bioscience).

Alkaline phosphatase staining

Alkaline phosphatase (AP) staining was performed with Leukocyte AP Kit (Sigma, 86R-1KT) according to the manufacturer's instructions. In brief, cells were rinsed with D-PBS and fixed in 4%

paraformaldehyde at room temperature for 20 min and rinsed with ultrapure water. Cells were then incubated with an alkaline-dye mixture at room temperature overnight. After staining, the dye was removed, and cells were rinsed with ultrapure water.

Preparation of BSA-conjugated palmitic acid

BSA-conjugated palmitic acid (Pal) was prepared as described in previous reports (Hannah et al., 2001) with modifications. In brief, Pal (FUJIFILM Wako Pure Chemical, 165-00102) was dissolved in ethanol (FUJIFILM Wako Pure Chemical, 057-00456) and precipitated by adding NaOH (FUJIFILM Wako Pure Chemical, 198-13765). The supernatant was evaporated under stream of nitrogen. The precipitate was reconstituted with 0.9%w/v NaCl (FUJIFILM Wako Pure Chemical, 191-01665) and 10%w/v fatty acid-free BSA (FUJIFILM Wako Pure Chemical, 017-15146) at a molecular ratio of 6:1. The solution was adjusted to pH 7.0 with HCl (FUJIFILM Wako Pure Chemical, 080-01066).

LIVE/DEAD assay

The LIVE/DEAD assay (Thermo Fisher Scientific, L3224))was performed according to the manufacturer's instructions. In brief, half of the culture medium was aspirated, calcein AM and ethidium homodimer-1 dissolved in D-PBS were added to the medium, and cells were incubated at 37 °C for 30 min. Medium and D-PBS were changed to culture medium and cells were examined using a microscope.

Capillary electrophoresis-mass spectrometry

Capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) was performed as previously described (Ohmura et al., 2015; Shiota et al., 2018; Yamamoto et al., 2014). Briefly, cells were rinsed with D-PBS and cultured under the conditions described above for 3 h. Cells were rinsed with ice-cold 5 % (w/v) mannitol (FUJIFILM Wako Pure Chemical, 137-00843) twice and collected with methanol (FUJIFILM Wako Pure Chemical, 138-06473) that contained 300 mM methionine sulfone and 300 mM 2-morpholinoethanesulfonic acid as internal and external standards, respectively. The protocols of hydrophilic metabolites extraction, as well as the instrument specifications, and measurement conditions were described previously (Koh et al., 2013). Briefly, metabolites were extracted from cells with a extraction solvent [methanol:water:chloroform = 1:0.5:1 (v/v/v)]. The extract was centrifuged at 20,000 *x g* and 4 °C for 15 minutes. The water and methanol layer was filtered using a 5 kDa cut-off filter (UFC3LCCNB, Human Metabolome Technologies, Tsuruoka, Japan). The filtrate was lyophilized and dissolved in deionized water containing 3-aminopyrrolidine

and trimesate (200 mM each) as reference compounds. Metabolites were analyzed using an Agilent CE-TOFMS system equipped with an Agilent G7100A CE instrument, and an Agilent 6530 Q-TOF LC/MS system (Agilent Technologies). Raw data were processed using MasterHands (Sugimoto et al., 2010). The metabolites were identified by matching m/z and corrected migration times with those in our standard library. The absolute concentration was quantified based on the ratio among peak areas of each metabolite, internal, and external standard compounds.

Liquid chromatography-mass spectrometry

Cells were rinsed with D-PBS and then collected with 0.25 mM trypsin-EDTA followed by pelleting at 300 g for 4 min. Cell pellets were stored for further analysis.

Lipid preparation

A comprehensive lipid analysis was done as described previously (Shindou et al., 2017; Yamamoto et al., 2018). Briefly, total lipids were extracted using the Bligh-Dyer method (Bligh and Dyer, 1959). Anionic phospholipids (PLs) including phosphatidic acid, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, lysophosphatidic acid, lysophosphatidylserine, lysophosphatidylinositol, lysophosphatidylglycerol, C1P, sphinganine-1-phosphate, and sphingosine-1-phosphate were condensed using a diethylaminoethyl-cellulose column (Santa Cruz Biotechnology) and subjected to methylation by trimethylsilyldiazomethane (Kielkowska et al., 2014). Samples of the organic layer were dried under a gentle stream of nitrogen and then dissolved in methanol for LC/MS/MS measurements.

MS analyses of phospholipids

The samples were analyzed using an electrospray ionization- triple-stage quadrupole mass spectrometer (TSQ Vantage AM, Thermo Fisher Scientific) interfaced with an UltiMate 3000 LC system (Thermo Fisher Scientific). The samples were subjected to LC-ESI-MS/MS analysis using the Waters X-Bridge C18 columns (3.5 μm, 150 mm × 1.0 mm i.d.). Samples were simply injected via the HTC PAL autosampler (CTC Analytics), and the PL fractions were separated by a linear gradient composed of different ratios of mobile phase A [isopropanol/methanol/water (5/1/4 v/v/v) supplemented with 5 mM ammonium formate and 0.05% ammonium hydroxide (28% in water)] and mobile phase B [isopropanol supplemented with 5 mM ammonium formate and 0.05% ammonium hydroxide (28% in water)]. The linear gradient increased from 40% to 60% B in 1 min, from 60% to 80% B for the next 8 minutes, from 80% to 95% B during the next 2 min, and was then kept at 95%

B for 19 min with a flow rate of 25 μL/min and a column temperature of 25 ℃. Lipid species were measured using selected reaction monitoring (SRM) in the positive ion mode. Characteristic fragments of individual lipids were detected via the product ion scan (MS/MS mode).

MS analyses of triacylglycerol

Analyses were performed on an LC/MS/MS system consisting of a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an electrospray ionization (ESI) source and an UltiMate 3000 system (Thermo Fisher Scientific). Lipid samples were separated using a Waters X-Bridge C18 column (3.5 μm, 150 mm × 1.0 mm i.d.) at 40°C, and a linear gradient composed of different ratios of mobile phase A [isopropanol/methanol/water (5/1/4 v/v/v) supplemented with 5 mM ammonium formate and 0.05% ammonium hydroxide (28% in water)], and mobile phase B [isopropanol supplemented with 5 mM ammonium formate and 0.05% ammonium hydroxide (28% in water)]: ratios of 60/40% (0 min), 40/60% (1 min), 20/80% (9 min), 5/95% (11-30 min), 95/5% (31-35 min) and 60/40% (35-45 min). The flow rate was 25 μL/min. The source and ion transfer parameters applied were as follows: spray voltage 4.0 kV. For positive ionization modes, the sheath gas and the capillary temperature were maintained at 10 and 320 °C, respectively. The Orbitrap mass analyzer was operated at a resolving power of 70,000 in full-scan mode (scan range 600–2000 m/z; automatic gain control (AGC) target 1e6) and of 17,500 in the Top 20 data-dependent MS2 mode (stepped normalized collision energy 20, 30 and 40; isolation window 4.0 m/z; AGC target 1e5) with a dynamic exclusion setting of 10.0 s. Identification of TAG molecular species was performed using the LipidSearch4.2 software (Mitsui knowledge industry CO., LTD.).

Liposome preparation

Liposomes were prepared as previously described (Mendez and Banerjee, 2017; Szoka and Papahadjopoulos, 1980). Briefly, lipids dissolved in chloroform (FUJIFILM Wako Pure Chemical, 038-02606) were dried in a stream of nitrogen gas to form a lipid film. The film was reconstituted in medium and sonicated with an ultrasonic bath (Branson, CPX2800H-J) to produce liposomes. One percent penicillin-streptomycin (Thermo Fisher Scientific, 15140122) was supplemented to the medium.

Flow cytometry

Cells were rinsed with D-PBS and incubated for 3 min with TrypLE Select at 37 °C. Cells were collected with AS103C and counted using Vi-CELL. They were then pelleted for 3 min at 300 rcf and resuspended in D-PBS supplemented with 2% FBS. A total of 1 x 10^6 cells were aliquoted and

supplemented with 10 µL of primary antibodies conjugated with phycoerythrin for 30 min on ice. Cells were rinsed with D-PBS and pelleted for 3 min at 300 rcf. They were then resuspended in D-PBS supplemented with 2% FBS and analyzed with Gallios 2L6C (Beckman Coulter). PE-labeled Isotype control antibody and PE-labeled antibody against TRA1-60 were purchased from Miltenyi Biotec.

Quantification and statistical analysis

All statistical analyses were carried out using SPSS (IBM) or GraphPad Prism (GraphPad). Values are presented as means ± S.D. Statistical significance was evaluated using the Student's *t*-test for comparisons between two groups and one-way ANOVA followed by Dunnett's multiple comparison test for multiple comparisons between more than two groups unless otherwise specified. A value of $p < 0.05$ was considered significant.

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