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Supplemental Information

Lipid Droplets Maintain Energy Homeostasis and Glioblastoma Growth via Autophagic Release of Stored Fatty Acids

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Supplemental Information

Transparent Methods

GBM patient samples

Autopsy and biopsy samples from GBM patients were obtained from the Department of Pathology of The Ohio State University (OSU) and University of California at Los Angeles (UCLA) Medical Center, respectively. GBM biopsies were fixed with 4% paraformaldehyde for 24 hr. One half of the biopsy was embedded in paraffin and the other half was incubated with 30% sucrose for 24 hr and further embedded in tissue-plus optimal cutting temperature (O.C.T.) compound (Cat# 23730571, Fisher). Cryosections derived from the latter were stained with BODIPY 493/503 (Cat# D-3922; Life Technologies, Grand Island, NY). The use of tissues from GBM patients was approved by the OSU and UCLA Institutional Human Care and Use Committees.

Cell lines

U87, T98, U251 GBM cell lines were cultured in Dulbecco's Modified Eagle's Media (DMEM, Cat# 15-013-CV, Corning Incorporated) supplemented with 5% FBS (Cat# SH3007103, Hyclone, GE Healthcare) in a humidified atmosphere of 5% CO_2/95% air at 37˚C. For the glucose withdrawal experiments, cells were washed once with 1x PBS and then placed into fresh DMEM medium containing 5% dialyzed FBS (Cat# 26400044, Gibco) with or without 25 mM glucose. Human astrocyte cells were maintained in Geltrex® matrix (#A1413202, Life Technologies) coated plates with DMEM supplemented with 1% of N-2 (#17502‐048, Life Technologies) and 10% of One Shot™ format FBS (#16000‐077, Life Technologies) at 37°C in a humidified atmosphere of 5% CO2. GBM patient-derived cells, GBM83, were cultured in neurobasal medium

supplemented with B-27 serum-free supplements (1x), heparin (2 mg/ml), EGF (50 ng/ml), and fibroblast growth factor (FGF, 50 ng/ml) (Ru et al., 2016).

Intracranial mouse model

Female athymic nude mice (6–8 weeks) obtained from OSU Target Validation Shared Resource were used to generate intracranial xenograft models. Cells ($1x10^5$ cells in 4 μl of PBS) were stereotactically implanted into mouse brains. Mice were observed every day and were sacrificed when they became moribund. All animal procedures were approved by the Subcommittee on Research Animal Care at Ohio State University Medical Center.

Thin-layer chromatography for lipids

TLC analysis for lipids was performed as previously described (Guo et al., 2009). Total cellular lipids were extracted by scraping cells from 10 cm culture dishes (10^6 cells) or by dissociating frozen tissues (4 mg) into 2 mL of PBS-containing protease inhibitor and 1 mM phenylmethylsulfonyl fluoride and adding 4 ml chloroform/methanol (2:1, v/v) containing 0.01% butylate hydroxytoluene (Sigma). The solution was vortexed and centrifuged at 1500x*g* for 5 min. The organic phase was collected, and 2.5 ml of chloroform was added to the remaining aqueous phase, vortexed, and centrifuged at 1500 x *g* for 5 min. The organic phase was mixed with the previous extraction. TLC was performed by spotting the cellular lipid extracts from 4 mg tissues or 10^6 cells. TG standard (Cat#10010509-1, Cayman Chemical) on a 5 cm x 10 cm silica gel aluminum sheet (Cat# 1168350001, EMD Chemicals) that was developed with hexane/diethyl ether/acetic acid (80:20:2, v/v/v). Lipids were visualized with iodine vapor and results recorded with a digital camera.

Western blotting analysis

Cultured cells were lysed and homogenized in RIPA buffer (Cat# NC9484499, Fisher Scientific) containing a phosphatase inhibitor (Cat# 04906845001, Roche), a protease inhibitor cocktail (Cat# 11836170001, Roche) and 1 mmol/L phenylmethanesulfonyl fluoride. Equal amounts of protein extracts were separated by 12% SDS-PAGE and then transferred to nitrocellulose (Cat# 162-0112, Biorad) or to PVDF membranes (Cat# 10600100, GE Healthcare) for LC3 detection. Membranes were blocked in Tris-buffered saline containing 0.01% Tween and 5% nonfat milk for 1 hr and then probed with the indicated primary antibodies, followed by the appropriate secondary antibodies (Cat #7074 or #7076; Cell Signaling) conjugated to horseradish peroxidase. The immunoreactivity was examined using the ECL kit (Cat# RPN2106, GE Healthcare). Antibodies to LC3 (Cat# 4108) and ATG5 (Cat# 8540) were purchased from Cell Signaling. Antibody to CPT1A (Cat# ab128568) was purchased from Abcam. Antibody to β-actin (Cat# A1978) was purchased from Sigma.

Cell proliferation assay

Cells ($4x10^4$) were seeded in 12-well plates and cultured overnight. Cells were washed once with 1x PBS and then placed into fresh DMEM medium supplemented with 5% dialyzed FBS (Cat# 26400044, Gibco) in the absence or presence of glucose (25 mM). Cell number was counted with a hemocytomer and cell death was determined by Trypan blue exclusion (Cat# 15250-061; Life Technologies).

Lentiviral transduction

Lentivirus vector containing ATG5 shRNA (TRCN0000330394, TRCN0000330392), CPT1A shRNA (TRCN0000036279, TRCN0000036282) and the non-mammalian shRNA control (SHC002) were purchased from Sigma. The 293FT cells were transfected with shRNA vector and packaging plasmids pCMV-R8.74psPAX2 and the envelope plasmid pMD2.G using

polyethylenimine (Cat# 23966, Polysciences). The supernatants were collected at 48 hr and concentrated using the Lenti-X Concentrator (Cat# 631232, Clontech) according to the manufacturer's protocol. Lentiviral transduction was performed according to Sigma MISSION protocol with polybrene (8 μg/mL; Cat# H9268, Sigma).

Lipid droplet staining and quantification

LDs were stained by incubating cells with 0.5 μM BODIPY 493/503 (Cat# D-3922, Life Technologies) or 0.1 μM Nile red (Cat# N1142, Life Technologies) for 30 min and visualized by confocal microscopy (Carl Zeiss LSM 510 Meta, 63x/1.4 NA oil, 1 μM wide z-stack). LDs were also detected by immunofluorescence staining with TIP47 (Cat# ab47638, Abcam). The nucleus was stained with the DAPI-containing antifade mounting reagent (Cat #P36935, Life Technologies) for tissues or fixed cells. Live cells were stained with Hoechst 33342 (5 μg/mL, 30 min) (Cat# H3570, Life Technologies) for nucleus observation. Live cells were stained with Lysotracker (Cat# L7528, Life Technologies) and Mitotracker (Cat# M2245, Life Technologies) for lysosome and mitochondrial observation, respectively. Thirty cells in each group were analyzed and particle numbers were quantified with the ImageJ software (NIH) in 3D stacks.

Immunofluorescence microscopy

Cells were seeded and cultured on glass coverslips. Cells were washed twice with 1x PBS and fixed with 4% paraformaldehyde/0.025% glutaraldehyde for 10 min followed by 5 min permeabilization with 0.1% Triton X-100. After blocking in 5% BSA for 10 min, cells were incubated with the primary antibody overnight at 4˚C, and then incubated with Alexa Fluor 488 goat anti-rabbit IgG (Cat# A-11034, Invitrogen) or Alexa Fluor 568 goat anti-rabbit IgG (Cat# A-11036, Invitrogen) for 30 min at 37˚C. Cells were then incubated with 0.5 μM BODIPY 493/503 for 30 min to stain lipid droplets. Coverslips were mounted with the antifade reagent containing

DAPI (Cat# P36935, Life Technologies) and visualized by confocal microscopy. Antibody to LC3 (Cat# 4108) was purchased from Cell Signaling, and antibody to LAMP1 (Cat# ab25630) was purchased from Abcam.

Transmission electronic microscopy (TEM)

Tumor tissues from GBM patients were first fixed in 2.5% glutaraldehyde/0.1 M phosphate (pH 7.4) for 10 min, and then cut into pieces less than 1 mm^3, followed by fixation in 2.5% glutaraldehyde/0.1M phosphate (pH 7.4) overnight at 4˚C. After fixation in 1% osmium tetroxide/phosphate buffer for 1 hr at room temperature, the tissue pieces were stained with 2% uranyl acetate/10% ethanol for 1 hr, followed by dehydration in increasing ethanol serial dilutions. The tissues were finally embedded in Eponate 12 resin. Ultra-thin sections (70 mm) were prepared on a Leica EM UC6 ultramicrotome and stained with 2% uranyl acetate and Reynold's lead citrate. TEM was performed on a FEI Tecnai G2 Spirit TEM at 80kV. Images were captured by an AMT 2 x 2 digital camera. These experiments were performed at the OSU Microscopy Core Facility.

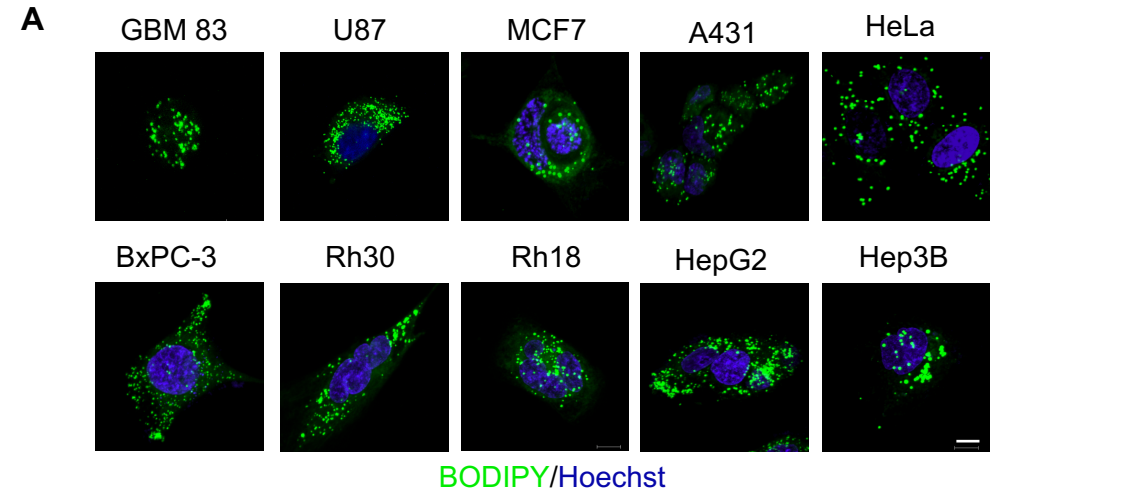
H&E staining

Paraffin tissue sections were deparaffinized in xylene and rehydrated in decreasing ethanol serial solutions. After washing with dH_2O, slides were stained with hematoxylin and eosin solutions in sequence, followed by washing with dH_2O. Slides were then dehydrated in increasing ethanol serial dilutions, immersed in xylene and mounted in Permount™ Mounting Medium (Cat# SP15-100, Fisher Scientific).

Statistical analysis

Statistical analysis was performed in Excel and GraphPad Prism 7. Cell proliferation, quantification of LDs and TGs, and co-localization were analyzed using the two-tailed t-test as well as by ANOVA, as appropriate. $P < 0.05$ was considered statistically significant.

Figure S1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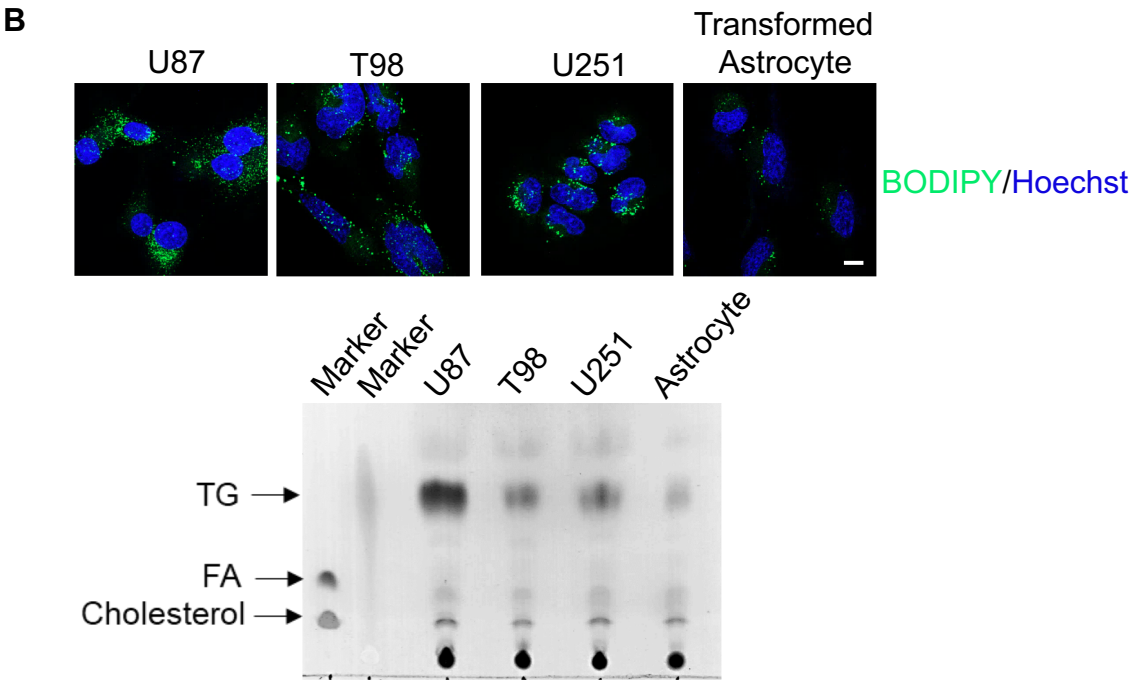
HeLa
BxPC-3
Rh30
Rh18
HepG2
Hep3B
BODIPY/Hoechst


B
U87
T98
U251
Transformed Astrocyte
BODIPY/Hoechst
Marker
Marker
U87
T98
U251
Astrocyte
TG
FA
Cholesterol

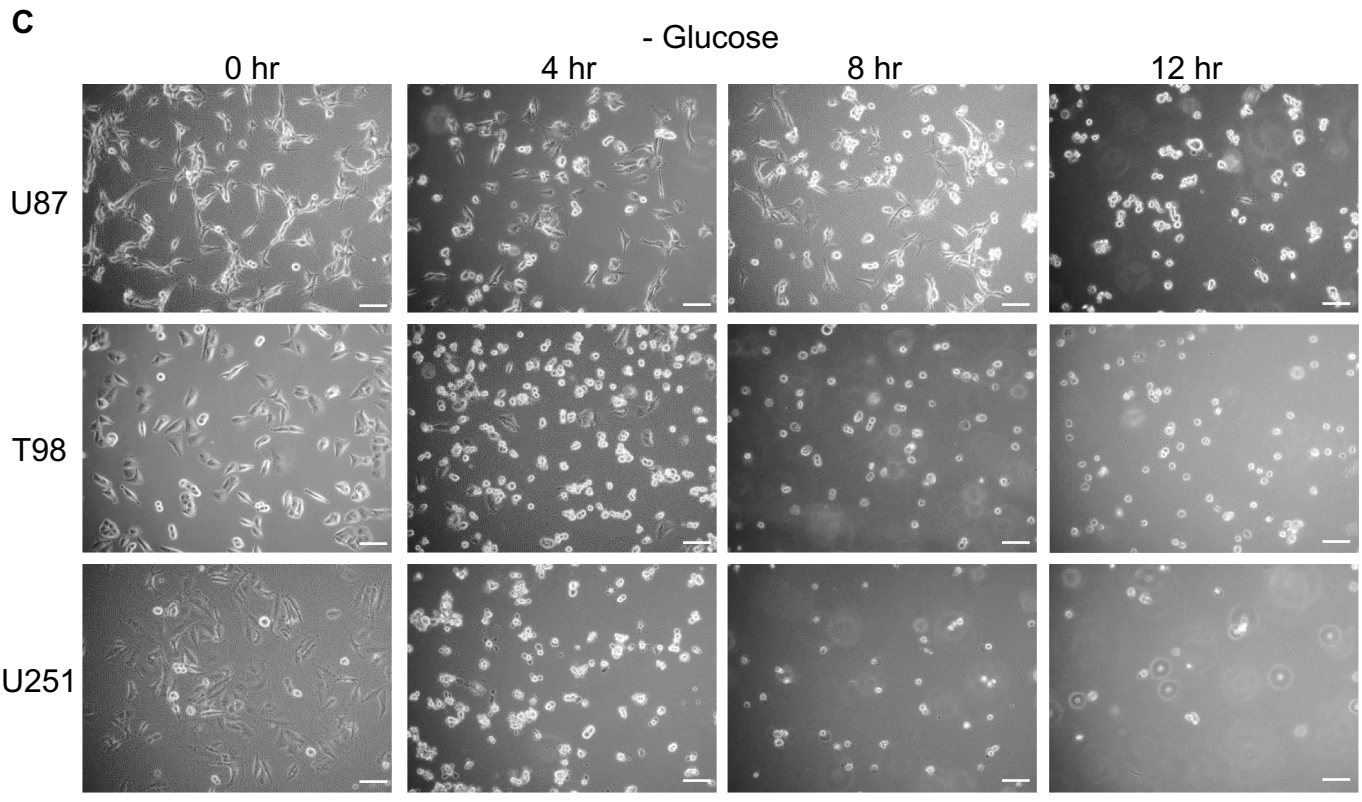

C
- Glucose
0 hr
4 hr
8 hr
12 hr
U87
T98
U251

Figure S1. Glucose withdrawal induces GBM cell death in a time-dependent manner. Related to Figures 1 and 2.

(A) Representative fluorescence images of different types of cancer cell lines stained with BODIPY 493/503 (green) and Hoechst 33342 (blue). Scale bar, 10 μm. MCF7, breast adenocarcinoma cell line; HepG2 and Hep3B hepatocellular carcinoma cell lines; A431, epidermoid carcinoma cell line; HeLa, cervical cancer cell line; BxPC-3, pancreas adenocarcinoma cell line; Rh18 and Rh30, rhabdomyosarcoma cell lines.

(B) Representative fluorescence images (upper panel) of GBM cells and transformed normal human astrocytes stained with BODIPY 493/503 (green) and Hoechst 33342 (blue). Scale bar, 10 μm. Total lipids were analyzed by TLC (lower panel). FA, fatty acids.

(C) Representative micrograph images of U87, U251 and T98 cells after withdrawal of glucose for 0 hr, 4 hr, 8 hr and 12 hr. Scale bar, 20 μm.

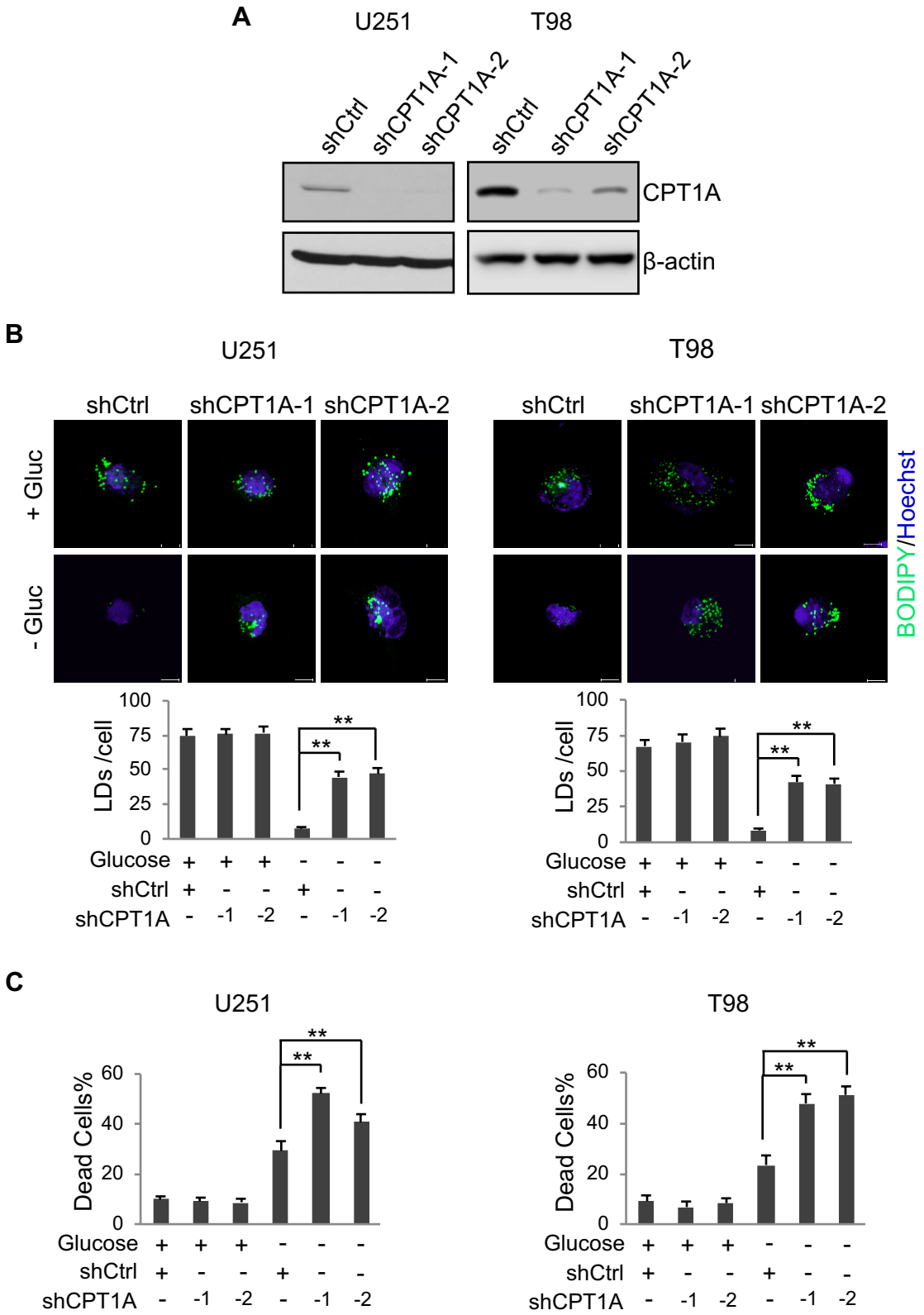


Figure S2. Genetic inhibition of CPT1A blocks glucose starvation-induced LD hydrolysis, while significantly enhancing GBM cell death. Related to Figure 3.

(A-C) U251 and T98 cells were transfected with CPT1A shRNA-expressing lentivirus for 24 hr and then cultured in glucose-free or 25 mM glucose medium for 4 hr. Cell lysates were analyzed by western blotting to determine CPT1A knockdown effects (A). The presence of lipid droplets was examined by confocal microscopy after staining with BODIPY 493/503 (green) and Hoechst 33342 (blue) (B). Quantification of LDs/cell (mean ± SEM, n = 30) was conducted using ImageJ. Cell death was determined by trypan blue staining after 3 hr glucose deprivation (mean ± SD, n = 3) (C). Significance was determined by one-way ANOVA. **p < 0.01.

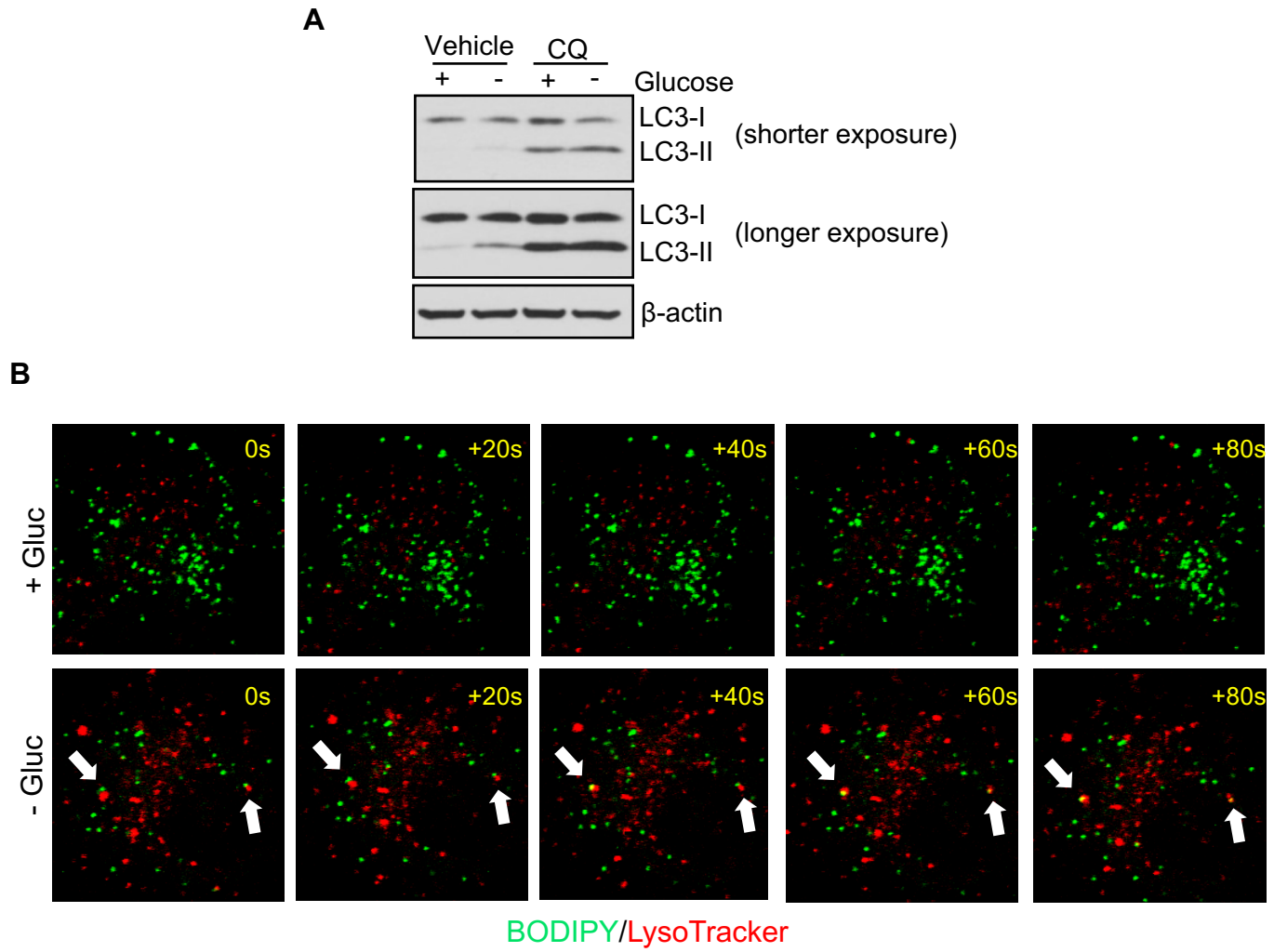
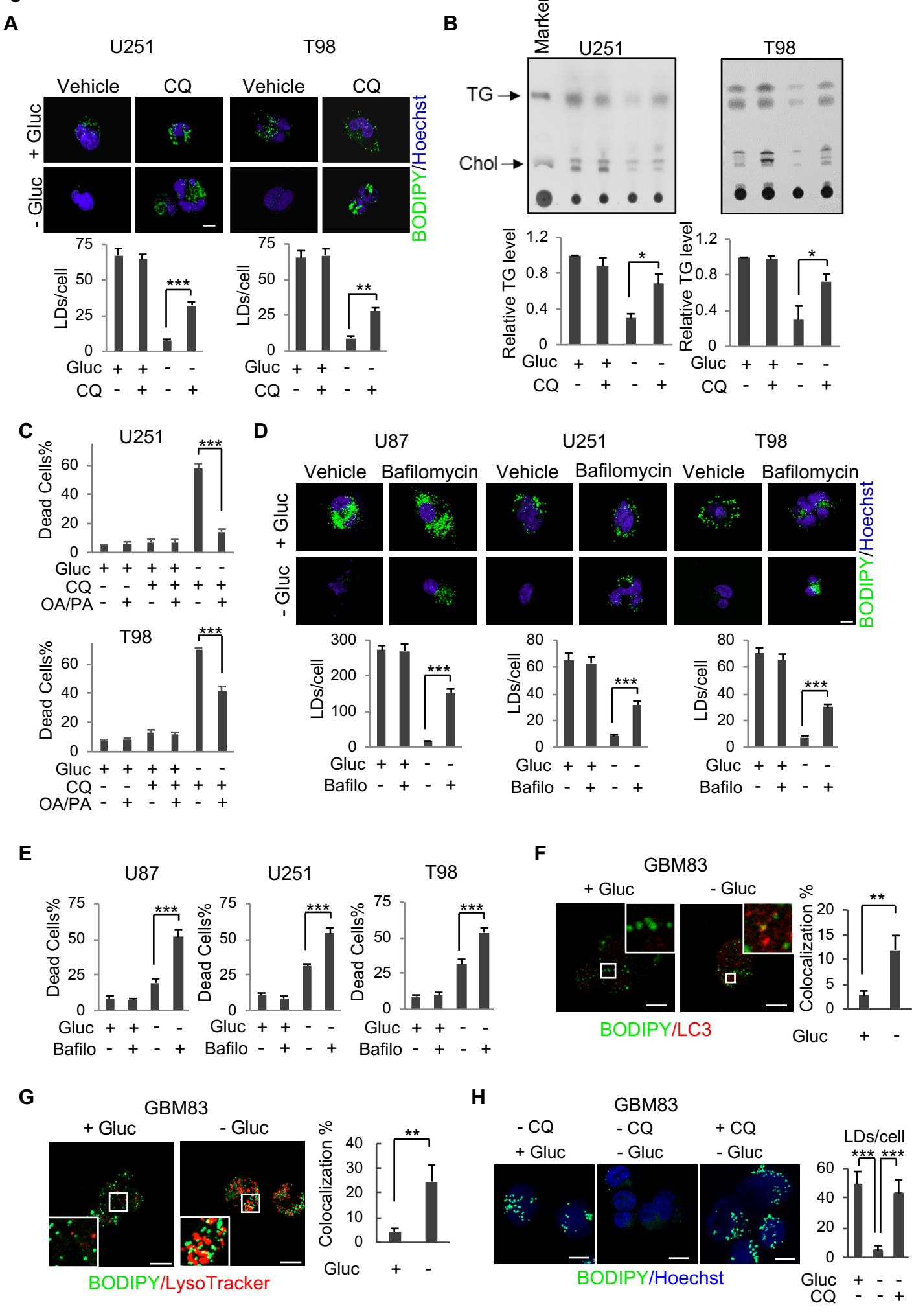


Figure S3. Glucose starvation induces autophagy to hydrolyze LDs in GBM cells. Related to Figure 4.

(A) Western blotting analysis of U87 cells in the absence or presence of glucose (25 mM) and CQ (5 μM) for 8 hr.

(B) Time-lapse imaging shows that glucose starvation induced the mobilization of LDs to the lysosomes in U87 cells. Cells were stained with BODIPY 493/503 (green) and LysoTracker (red) after glucose starvation for 7 hr and then observed by confocal microscopy in 20 sec intervals in a 5% CO_2 and 37°C chamber. Arrows indicate the mobilization and colocalization events of LDs and lysosomes.

Figure S4


A
U251
T98
Vehicle
CQ
Vehicle
CQ
+ Gluc
- Gluc
BODIPY/Hoechst
LDs/cell
Gluc
CQ
B
Marker
U251
T98
TG
Chol
Relative TG level
Gluc
CQ
C
U251
T98
Dead Cells%
Gluc
CQ
OA/PA
D
U87
U251
T98
Vehicle
Bafilomycin
+ Gluc
- Gluc
BODIPY/Hoechst
LDs/cell
Gluc
Bafilo
E
U87
U251
T98
Dead Cells%
Gluc
Bafilo
F
GBM83
+ Gluc
- Gluc
BODIPY/LC3
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BODIPY/LysoTracker
Colocalization %
Gluc
H
GBM83
- CQ
+ Gluc
- CQ
- Gluc
+ CQ
- Gluc
BODIPY/Hoechst
LDs/cell
Gluc
CQ

Figure S4. Inhibition of autophagy blocks glucose starvation-induced TG/LD hydrolysis, while significantly increasing GBM cell death. Related to Figure 4.

(A and B) U251 and T98 cells were treated with/without CQ (5 μM) in glucose-free or 25 mM glucose medium. After 4 hr, cells were stained with BODIPY 493/503 (green) and Hoechst 33342 (blue) and then observed by confocal microscopy (A). Scale bar: 10 μm. Quantification of LDs/cell (mean ± SEM, n = 30) was conducted by examining over 30 cells. Total lipids were analyzed by TLC and relative TG levels were determined by ImageJ (mean ± SD, n = 3) (B).

(C) U251 and T98 cells were cultured in medium with/without palmitic acid (PA, C16:0, 5 μM) and oleic acid (OA, C18:1, 5 μM) mixtures (1:1) for 24 hr, and then treated with/without CQ (5 μM) in the absence or presence of glucose (25 mM) for 3 hr to determine cell death by trypan blue staining (mean ± SD, n = 3) (C).

(D and E) GBM cells were treated with/without Bafilomycin (100 nM) in glucose-free or 25 mM glucose medium for 8hr (U87 cells) or 4 hr (U251 and T98 cells). Cells were stained with BODIPY 493/503 (Green) and Hoechst 33342 (blue) and then observed by confocal microscopy. Scale bar: 10 μm. Quantification of LDs/cell was determined same as in panel A. Cell death was determined by trypan blue staining after 8hr (U87) or 4 hr (U251 and T98) treatment (E).

(F and G) Representative fluorescence images of GBM patient-derived primary GBM83 cells stained with BODIPY 493/503 (green) and anti-LC3 antibody (red) (F), or LysoTracker (red) (G) in the absence or presence of glucose (5 mM) for 4 hr. The co-localization was quantified by ImageJ (mean ± SEM, n = 10 cells).

(H) GBM83 cells were treated with/without CQ (5 μM) in glucose-free or 5 mM glucose medium. After 12 hr, cells were stained with BODIPY 493/503 (green) and Hoechst 33342 (blue) and then observed by confocal microscopy. Scale bar: 10 μm. LDs/cell were quantified by ImageJ (mean ± SEM, n = 30 cells).

Significance was determined by one-way ANOVA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.