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Supplemental information

SREBP-1 upregulates lipophagy to maintain cholesterol homeostasis in brain tumor cells

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Fig. S1

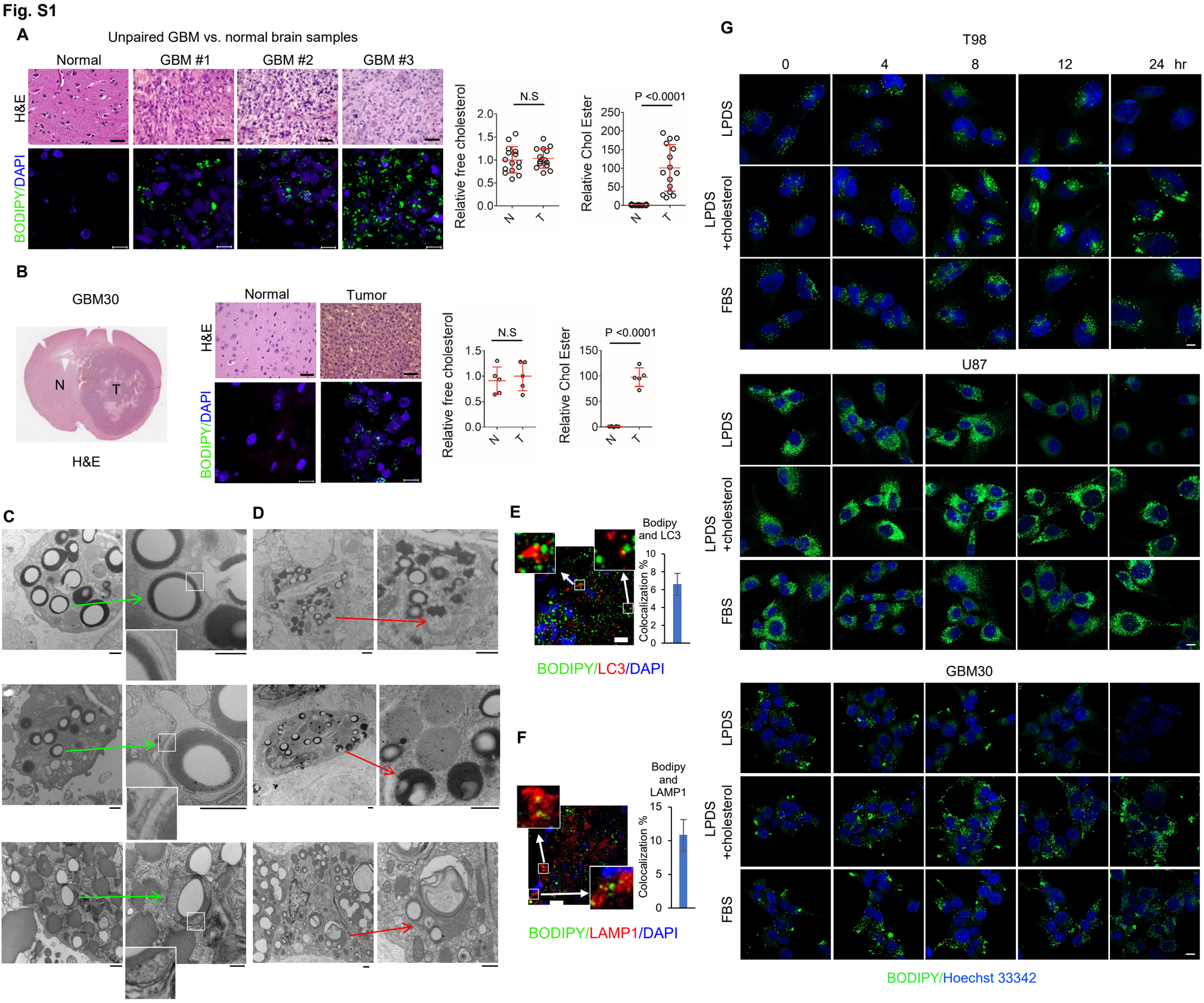

A
Unpaired GBM vs. normal brain samples
Normal
GBM #1
GBM #2
GBM #3
H&E
BODIPY/DAPI
Relative free cholesterol
N.S
Relative Chol Ester
P <0.0001
N
T
B
GBM30
N
T
H&E
Normal
Tumor
H&E
BODIPY/DAPI
Relative free cholesterol
N.S
Relative Chol Ester
P <0.0001
C
D
E
Bodipy and LC3
Colocalization %
BODIPY/LC3/DAPI
F
Bodipy and LAMP1
Colocalization %
BODIPY/LAMP1/DAPI
G
T98
0
4
8
12
24 hr
LPDS
LPDS +cholesterol
FBS
U87
GBM30
BODIPY/Hoechst 33342

Figure S1 (related to Figure 1). GBM cells contain abundant LD-bound CEs that are present in autophagosomes and lysosomes in tumor tissues.

A) Representative images of H&E or BODIPY 493/503 (green)/DAPI (blue) staining of GBM tumor and normal brain tissues from unpaired human patient autopsies. Free cholesterol and cholesteryl esters (CE) in GBM tumor tissues versus normal brain tissues (n = 15) were determined by cholesterol/CE measuring kit (mean ± SD). Statistical significance was analyzed by an unpaired Student's t test. N.S, not significant. Scale bars, 10 μm in fluorescence images and 50 μm in H&E images.

B) Primary GBM30 orthotopic mouse model stained by H&E or BODIPY 493/503 (green)/DAPI (blue). Free cholesterol and CE in normal mice brain versus tumor tissues were determined by cholesterol/CE measuring kit (mean ± SD, n = 5). Statistical significance was analyzed by an unpaired Student's *t*-test. N.S, not significant. Scale bars, 10 μm in fluorescence images and 50 μm in H&E images.

C, D) Representative transmission electron microscopy images of tumor tissues from GBM patient biopsies. Green arrow indicates the double membrane vesicle that engulfs lipid droplets (LDs) (C); red arrow shows that LD is entrapped in the lysosome (LY) (D). Scale bars, 500 nm.

E, F) Representative confocal fluorescent images (left panels) of co-staining of BODIPY 493/503 (green) with LC3 (red) (E), or with LAMP1 (red) (F) of GBM patient biopsies; the colocalization (right panels) of BODIPY-stained LDs with LC3-stained puncta (E), or LAMP1-stained lysosomes (F) was quantified by ImageJ software from 10 areas (mean ± SD). Scale bar, 10 μm.

G) Representative confocal images of BODIPY 493/503 (green) staining in live T98, U87 and GBM30 cells cultured in 5%FBS or 5% LPDS media in the absence or presence of supplemental

cholesterol (5 μg/ml) for indicated time. Nuclei were stained with Hoechst 33342 (blue). Scale bar, 10 μm.

Fig. S2

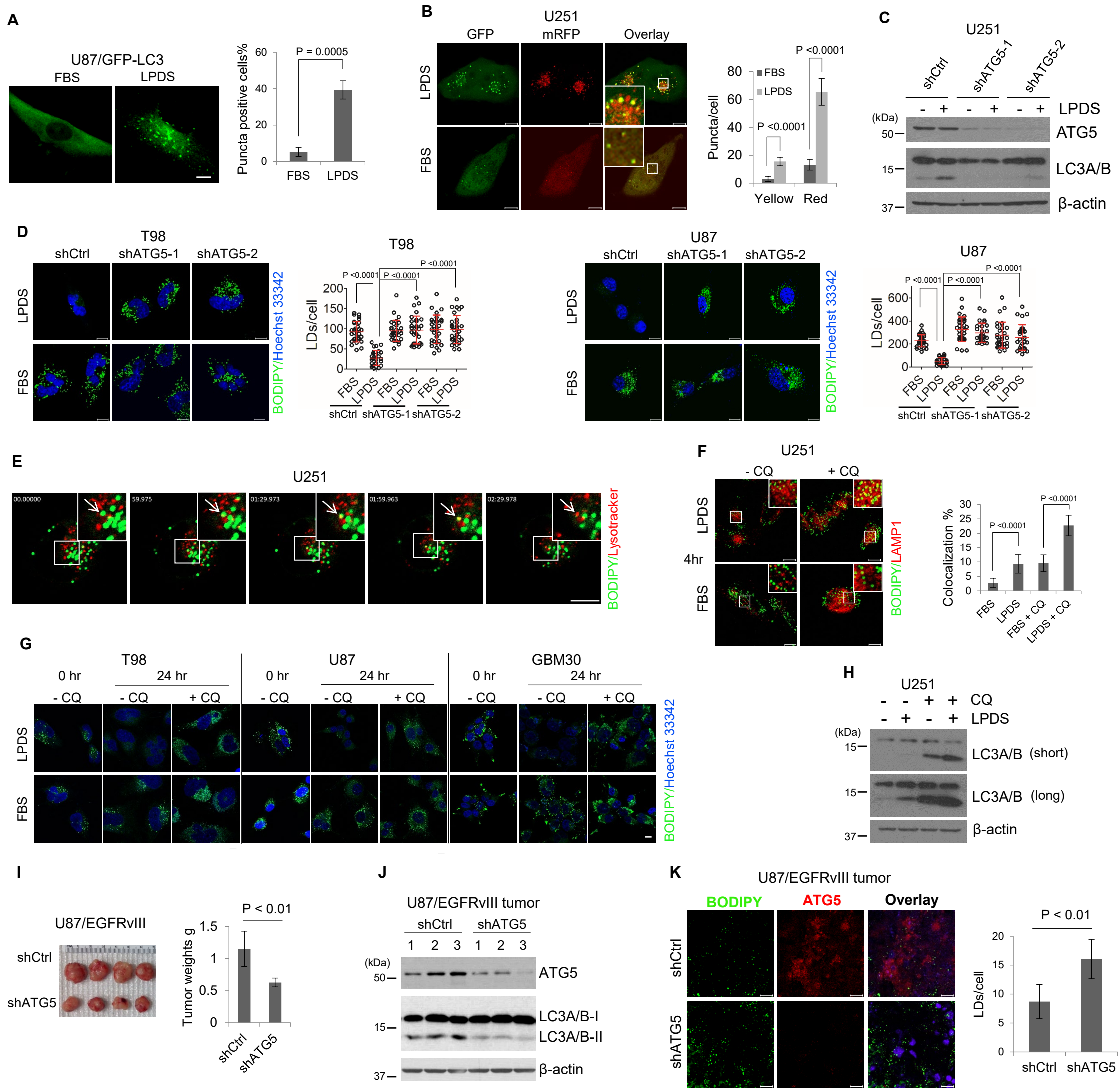

A
U87/GFP-LC3
FBS
LPDS
Puncta positive cells%
P = 0.0005
B
U251
GFP
mRFP
Overlay
LPDS
FBS
Puncta/cell
P <0.0001
FBS
LPDS
Yellow
Red
C
U251
shCtrl
shATG5-1
shATG5-2
LPDS
(kDa)
ATG5
LC3A/B
β-actin
D
T98
shCtrl
shATG5-1
shATG5-2
BODIPY/Hoechst 33342
LDs/cell
U87
E
U251
BODIPY/Lysotracker
F
U251
- CQ
+ CQ
4hr
BODIPY/LAMP1
Colocalization %
G
T98
U87
GBM30
0 hr
24 hr
H
U251
CQ
LPDS
LC3A/B (short)
LC3A/B (long)
I
U87/EGFRvIII
shCtrl
shATG5
Tumor weights g
P < 0.01
J
U87/EGFRvIII tumor
ATG5
LC3A/B-I
LC3A/B-II
K
U87/EGFRvIII tumor
BODIPY
ATG5
Overlay
LDs/cell

Figure S2 (related to Figure 1). Extracellular cholesterol depletion induces autophagy-mediated hydrolysis of LDs in GBM cells.

A) Representative confocal images of GFP-LC3 puncta in U87/GFP-LC3 cells cultured in 5% FBS or 5% LPDS for 24 hr (left panel). Puncta was quantified by ImageJ software, and cells containing over 30 puncta were counted as positive cells (mean ± SD, right panel). Statistical significance was analyzed by an unpaired Student's t test. Scale bar, 10 μm.

B) Representative confocal images of mRFP-GFP-LC3 expression in U251 cells cultured in 5% FBS or 5% LPDS media for 24 hr (left panels). The number of mRFP-GFP (yellow) and mRFP (red) signal in each condition was quantified by ImageJ software from over 30 cells (mean ± SD) (right panels). Scale bars, 10 μm. Statistical significance was analyzed by one-way ANOVA.

C) A representative western blot of U251 cells with shRNA silencing of ATG5 in comparison with shRNA control cultured in 5% FBS or 5% LPDS for 24 hr.

D) Representative confocal images of BODIPY 493/503 (green) staining in live T98 and U87 cells with shRNA silencing of ATG5 in comparison with shRNA control cells cultured in 5% FBS or 5% LPDS for 24 hr. Nuclei were stained with Hoechst 33342 (blue). LDs were quantified by ImageJ software from 30 cells (mean ± SD. Statistical significance was analyzed by one-way ANOVA. Scale bars, 10 μm

E) Representative time-lapse images of co-staining of BODIPY 493/503 (green) and Lysotracker (red) in live U251 cells cultured in 5% LPDS after 4 hr. Scale bar, 10 μm.

F) Representative confocal images of co-staining of BODIPY 493/503 (green) with LAMP1 (red) in U251 cells cultured in 5% FBS or 5% LPDS media in the absence or presence of CQ (5 μM) for 4 hr. The colocalization (yellow) of BODIPY-stained LDs with LAMP1 was quantified by

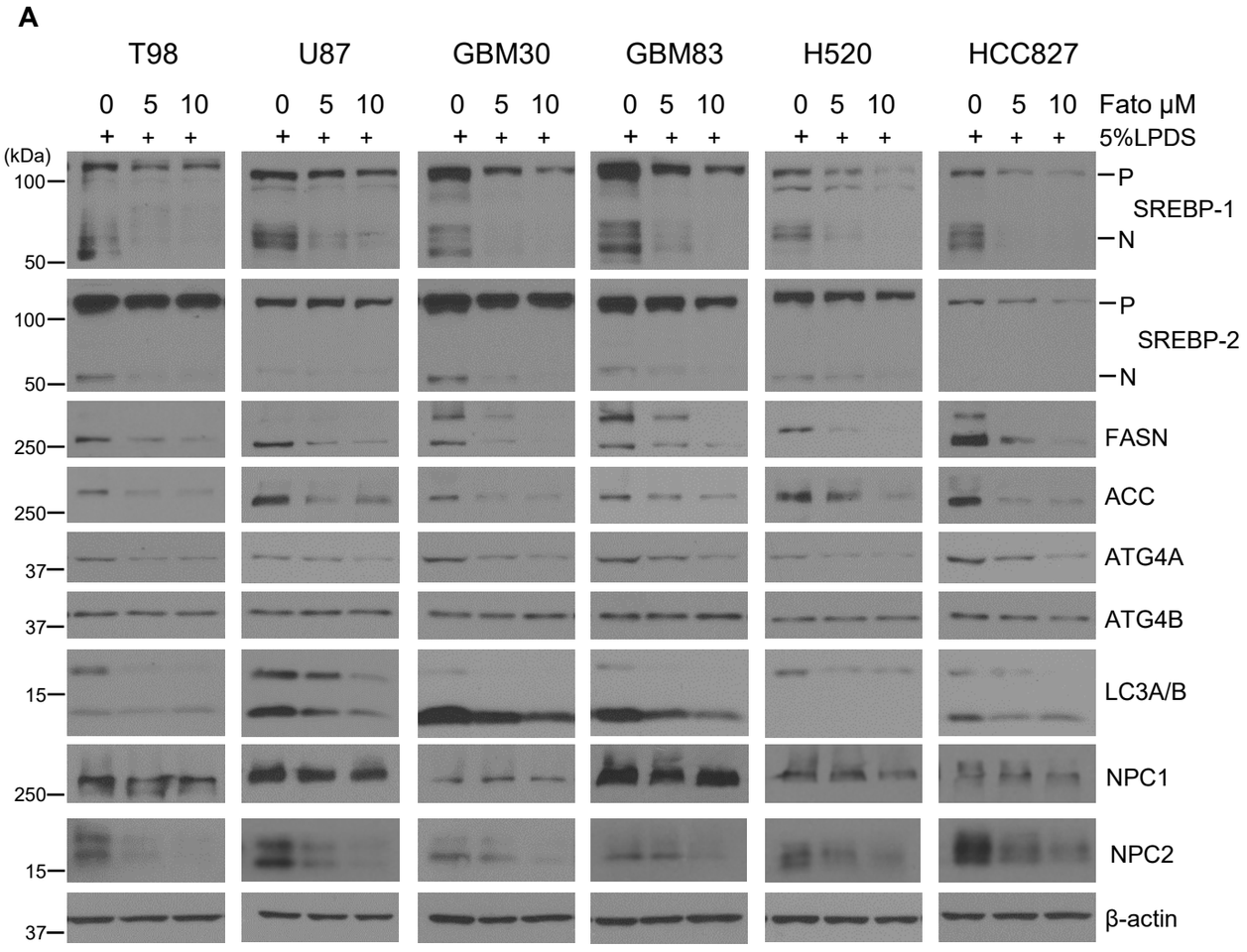
ImageJ software from 30 cells (mean ± SD). Statistical significance was analyzed by one-way ANOVA. Scale bars, 10 μm.

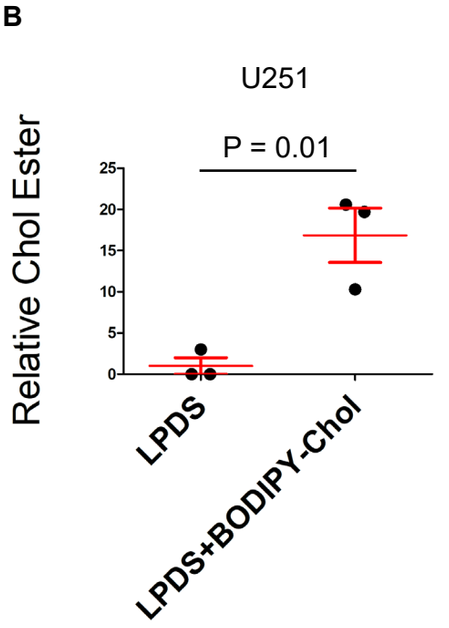
G) Representative confocal images of BODIPY 493/503 (green) staining in live T98, U87 and GBM30 cells cultured in 5% LPDS media in the absence or presence of CQ (5 μM) for 0 and 24 hr. Nuclei were stained with Hoechst 33342 (blue). Scale bars, 10 μm.

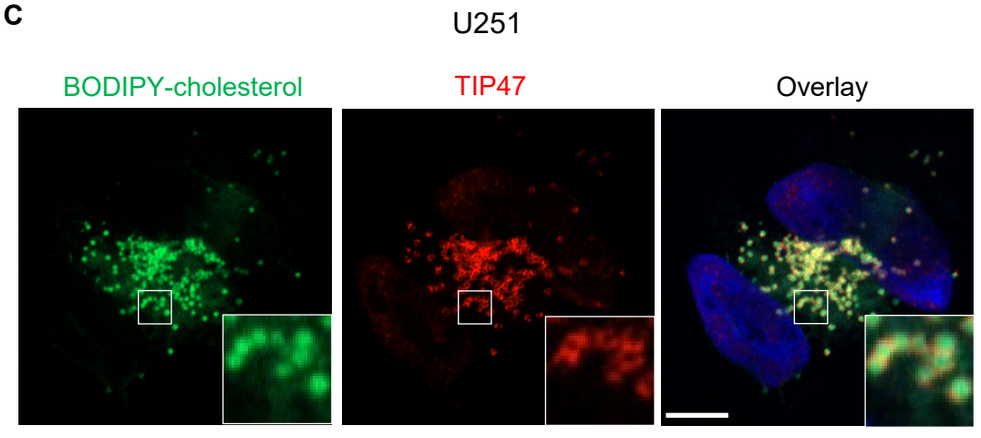
H) A representative western blot of U251 cells cultured in 5% FBS or 5% LPDS in the absence or presence of CQ (5 μM) for 24 hr.

I-K) Mouse subcutaneous tumors of U87/EGFRvIII cells with shRNA silencing of ATG5 in comparison with shRNA control. Implanted tumors were excised and weighed (I), a representative western blot shows expressions of ATG5 and LC3B proteins in shCtrl and shATG5 tumors (J). BODIPY 493/503 (green) and anti-ATG5 antibody were used to stain the sections of tumors tissues and nuclei were stained with DAPI (blue). LDs were quantified by ImageJ software (mean ± SD). Statistical significance was analyzed by an unpaired Student's t test. Scale bars, 10 μm.

Fig. S3


A
T98
U87
GBM30
GBM83
H520
HCC827
0 5 10
Fato μM
5%LPDS
(kDa)
100
50
P
SREBP-1
N
SREBP-2
250
FASN
ACC
37
ATG4A
ATG4B
15
LC3A/B
NPC1
NPC2
β-actin


B
U251
P = 0.01
Relative Chol Ester
25
20
15
10
5
0
LPDS
LPDS+BODIPY-Chol


C
U251
BODIPY-cholesterol
TIP47
Overlay

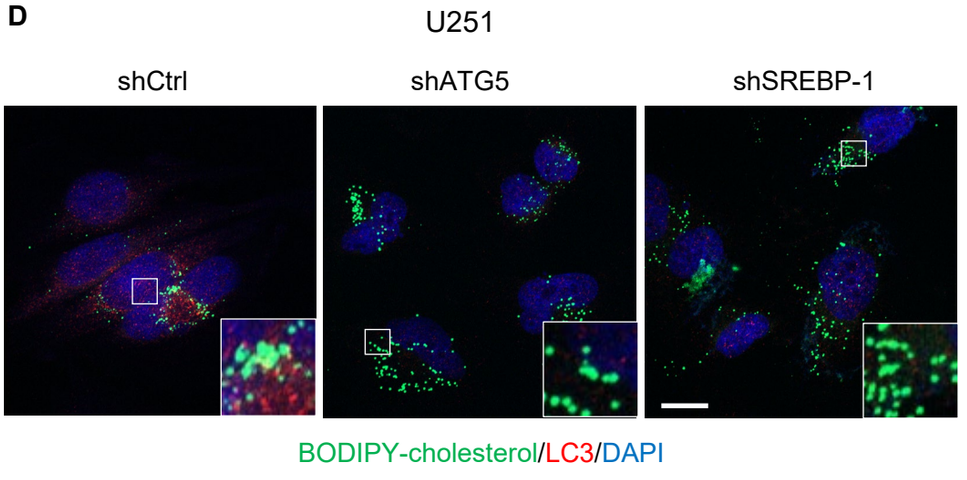

D
U251
shCtrl
shATG5
shSREBP-1
BODIPY-cholesterol/LC3/DAPI

Figure S3 (related to Figure 2). SREBP-1 inactivation is associated with reduced autophagic protein levels in GBM cells.

A) Representative western blots of T98, U87, GBM30, GBM83 (GBM), H520 and HCC827 (lung cancer) cells cultured in 5%LPDS in the absence or presence of SREBP inhibitor Fatostatin at indicated doses for 40 hr.

B) Relative cholesteryl ester levels in U251 cells cultured in 5%LPDS or 5%LPDS supplemented with BODIPY-cholesterol (20 μg/ml) for 24 hr. Statistical significance was analyzed by an unpaired Student's t-test.

C) Representative confocal images of U251 cells cultured with BODIPY-cholesterol (20 μg/ml) for 24 hr in 5% FBS culture medium (green) and stained with TIP47 (red). Nuclei were stained with DAPI (blue). Scale bar, 10 μm.

D) Representative confocal images of U251 cells with shRNA knockdown of ATG5 or SREBP-1 cultured with BODIPY-cholesterol (20 μg/ml) (green) in 5% FBS medium for 24 hr, then switched medium to 5% LPDS for 4 hr and stained with LC3 (red). Nuclei were stained with DAPI (blue). Scale bar, 10 μm.

Fig. S4

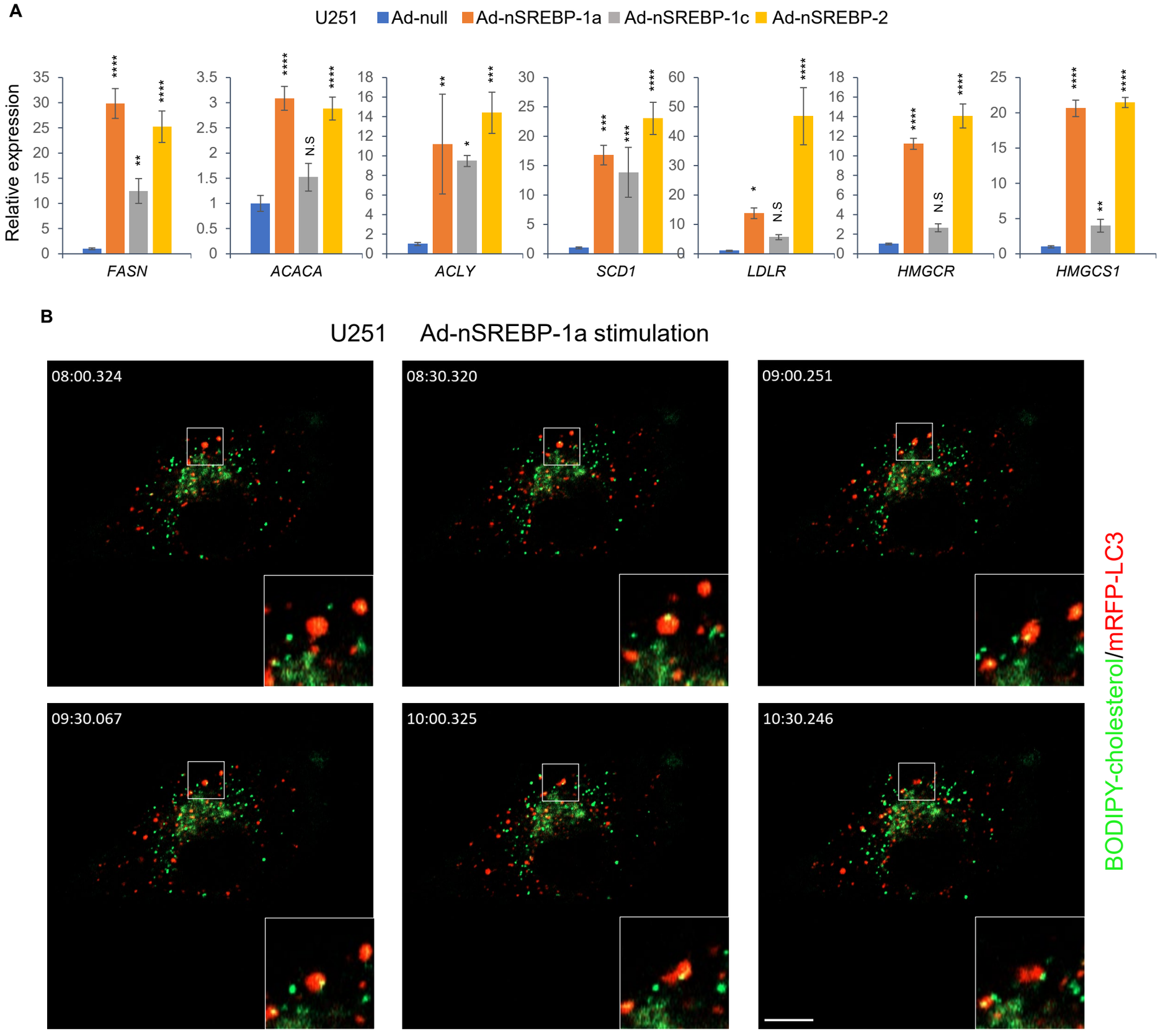

A
U251
Ad-null
Ad-nSREBP-1a
Ad-nSREBP-1c
Ad-nSREBP-2
Relative expression
FASN
ACACA
ACLY
SCD1
LDLR
HMGCR
HMGCS1
N.S
B
U251 Ad-nSREBP-1a stimulation
08:00.324
08:30.320
09:00.251
09:30.067
10:00.325
10:30.246
BODIPY-cholesterol/mRFP-LC3

Figure S4 (related to Figure 3). Overexpression of N-terminal SREBP isoforms results in greater lipogenic and LDLR expression in GBM cells.

A) Real-time PCR analysis of mRNA expression (mean ± SD) of lipogenic and LDLR genes in U251 cells with overexpression of N-terminal SREBP-1a (Ad-nSREBP-1a), -1c (Ad-nSREBP-1c), -2 (Ad-nSREBP-2) or Ad-null via adenovirus-mediated vector cultured in 5% FBS for 48 hr. Statistical significance was analyzed by one-way ANOVA. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$. ****, $p < 0.0001$. N.S, not significant.

B) Representative time-lapse images (2.5 minutes from 8' to 10'30'') of BODIPY-cholesterol-formed LDs (green) (20 μg/ml for 12 hr in 5% FBS medium) and RFP-LC3-formed puncta (red) in live U251-RFP-LC3 stable cells with overexpression of N-terminal SREBP-1a via adenovirus-mediated vector for 8 hours. Scale bar, 10 μm.

Fig. S5

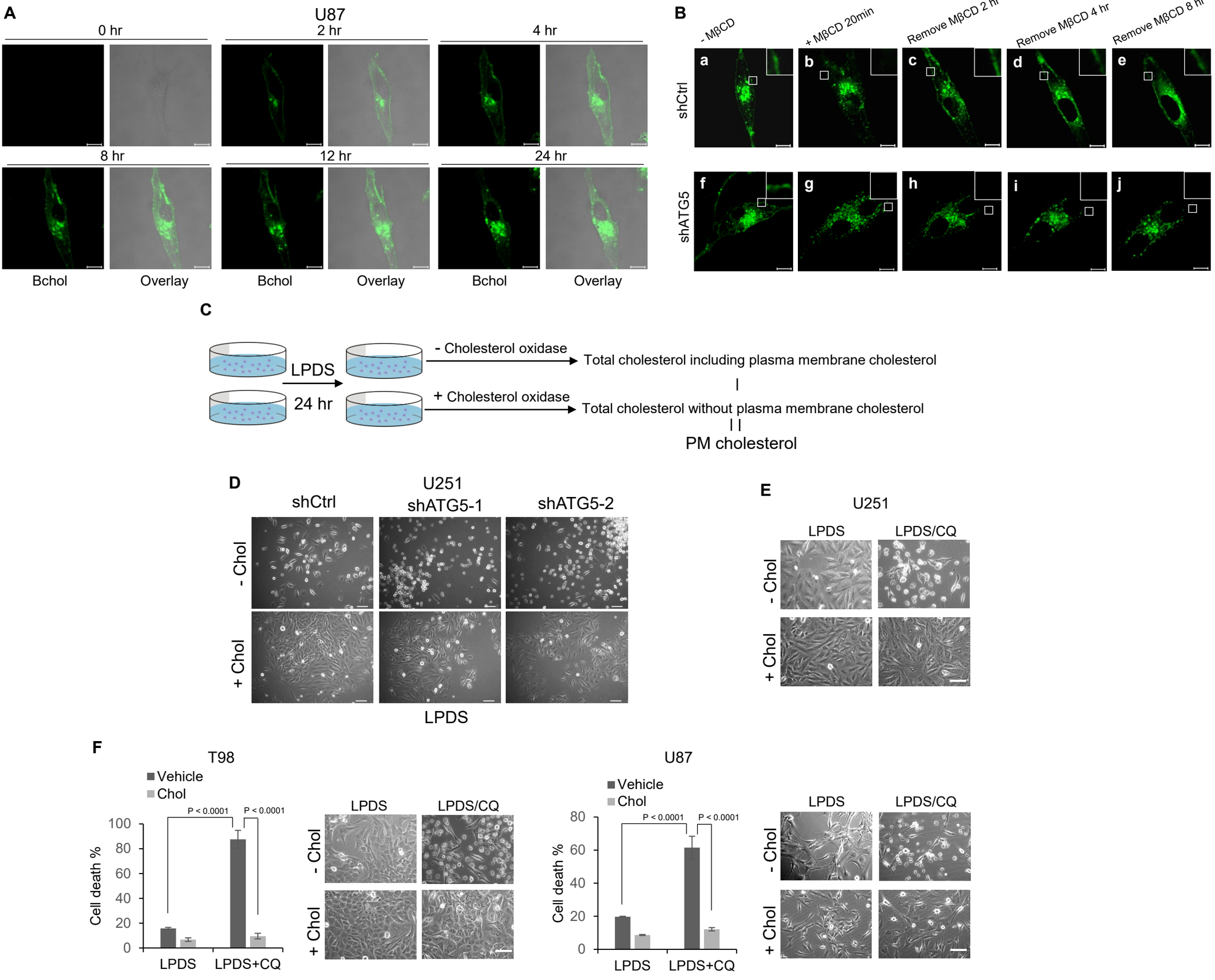

A
U87
0 hr
2 hr
4 hr
8 hr
12 hr
24 hr
Bchol
Overlay
B
U87
- MβCD
+ MβCD 20min
Remove MβCD 2 hr
Remove MβCD 4 hr
Remove MβCD 8 hr
shCtrl
shATG5
C
LPDS
24 hr
- Cholesterol oxidase
+ Cholesterol oxidase
Total cholesterol including plasma membrane cholesterol
Total cholesterol without plasma membrane cholesterol
PM cholesterol
D
U251
shCtrl
shATG5-1
shATG5-2
- Chol
+ Chol
LPDS
E
U251
LPDS
LPDS/CQ
- Chol
+ Chol
F
T98
Vehicle
Chol
P < 0.0001
P < 0.0001
Cell death %
LPDS
LPDS+CQ
LPDS/CQ
U87

Figure S5 (related to Figure 4). Lipophagy of LDs releases cholesterol to maintain membrane cholesterol homeostasis and GBM survival upon extracellular cholesterol depletion.

A) Representative confocal images of U87 cells cultured in serum-free media with BODIPY-cholesterol (20 μg/ml) after serum starvation for 24 hr and imaged by confocal and light microscopy at the indicated time. Scale bars, 10 μm.

B) U87 cells in which ATG5 was knocked down by a shRNA-expressing lentivirus versus cell treated with an shRNA control were cultured in serum-free media with BODIPY-cholesterol (20 μg/ml) for 24 hr after serum starvation for 24 hr (a and f), then treated with MβCD (4 μM) for 20 min (b and g), followed by removal of MβCD and observed by confocal microscopy at 2, 4 and 8 hr (c-e, h-J). Inserts are the amplification of membrane labeling by BODIPY-cholesterol. Scale bars, 10 μm.

C) A schematic diagram illustrating the procedure of biochemical measurement of plasma membrane (PM) cholesterol level.

D) Representative bright field images of U251 cells with shRNA silencing of ATG5 in comparison with shRNA control cultured in 5% LPDS in the absence or presence of supplemental cholesterol (5 μg/ml) for 3 days. Scale bars, 100 μm.

E) Representative brightfield imagesof U251, cells cultured in 5%LPDS in the absence or presence of CQ (5 μM) with/without supplemental cholesterol (5 μg/ml) for 3 days. Scale bar, 100 μm.

F) Cell death percentile (left panel) and representative brightfield images (right panel) of T98 and U87 cells cultured in 5%LPDS in the absence or presence of CQ (5 μM) with/without supplemental cholesterol (5 μg/ml) for 3 days. Statistical significance was analyzed by one-way ANOVA. Scale bar, 100 μm.

Fig. S6

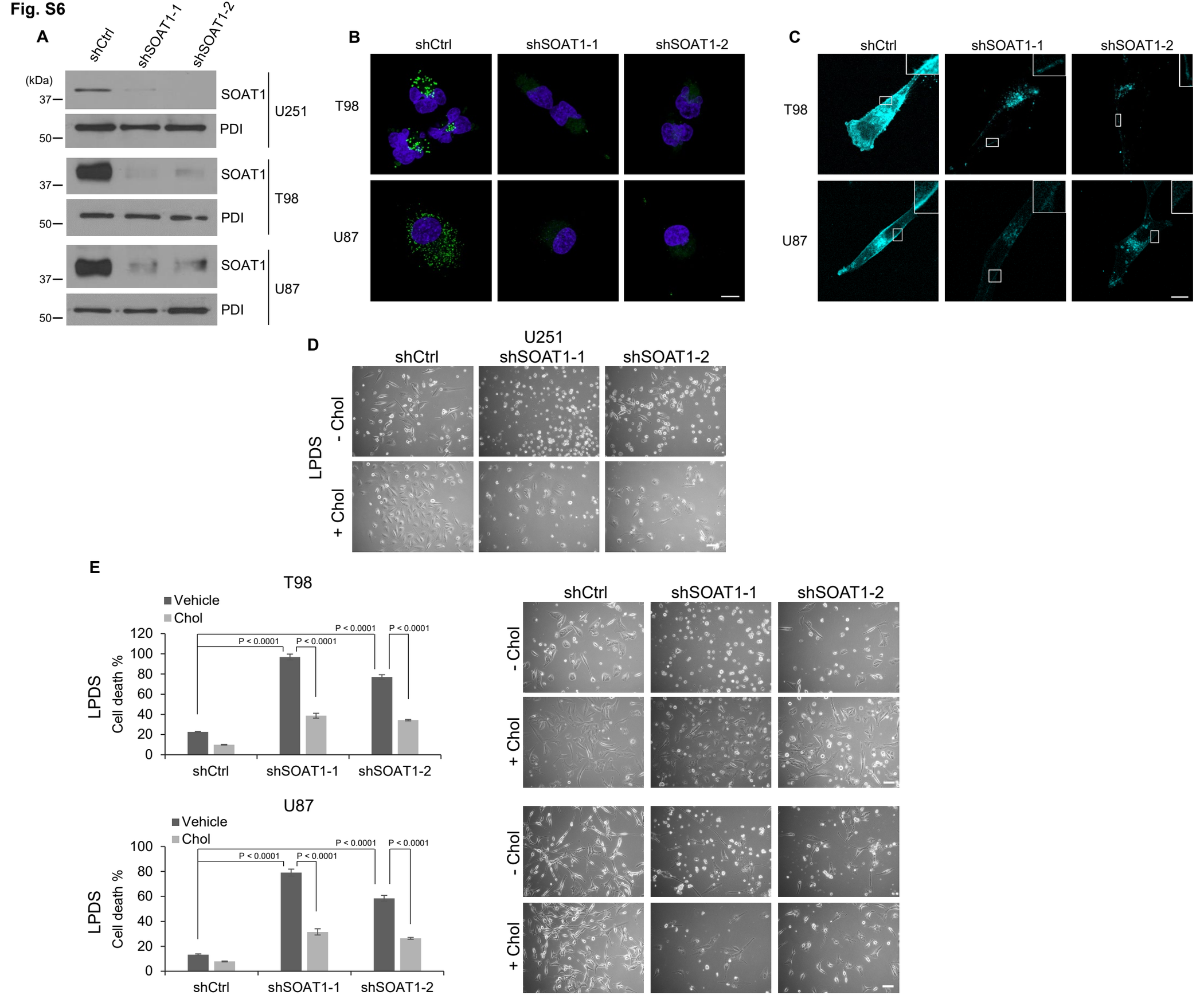

A
shCtrl
shSOAT1-1
shSOAT1-2
(kDa)
37
50
SOAT1
PDI
U251
T98
U87
B
C
D
U251
LPDS
- Chol
+ Chol
E
T98
Vehicle
Chol
Cell death %
P < 0.0001
shCtrl
shSOAT1-1
shSOAT1-2

Figure S6 (related to Figure 4). GBM cells depleted of LD-bound CE storage fail to maintain proper membrane cholesterol levels and are sensitive to exogenous cholesterol depletion

A) Representative western blots of expression of SOAT1 in U251, T98 and U87 cells with shRNA silencing of SOAT1 in comparison with shRNA control cells.

B) Representative confocal images of BODIPY 493/503 (green) staining in live T98 and U87 cells with shRNA silencing of SOAT1 in comparison with shRNA control cultured in 5% FBS media for 24 hr and then split in 35 mm glass-bottom dish cultured in 5% FBS media for 24 hr. Nuclei were stained with Hoechst 33342 (blue). Scale bar, 10 μm.

C) Filipin staining and fluorescent microscopy images of T98 and U87 with shRNA silencing of SOAT1 in comparison with shRNA control cells cultured in 5% LPDS for 24 hr. Scale bar, 10 μm.

D) Representative bright field images of U251, cells with shRNA silencing of SOAT1 in comparison with shRNA control cultured in 5% LPDS in the absence or presence of supplemental cholesterol (5 μg/ml) for 3 days. Scale bars, 100 μm.

E) Cell death percentile (left panel) and representative bright field images (right panel) of T98 and U87 cells with shRNA silencing of SOAT1 in comparison with shRNA control cultured in 5% LPDS in the absence or presence of supplemental cholesterol (5 μg/ml) for 3 days. Statistical significance was analyzed by one-way ANOVA. Scale bars, 100 μm.