SUPPLEMENTAL INFORMATION

SUPPLEMENTAL MATERIALS AND METHODS

Treatment of hepatocytes with lipids. 1–palmitoyl-sn-glycero-3-phosphocholine (lysophosphatidylcholine, LPC; #L5254), palmitate (PA, #P5585) and oliate (OA, #O7501) were obtained from Sigma-Aldrich Inc. (St. Louis, MO). LPC was dissolved in DMSO and DMEM (1:1) at a stock concentration of 25 mM [1]. Cells were cultured in media containing the desired concentration of LPC. PA was dissolved in isopropanol at a stock concentration of 160 mM [2]. Cells were cultured in media containing PA and 1% BSA to maintain the physiological ratio between bound and unbound FFA [2]. The concentration of DMSO or isopropanol was $\leq$ 0.2% in the media; the corresponding concentration of DMSO or isopropanol was used as a vehicle control.

Quantitation of cell death. Apoptotic cells were evaluated by both morphological and biochemical approaches as previous described by us[1]. Briefly, Huh-7 cells were incubated with either 2 µg/ml of 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) for 30 min at 37 °C or 1 mg/ml of propidium iodide (a fluorescent dye exclusion marker for cell viability, analogous to trypan blue), and analyzed by fluorescence microscopy (Nikon Eclipse TE200; Nikon, Tokyo, Japan). Following DAPI staining, apoptosis was quantified by assessing the morphologic changes of nuclear chromatin condensation and fragmentation, and was expressed as a percentage of total cells. Cell survival was also examined by propidium idodide nuclear labeling to avoid the potential pitfalls of assessing apoptosis in cells without caspase 9 (e.g, absence of classic nuclear fragmentation due to loss of caspase directed cascades). Cell viability by propidium iodide exclusion was expressed as a percentage of total cell number. For caspase 3/7 activity, a biochemical hallmark of apoptosis, cells were plated in 96-well plates. The assay was

performed using the commercially available Apo-One homogeneous caspase 3/7 assay (Promega, Madison, WI) according to the manufacturer's instruction.

Quantitative real-time PCR. Total cellular RNA was extracted using Trizol reagent (Invitrogen, Camarillo, CA) and was reverse-transcribed into complementary DNA with Moloney murine leukemia virus reverse transcriptase (Invitrogen, Camarillo, CA) and random primers (Invitrogen, Camarillo, CA) as previously described [3]. Quantification of the complementary DNA template was performed by quantitative real time PCR (qRT-PCR; LightCycler; Roche Applied Science, Indianapolis, IN) using SYBR green (Invitrogen) as a fluorophore. PCR primers were as follows: for human ACC1 (NM_198834.1): forward 5'-GTCTTTGCCAACTGGAGAGG -3' and reverse 5'- CAAACTTCAGCACTTGGTCGT -3', for human ACC2 (NM_001093.3): forward 5'- CAGTTCGCCGACTTCCAT -3' and reverse 5'-TCTTCCACTCCAGGATGTCA -3', for human ACOX1 (NM_007292.4): forward 5'-GGCACCACTGCTCAGAAAG -3' and reverse 5'- CGTAGGTGCCAATTATCTGGA -3', for human CPT1A (NM_001876.3): forward 5'- GACAATACCTCGGAGCCTCA -3' and reverse 5'- AATAGGCCTGACGACACCTG -3', for human CPT2 (NM_000098.2): forward 5'-TGACCAAAGAAGCAGCAATG -3' and reverse 5'- GAGCTCAGGCAAGATGATCC -3', human SHH (NM_000193): forward 5'- GATGTCTGCTGCTAGTCCTCG -3' and reverse 5'-AGAAGCAGGGTCAAGAGTGGTGAA -3', for human Gli2 (NM_005270): forward 5'-TGGCCGCTTCAGATGACAGATGTTG -3' and reverse 5'-CGTTAGCCGAATGTCAGCCGTGAAG -3', for mouse SHH (NM_009170): forward 5'-CTGGCCAGATGTTTTCTGGT -3' and reverse 5'- TAAAGGGGTCAGCTTTTTGG -3', for mouse Gli2 (NM_001081125): forward 5'- CCCATCACCATTCATAAGC -3' and reverse 5'-CTGCTCCTGTGTCAGTCCAA -3'. As an internal control, primers for 18S ribosomal RNA

(rRNA) were used (Ambion, Austin, TX). The relative mRNA expression levels were expressed as the ratio of target mRNA /18S rRNA per each sample as previously described [3].

GLI reporter assay. GLI reporter activity was quantified with a reporter construct containing eight copies of a consensus GLI-binding site (8×-GLI) downstream of a luciferase gene (pδ51LucII plasmid; δ-crystalline promoter). The 8×-GLI reporter was kindly provided by M. Fernandez-Zapico (Division of Oncology Research, Mayo Clinic, Rochester, MN). The plasmid was transfected into Huh-7 or shC9 cells (0.5 μg/well), using FuGene HD (Roche Diagnosis, Basel, Switzerland) [4]. Cells were cotransfected with 50 ng of a plasmid expressing Renilla luciferase under the control of cytomegalovirus (pRL-CMV; Promega). After twenty four hours of transfection, cells were treated as indicated. Cells were lysed and both firefly and Renilla luciferase activities quantified using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions. Firefly luciferase activity was normalized to Renilla luciferase activity to control for transfection efficiency and cell numbers. Data (firefly/Renilla luciferase activity) are presented as fold change to vehicle-treated cells transfected with the 8×-GLI/pRL-CMV reporter constructs [44].

Antibodies and reagents. Antibodies used were obtained from the following sources: goat anti-caspase 2 (1:50; sc-626-G), mouse anti-caspase 9 p10 (1:50; sc-17784), mouse anti-phospho-c-Jun (Ser63) (1:1000; sc-822), mouse anti-c-Jun (1:1000; sc-44), Lamin B (1:1000; sc-6216) and rabbit anti-Sonic Hedgehog (1:300; sc-9024, Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-Ubiquitin (1:500; Z0458, Dako, Carpinteria, CA); rabbit anti-GLI2 (1:500; ARP31885, Aviva Systems Biology, San Diego, CA). The JNK inhibitor SP600125 (#420119) and the pan-caspase inhibitor Z-VAD-fmk (#219007) were obtained from Calbiochem (San Diego, CA). GANT61 (#3191) was obtained from Tocris bioscience (Ellisville, MO). GDC-0449

(#S1082) was obtained from Selleck Chemicals (Houston, TX). BSA, Bradford reagent, and other chemicals were all obtained from Sigma-Aldrich (St. Louis, MO).

Statistical analysis. All data represent at least three independent experiments and are expressed as the mean ± SEM. Differences between groups were compared using Student's *t*-test and one-way analysis of variance with a *post hoc* Dunnett test; significance was accepted at p values <0.05.

SUPPLEMENTAL REFERENCES

[1] Kakisaka K, Cazanave SC, Fingas CD, Guicciardi ME, Bronk SF, Werneburg NW, et al. Mechanisms of lysophosphatidylcholine-induced hepatocyte lipoapoptosis. American journal of physiology Gastrointestinal and liver physiology 2012;302:G77-84.

[2] Cazanave SC, Mott JL, Elmi NA, Bronk SF, Werneburg NW, Akazawa Y, et al. JNK1-dependent PUMA expression contributes to hepatocyte lipoapoptosis. J Biol Chem 2009;284:26591-26602.

[3] Barreyro FJ, Kobayashi S, Bronk SF, Werneburg NW, Malhi H, Gores GJ. Transcriptional regulation of Bim by FoxO3A mediates hepatocyte lipoapoptosis. J Biol Chem 2007;282:27141-27154.

[4] Fingas CD, Bronk SF, Werneburg NW, Mott JL, Guicciardi ME, Cazanave SC, et al. Myofibroblast-derived PDGF-BB promotes Hedgehog survival signaling in cholangiocarcinoma cells. Hepatology 2011;54:2076-2088.

SUPPLEMENTAL TABLES

Supplemental Table 1. Oligonucleotide sequences used for EMSA

Oligonucleotide	Oligonucleotide sequence (5'-3')
AP-1 BS1	F: TGAAGACGAAG**TGACTCA**ACAAAGGCAGGC
AP-1 BS2	F: TCAGGAAATGCCC**TGAGTCA**CCCAAGCTCA
AP-1 BS1 mutant	F: TGAAGACGAAGaactgcaACAAAGGCAGGC
AP-1 BS2 mutant	F: TCAGGAAATGCCCaactgcaCCCAAGCTCA

Bold sequences indicates the putative AP-1 binding site within the promoter region of human *SHH* gene; small charcters indicates the mutated nucleotides.

Supplemental Table 2. Primers used for chromatin immunoprecipitaion assay

Promoter region	From TSS*	Primer sequence (5'-3')	Product size
AP-1 BS1	-4564 -4415	F: CCTAAGAAGAGGTGTCCATT R: TCCTAAGGCAGGCGAT	149
AP-1 BS2	-2683 -2578	F: GGGCTGTCTGGAGATGGA R: CGTGGCTTGGCATTGAG	105
Unrelated Sequence	-8199 -8096	F: CCTGTGAGTGGACGGTAA R: TGCCTATGCTCCCTTGTTGC	103

*TSS: transcription start site

SUPPLEMENTAL FIGURES

Supplemental Figure 1. Regulatory genes for lipolysis are not down regulated in shC9 cells treated with palmitate. Wild type (WT) and shC9 cells were incubated with vehicle (Veh), PA at 800 μM or OA at 800 μM for 16 hours, respectively. Each mRNA was quantified by real-time PCR, normalized to 18S rRNA, and expressed as fold change over vehicle. ACACA or ACACB, acetyl-CoA carboxylase alpha or beta; CPT1A carnitine palmitoyltransferase 1A; CPT2, carnitine palmitoyltransferase 2; ACOX1 acyl-CoA oxidase 1, palmitoyl. All data are expressed as mean ± SEM for at least three experiments; *p<0.01.

Supplemental Figure 2. Knockdown of caspase 9 by shRNA reduces palmitate- and LPC-induced cell death. (A, B and C) Wild type (WT) and shC9 cells were incubated with vehicle (Veh), PA at 800 μM or LPC at 85 μM for 16 hours, respectively. Cell death was assessed by morphological criteria after DAPI (apoptotic nuclei) or after propidium iodide (PI) staining (dye exclusion assay), or biochemically by measuring caspase 3/7 catalytic activity using a commercial assay; in the latter assay, the data are expessed as fold change over untreated cells. All data are expressed as mean ± SEM for at least three experiments; *p<0.01.

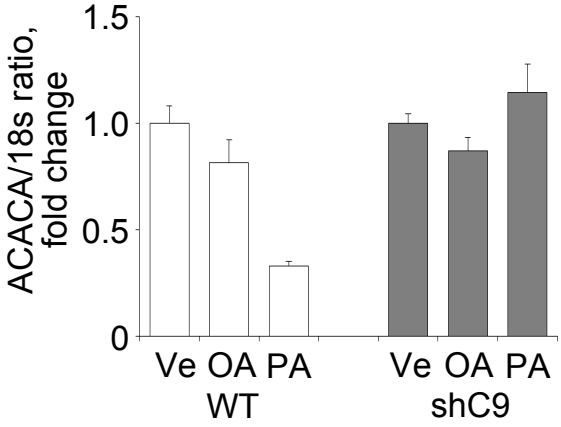
Supplemental Figure 3. Lipid loading and cell death in caspase $3^{-/-}$ primary mouse hepatocytes treated with palmitate (PA) or lysophosphatidylcholine (LPC). (A) Mouse primary hepatocytes isolated from wild type (WT) or $C3^{-/-}$ mice were incubated with PA 0, 100, or 300 μM for 12 hours. Lipid droplets were stained with Bodipy 505/515, imaged by fluorescent microscopy, and lipid droplet area measured using Metafluor quantitative fluorescence software and expressed as fold change over vehicle treated cells at upper panels. Mouse primary hepatocytes treated with PA 300 μM or OA 300 μM for 12 hours, respectively, were imaged by confocal microscopy and the respective images depicted in the bottom panel.

(B) Cells were treated with 400 μM PA or for 16 hours or 42.5 μM LPC for 16 hours. Apoptosis was assessed by morphological criteria after DAPI staining, and was expressed as a percentage of total cell number. All data are expressed as mean ± SEM for at least three experiments; *p<0.01.

Supplemental Figure 4. Palmitate (PA) induced accumulation of ubiquitin in shC9 cells. Wild type (WT) and shC9 cells were incubated with vehicle (Veh), OA at 400 μM or PA at 400 μM for 16 hours, respectively. Immunocytochemistry was performed for ubiquitin chains. The respective images obtained by confocal microscopy are presented in upper panel. The number of punctate ubiquitin staining cells were expressed as a percentage of total cell number in the bottom panel. All data are expressed as mean ± SEM for three experiments; *p<0.01.

Supplemental Figure 5. An autocrine SHH survival pathway in caspase $3^{-/-}$ primary mouse hepatocytes during lipotoxic stress. (A) Mouse primary hepatocytes obtained from $C3^{-/-}$ mice ($C3^{-/-}$) were pre-incubated for 12 hours with vehicle, the Gli1/2 inhibitor GANT61 at 5 μM, or the Smoothened antagonist GDC-0449 at 20 μM. After pre-incubation, $C3^{-/-}$ cells were incubated with Vehicle (Veh), PA at 400 μM for 12 hours or LPC at 42.5 μM for 6 hours. Mouse *Gli2* mRNA was quantified by real-time PCR, normalized to 18S rRNA, and expressed as fold change over vehicle. (B) After pre-incubation with GANT61 or GDC-0449, $C3^{-/-}$ mouse hepatocytes were incubated for 16 hours with PA at 400 μM or LPC at 42.5 μM, respectively. Cell death was quantified as propidium iodide positive (PI) cells expressed as a percentage of total cell number. All data are expressed as mean ± SEM for three experiments; *p<0.01, ** p<0.05.

Suppl. Fig.1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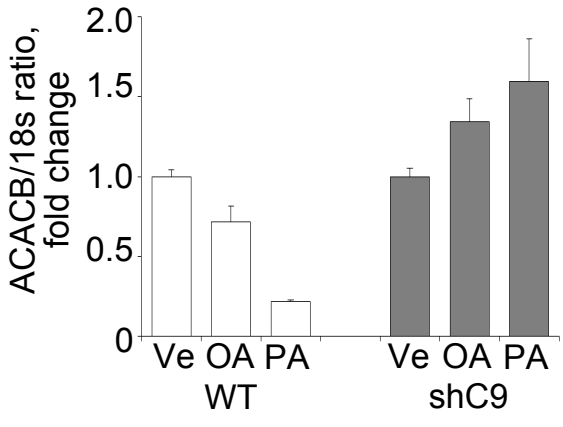
0.5
0
Ve OA PA
WT
Ve OA PA
shC9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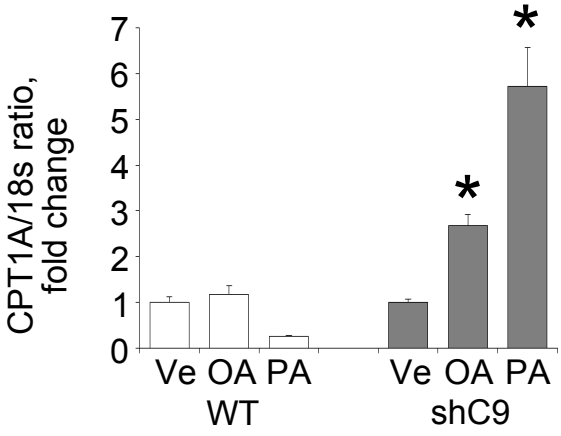
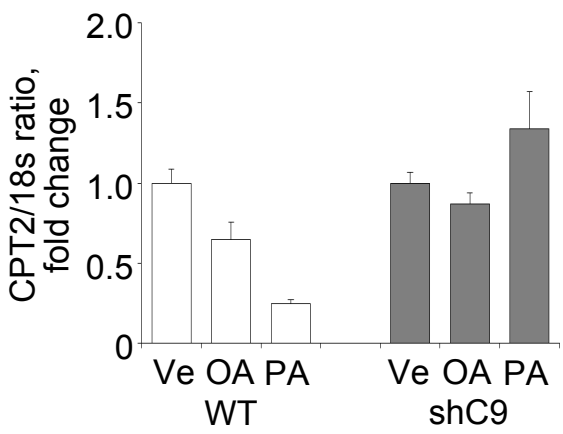
1.0
0.5
0
Ve OA PA
WT
Ve OA PA
shC9


CPT1A/18s ratio, fold change
7
6
5
4
3
2
1
0
*
*
Ve OA PA
WT
Ve OA PA
shC9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1.0
0.5
0
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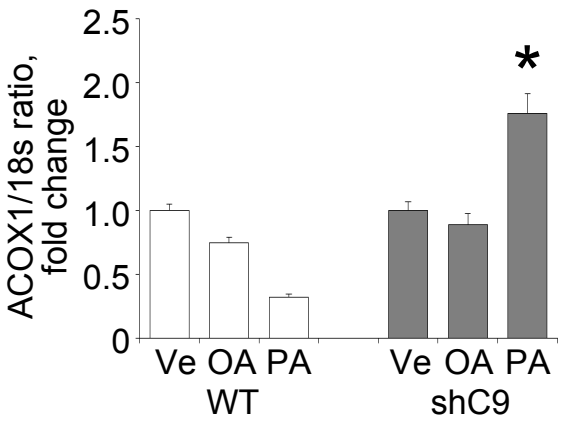
WT
Ve OA PA
shC9


ACOX1/18s ratio, fold change
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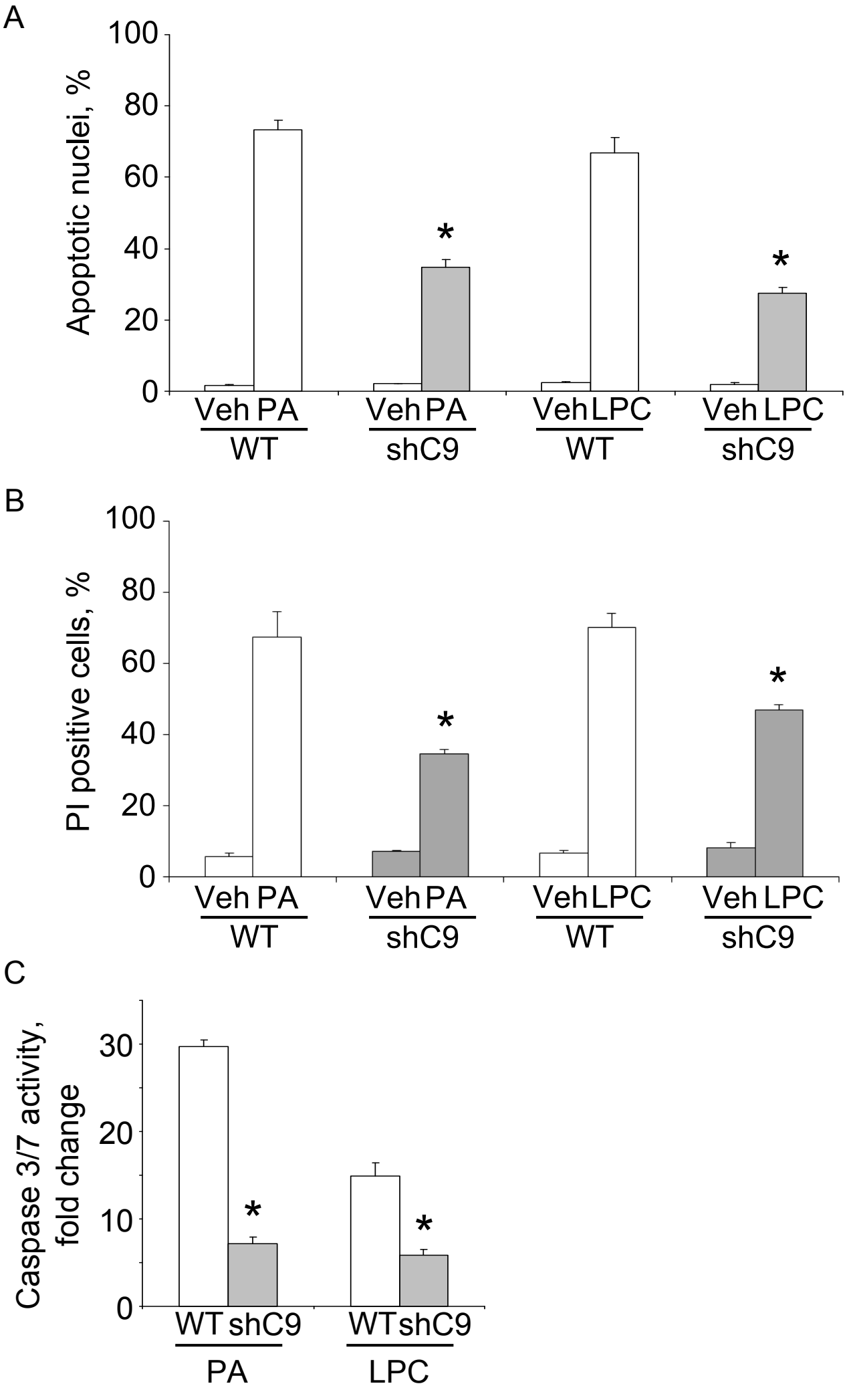
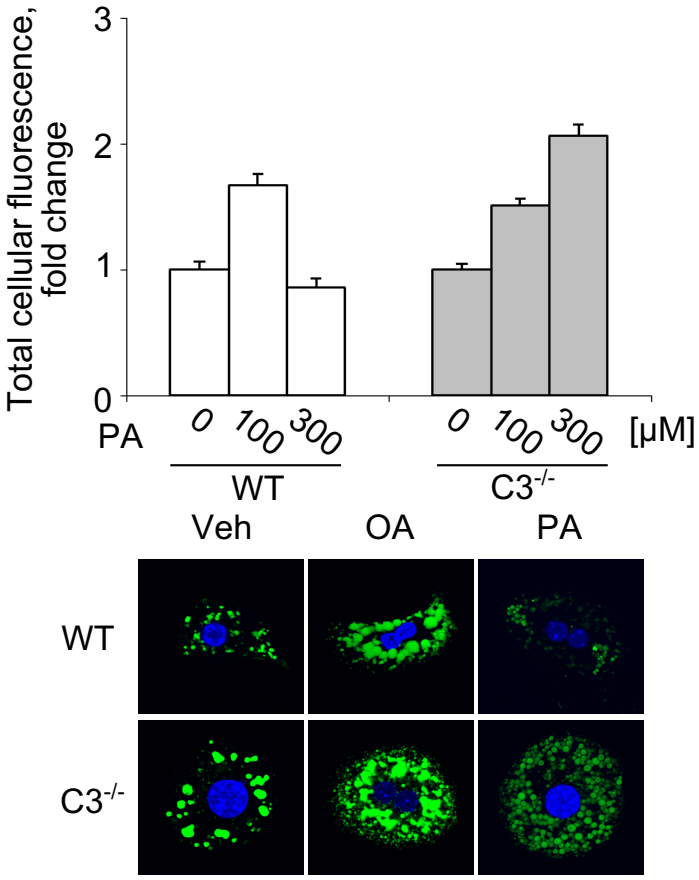
1.5
1.0
0.5
0
*
Ve OA PA
WT
Ve OA PA
shC9

Suppl. Fig. 2

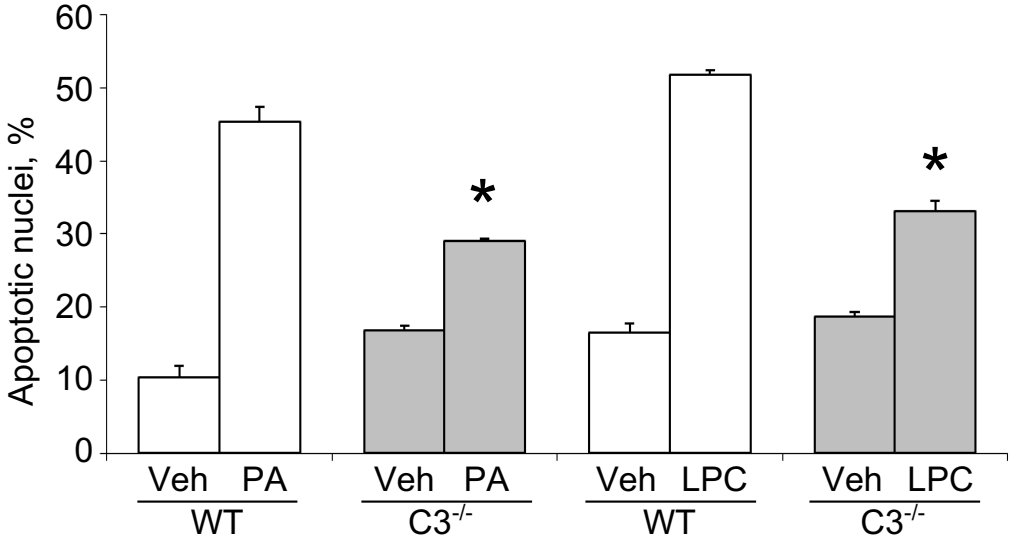

A
Apoptotic nuclei, %
100
80
60
40
20
0
*
*
Veh PA
Veh PA
Veh LPC
Veh LPC
WT
shC9
WT
shC9
B
PI positive cells, %
100
80
60
40
20
0
*
*
Veh PA
Veh PA
Veh LPC
Veh LPC
WT
shC9
WT
shC9
C
Caspase 3/7 activity, fold change
30
20
10
0
*
*
WT shC9
WT shC9
PA
LPC

Suppl. Fig.3

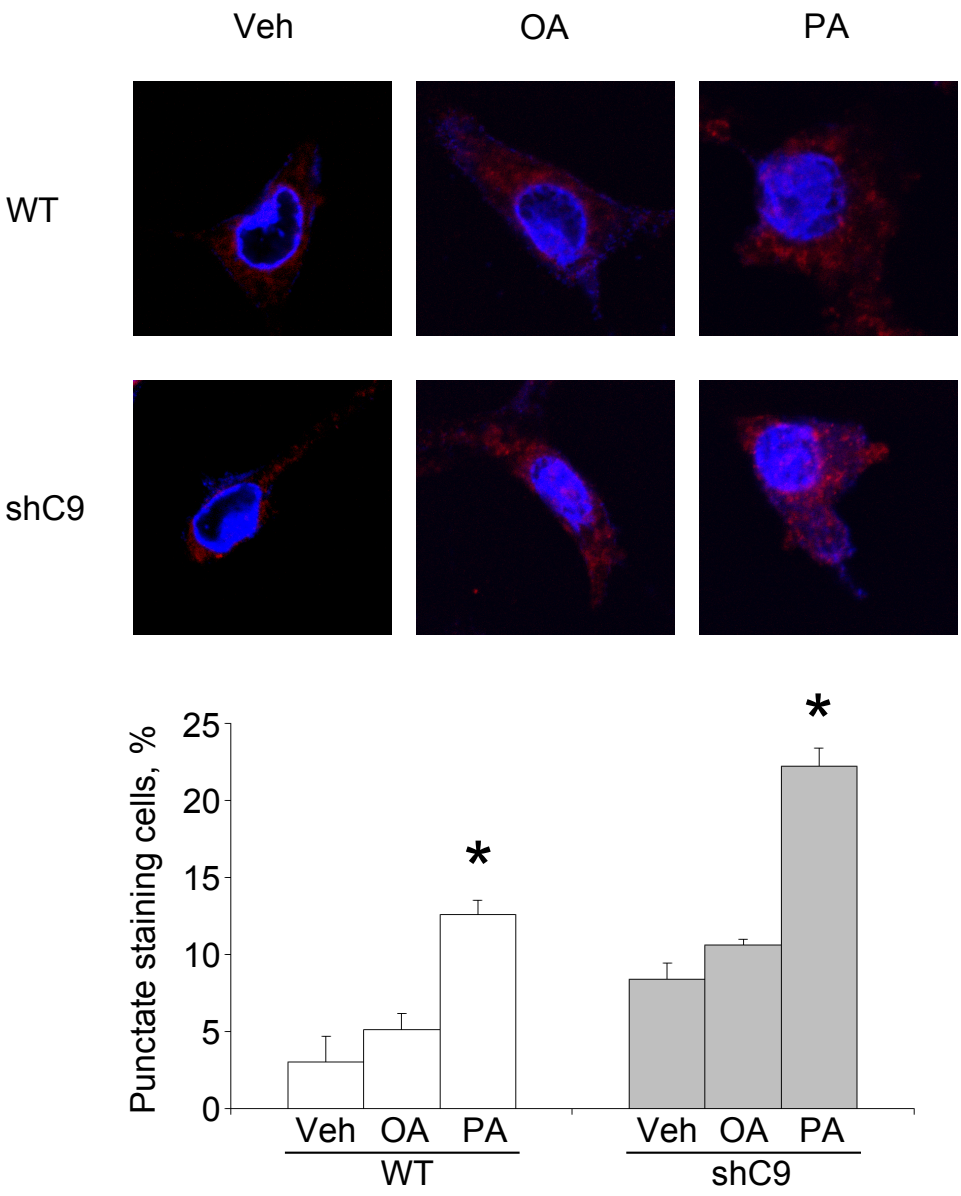
A



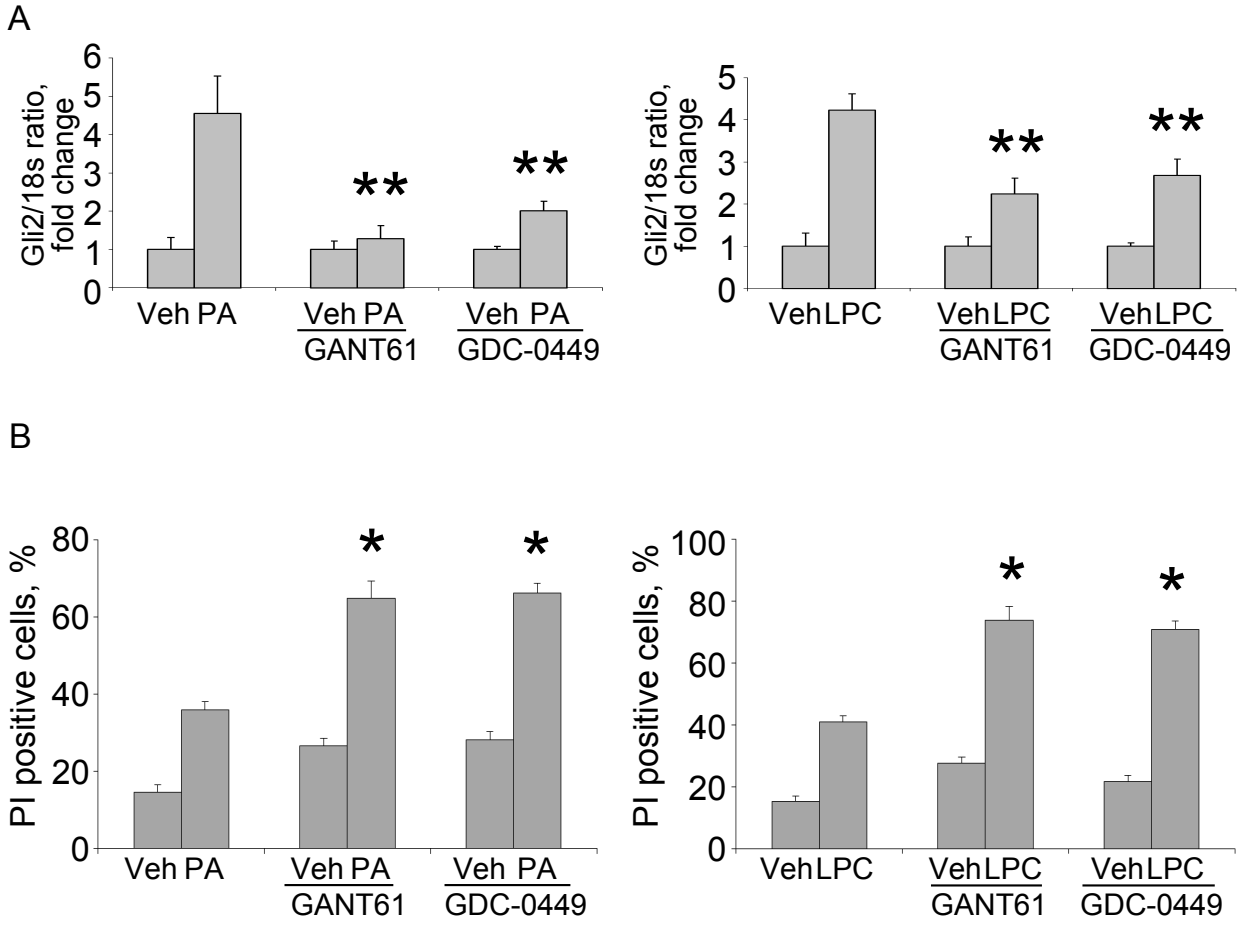
B



Suppl. Fig.4


Veh
OA
PA
WT
shC9
Punctate staining cells, %
25
20
15
10
5
0
*
*
Veh
OA
PA
WT
Veh
OA
PA
shC9

Suppl. Fig.5


A
Gli2/18s ratio, fold change
6
5
4
3
2
1
0
**
**
Veh PA
Veh PA
GANT61
Veh PA
GDC-0449
Gli2/18s ratio, fold change
5
4
3
2
1
0
**
**
VehLPC
VehLPC
GANT61
VehLPC
GDC-0449
B
PI positive cells, %
80
60
40
20
0
*
*
Veh PA
Veh PA
GANT61
Veh PA
GDC-0449
PI positive cells, %
100
80
60
40
20
0
*
*
VehLPC
VehLPC
GANT61
VehLPC
GDC-0449