ATP-citrate lyase (ACLY)-knockdown induces growth arrest and apoptosis through different cell- and environment-dependent mechanisms

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Abbreviations: ACLY, ATP-Citrate Lyase; FASN, Fatty-acid Synthase; ACACA, acetyl-CoA-carboxylase; ACSS2, Acyl-CoA synthetase short-chain family member 2; LR, Lipid reduced; FA, Fatty acid.

Keywords: ATP-Citrate Lyase; Tumour metabolism; *In vivo* fatty-acid synthesis, cholesterol synthesis.

Running Title: ATP-citrate lyase induces growth-arrest in cancer cells.

Disclosure of Potential Conflicts of Interest

There are no potential conflicts of interest.

Abstract

ATP-citrate lyase (ACLY) is a cytosolic enzyme that catalyzes generation of acetyl-CoA, that is a vital building block for fatty-acid, cholesterol and isoprenoid biosynthesis. ACLY is up-regulated in several types of cancer and its inhibition induces proliferation arrest in certain cancer cells. As ACLY is involved in several pathways its down-regulation may affect multiple processes. Here, we have shown that shRNA mediated ACLY-silencing in cell lines derived from different types of cancers induces proliferation/cell cycle arrest and apoptosis. However, this anti-proliferative effect of ACLY-knockdown was observed only when cells were cultivated under lipid-reduced growth conditions. Proliferation arrest induced by ACLY-silencing was partially rescued by supplementing the media with fatty acids and/or cholesterol. This indicates that the ACLY-knockdown-mediated growth arrest might be the result of either fatty acid or cholesterol starvation or both. In the absence of ACLY the cancer cells displayed elevated expression of SREBP-regulated downstream genes involved in *de novo* fatty acid and cholesterol biosynthesis. Furthermore, ACLY-suppression resulted in elevated expression of acyl-CoA synthetase short-chain family member 2 (ACSS2), an enzyme that also produces acetyl-CoA using acetate as a substrate. Acetate supplementation partially rescued the cancer cells from ACLY-suppression-induced proliferation arrest. We also observed that the absence of ACLY enhanced ACSS2-dependent lipid synthesis. These findings provide new insights into the role of ACLY in cancer cell growth and give critical information regarding the effects of ACLY-silencing on different pathways. This information is crucial in understanding the possible application of ACLY inhibition in cancer therapeutics.

Introduction

ATP-citrate lyase (ACLY) is a cytosolic enzyme that connects glucose/glutamine metabolism to *de novo* lipid synthesis (1-5). ACLY converts citrate into acetyl CoA, a precursor for fatty acid and mevalonate synthesis pathways (6-8). Both of these pathways are associated with cancer cell growth and transformation (9-11) .

In the fatty acid synthesis pathway, acetyl-CoA is carboxylated to malonyl-CoA by acetyl-CoA-carboxylase (ACACA). Both acetyl-CoA and malonyl-CoA are then used in a condensation reaction by the main lipogenic enzyme fatty acid synthase (FASN) to produce long-chain fatty acids (FA) (9) **(figure 1).** The FA-synthesis pathway is up-regulated in many cancer types. This up-regulated FA-synthesis fuels membrane biogenesis in rapidly proliferating cancer cells and renders membrane lipids more saturated (12). This affects fundamental cellular processes including signal transduction, gene expression, ciliogenesis, and therapy response (12-14).

Another pathway that uses acetyl CoA as a substrate is the mevalonate pathway. As shown in **figure 1** this pathway leads to the synthesis of farnesyl pyrophosphate (FPP), which is the branch point for several pathways leading to various end-products. One of these end-products is cholesterol. *De novo* cholesterol synthesis is also shown to be elevated in cancer cells (10). Cholesterol is reported to be required for the growth of cancer cells (10, 15). Additionally, FPP can also be converted into geranylgeranyl pyrophosphate (GGPP) (16). Both FPP and GGPP are respectively involved in farnesylation and geranylgeranylation of a variety of proteins **(figure 1)** (16). Farnesylation and geranylgeranylation are required for the ability of Ras and Rho proteins to induce malignant transformation, invasion, and metastasis (11, 17). Hence, ACLY is involved in multiple pathways that are known to be relevant in several types of cancers.

Distinctive elevation of ACLY expression and activity has been reported in lung, prostate, bladder, breast, liver, stomach, and colon tumors (18-24). Moreover, inhibition of ACLY by either RNAi or pharmacological inhibitors results in growth-arrest in tumor cells, both *in vitro* and *in vivo* (5, 18, 25, 26). However, the exact mechanism by which this growth arrest is implemented is not completely understood.

Lipogenesis is a highly coordinated process and perturbation at different steps of the lipogenic pathway can bring about major changes in expression and activity of other metabolic genes. Therefore, for therapeutic exploitation of this pathway it is very important to understand the effects of blocking one metabolic enzyme on expression and function of other metabolic genes. Additionally, in drug discovery it

is essential to consider the alternate mechanisms/pathways that may compensate for the inhibition of any enzyme/pathway.

It is known that ACLY is not the only source of acetyl CoA production in mammalian cells. The cytosolic enzyme acyl-CoA synthetase short-chain family member 2 (ACSS2) also produces acetyl-CoA using acetate as a substrate (27) **(figure 1).** Previous studies have speculated that in the absence of ACLY, ACSS2 becomes more relevant (5). It was also suggested that ACSS2 may help the cells in escaping ACLY-silencing induced effects provided its substrate is available (5, 28). However, the effect of ACLY-knockdown on the expression of ACSS2 has not been studied in detail.

Here, we investigated the effects of shRNA mediated ACLY-silencing in cell lines derived from different types of cancer. Our data clearly shows that ACLY-suppression induced proliferation arrest in multiple cancer cell lines. However, this effect was mainly observed when cancer cells were cultivated under lipid-reduced (LR) growth conditions. We observed that supplementation of oleic acid or cholesterol partially rescued the cancer cells from anti-proliferative effects induced by ACLY-knockdown. It indicates that the anti-proliferative effects of ACLY-silencing are mediated, through fatty-acid or cholesterol synthesis pathways. It was also observed that ACLY-suppression induces up-regulation of the genes involved in both of these pathways which could be a compensatory mechanism adopted by the cancer cells to overcome ACLY-deficiency. We found that upon ACLY-silencing the expression level of ACSS2 was markedly enhanced. Additionally, acetate supplementation partially rescued the cancer cells from ACLY-knockdown induced growth arrest, by increasing ACSS2-dependent lipid synthesis. Collectively, our data provide new understanding of the involvement of ACLY in cancer cell growth and it demonstrates the impact of ACLY-silencing on different pathways that are cross-linked through ACLY.

Materials and Methods

Cell lines and treatments

All the cell lines were obtained from the American Type Culture Collection. All the cell lines were authenticated. Farnesyl pyrophosphate (FPP), geranylgeranyl-pyrophosphate (GGPP), oleic acid and cholesterol were purchased from Sigma. Simvastatin was purchased from Merck Sharp. Soraphen A was received from Dr. R. Jansen, Helmholtz-Zentrum f. Infektionsforschung, Mikrobielle Wirkstoffe, Braunschweig (29, 30). siRNAs were purchased from Ambion. For Lipid reduced conditions the media were supplemented with HyClone lipid-reduced FBS (Thermo scientific).

Cloning of shRNA cassettes into an inducible expression vector

The TRC has developed a tightly regulated inducible shRNA expression system based on the lac operon, using IPTG (isopropyl-β-D-thio-galactoside) as inducer. The optimal IPTG concentration needed for maximal induction was found to be between 0.5 and 1.0 mM for most cell lines (manuscript in preparation). The inducible vector is available through the consortium (vector number TRC904) or at Sigma-Aldrich under the name pLKO-puro-IPTG-1xLacO. The sequences for the hairpins were selected from the TRC library and cloned into TRC904. For ACLY silencing CGTGAGAGCAATTCGAGATTA (shACLY-17) and CACCGAGTG-AAGTCGATAAAC (shACLY-86) were used. As a control a non-targeting TRC904: CAACAAGAT-GAAGAGCACCAA (shMock) was utilized recombinant lentiviruses were produced by the transfection of 293FT cells.

Lentiviral transduction and selection

Cells were plated in 24-well plates. Next day cells were infected at a 1:25 dilution of virus in the presence of 8 μg/ml polybrene. Virus-containing medium was then replaced by normal growth medium. 24 hours after infection, puromycin selection was started to obtain stable cell populations.

Immunoblotting analysis

Following antibodies were used for immunoblots ACLY (monoclonal rabbit, ab40793, Abcam), ß-Actin (monoclonal mouse, Sigma), SREBP-1 (Active Motif, monoclonal mouse, 39940) or SREBP2 (Active Motif, monoclonal mouse, 39942).

Proliferation assay

The cell lines transduced with IPTG-inducible ACLY-shRNA (shACLY) constructs were cultivated for 48 hours in normal medium ± 0.5 mM IPTG (Sigma Cat. No. I6758). The cells were seeded in normal or lipid-reduced medium ± IPTG. Growth curves were constructed by imaging plates using the *Incucyte system* (Essen Instruments).

RNA isolation and Real-time quantitative PCR (RTQ-PCR)

Total RNA from cultured cells was extracted and 3 µg of total RNA served as template for cDNA synthesis using Oligo dT primers and Superscript III reverse transcriptase. RTQ-PCR was performed on an ABI Prism 7900-HT Sequence Detection System (Applied Biosystems) using a qPCR core kit w/o dUTP (Eurogentec). Validated pre-designed Taqman Gene Expression Assays (Applied Biosystems) corresponding to the housekeeping genes TFRC (Hs00951083_m1) and PGK1 (Hs00943178_g1) were used to generate standard curves on serial dilutions of cDNA. After normalization by TFRC or PGK1 the relative expression values were calculated. The other Taqman Gene Expression assays used in this study are: ACLY (Hs00982738_m1), ACSS2 (Hs00218766_m1), FASN (Hs01005622_m1), ACACA (Hs01046047_m1), HMGCR (Hs00168352_m1).

Examination of Cell Cycle Regulation by Flow Cytometry

Corresponding cell lines were pre-induced for 48 hours with IPTG in normal growth conditions to ensure silencing of ACLY. The cells were then seeded in lipid-reduced growth conditions and were incubated for 24-hours ±IPTG. Cell cycle analysis was performed by use of the cell cycle kit according to the manufacturer's protocol (Guava Technologies).

Apoptosis assay

Corresponding cell lines were pre-induced for 48 hours with IPTG in normal growth conditions to ensure silencing of ACLY. The cells were then seeded in lipid-reduced growth conditions and were incubated for 72-hours ± IPTG. Apoptosis was assessed by using the *Guava Nexin* kit and the *Guava PCA system* (Guava Technologies) according to the manufacturer's protocol.

siRNA transfections

Cells were transfected with individual siRNA duplexes at a final concentration of 25 nM using HiPerFect (Qiagen) transfection reagent, following the manufacturer's reverse-transfection protocol.

Lipid synthesis

PC3M cells were seeded in 24-well plates and incubated for 72 hours. 2 μCi/ml of [1-^{14}C] acetate (36 μM final concentration) was added to the cells, and cells were incubated at 37°C for 2 hours. Lipids were extracted using a methanol/chloroform method. Lipids were dissolved in 500 μl hexane. Samples were counted for ^{14}C using a *Tri-Carb liquid scintillation counter* (PerkinElmer).

Statistical analysis

The results were analyzed by Students't test where applicable. P values <0.05 were considered statistically significant. The data presented represent means ± S.D as indicated in the corresponding figure legends.

Results

ACLY knockdown induces proliferation arrest by affecting cell cycle progression and induction of apoptosis in various cancer cell lines

To assess the effects of ACLY knockdown (KD) on tumor cells, we generated two independent expression constructs containing IPTG-inducible short hairpin RNAs (shRNAs) against ACLY (shACLY-17 and 86) as described in materials and methods. PC3M, HOP62 and HepG2 cell lines were transduced with these constructs. QPCR analysis revealed that IPTG induction (48hrs) in PC3M-shACLY-17 and 86 cells reduced ACLY-expression by 5 and 6 fold respectively **(Figure 2a)**. This ACLY-silencing was confirmed by Western blot analysis performed after 72 hours of IPTG induction **(Figure 2b)**. There was negligible effect on ACLY-expression on mRNA and protein level in PC3M-shMock control cell line **(Figure 2a-b)**. IPTG-induction in HepG2 and HOP62 derived shACLY-17 cell lines also resulted in decrease in expression of ACLY on mRNA and protein levels (**supplementary figure 1-2**).

The shRNA-mediated silencing of ACLY showed no effects on growth rates of PC3M- shACLY-17 and 86 cells when cultivated under normal growth conditions (**Figure 2c, supplementary figure 3a-c**). However, these cell lines exhibited reduced growth rates upon ACLY-suppression in lipid-reduced (LR) growth conditions (**Figure 2c, supplementary figure 3b-c**). IPTG-induction of mock shRNA in PC3M-shMock cells did not display any effect on proliferation in either normal or LR growth conditions **(Figure 2c, supplementary figure 3a and 3d**). The observed reduction in growth rates induced by ACLY-KD was not limited to PC3M derived cell lines. Similar effects were also observed in shACLY-17 cell lines derived from HepG2 and HOP62 cells **(Figure 3)** in LR-growth conditions. HOP62-shACLY-17 cells also displayed reduced rate of proliferation upon ACLY-silencing in normal growth conditions **(Figure 3).** However, these effects were much more enhanced in LR-conditions and were followed by cell death after 48 hours of ACLY-silencing in HOP62-shACLY-17 cells **(Figure 3)**.

It has been previously reported that ACLY deficiency induces apoptosis in cancer cells (31). To quantify the percentage of apoptotic cells in ACLY-KD cell population, we performed annexin-V and 7-amino actinomycin D [7-AAD] staining. It was observed that in LR-growth conditions ACLY down-regulation induces apoptosis in PC3M-derived shACLY cells, increasing the number of cells in early and late apoptotic stages (**Figure 4a**). Similar trends in induction of apoptosis were observed upon ACLY-silencing in shACLY cells derived from HOP62 and HepG2 cells **(supplementary figure 4)**. The induction of apoptosis was observed after 72 hours of ACLY-silencing.

The effects of ACLY-knockdown on cell cycle progression were also investigated. ACLY-silencing resulted in a decrease in the percentage of cells in the S phase accompanied by an increase in the percentage of cells in G0/G1 phase (**Figure 4b**). The cell cycle arrest was observed after 24 hours of ACLY-knockdown.

ACLY knockdown induces proliferation arrest in cancer cells by targeting multiple downstream pathways

As ACLY is involved in FA and mevalonate synthesis pathways hence, its knockdown may affect multiple pathways. Therefore, to elucidate which pathways is most affected by ACLY silencing, different cell populations undergoing ACLY-silencing induced proliferation arrest, were exposed to fatty-acids (oleic acid), cholesterol or the isoprenoid intermediates; farnesyl-pyrophosphate (FPP)/ geranylgeranyl-pyrophosphate (GGPP).

Exposure to exogenous cholesterol partially rescued PC3M-shACLY-17 cells from proliferation/cell cycle arrest and apoptosis induced by ACLY-knockdown **(Figure 5a-c)**. Oleic acid also slightly improved the proliferation rate and helped the cells escape from induction of apoptosis **(Figure 5a-c)**. FPP supplementation did not recover the cells from anti-proliferative effects of ACLY-suppression in PC3M-shACLY-17 cells **(Figure 5a-c)**. GGPP was also ineffective **(supplementary figure 5).** Treatment of PC3M- shACLY-17 cells with oleic acid (100 μM), cholesterol, GGPP or FPP (20 μM) in absence of IPTG has negligible effect on proliferation **(supplementary figure 6).**

Interestingly, different cell lines depict different rescue profiles. In contrast to PC3M cells, HepG2-shACLY-17 cells completely recovered from ACLY-silencing induced growth arrest by oleic acid supplementation whereas cholesterol supplementation had only a slight effect **(Figure 5a)**. It suggests that fatty acid supplementation is more effective in HepG2-shACLY-17 cells.

Oleic acid or cholesterol supplementation prevented cell death that follows ACLY-KD after 48 hours in HOP62 cells but did not reverse the proliferation arrest induced by ACLY-silencing **(Figure 5a)**. The effect of cholesterol supplementation on cell-viability is more long-lasting than that of oleic acid supplementation in HOP62- shACLY-17 cells.

These results confirm our hypothesis that ACLY silencing affects both the cholesterol and the FA-synthesis pathways. Though, the extent to which a certain pathway is affected may vary among different cell lines.

Next we sought to determine the rescue potential of oleic acid and cholesterol when their respective pathways were specifically blocked. Soraphen A is a highly potent inhibitor of ACACA, hence it blocks *de novo* lipid synthesis and induces apoptosis in cancer cells (32). The effects of soraphen A are largely reversed by supplementation of medium with fatty acids (32). In line with these findings, treatment of

PC3M cells with soraphen A resulted in cell death **(figure 5d),** and these effects were counteracted by exogenous oleic acid. **(figure 5d).** As expected cholesterol addition had no effect on soraphen A induced cell death in PC3M cells **(supplementary figure 7).** Specific blockade of the mevalonate pathway by statin, namely simvastatin also induced cell death in PC3M cells that was rescued by incubation of cells with cholesterol **(figure 5e)** but was unaffected by oleic acid supplementation **(supplementary figure 8)**. It shows that the blockade of other lipogenic enzymes lying downstream of ACLY in cholesterol or FA-synthesis pathways may affect their respective pathways.

ACLY-silencing influences the expression profile of downstream lipogenic genes regulated by sterol regulatory element binding proteins (SREBPs)

We examined the effect of ACLY-silencing on expression levels of downstream lipogenic genes in different cancer cells cultivated in LR-growth conditions. We found that upon ACLY-suppression the expression of FASN that is one of the main players in the fatty-acid synthesis pathway was significantly increased by 1.4, 1.3 and 1.3-folds in PC3M, HepG2 and HOP62 cells respectively **(Figure 6a)**.

The expression profile of HMGCR that is the rate-controlling enzyme of mevalonate pathway is also affected by ACLY depletion. We found that shRNA mediated silencing of ACLY significantly enhanced the expression of HMGCR by 1.4-fold in PC3M- shACLY-17 cells and by 2-folds in HepG2 and HOP62-derived cells **(Figure 6b).**

Expression of FASN and HMGCR is regulated by common family of transcription factors designated as sterol regulatory element binding proteins (SREBPs) (33-35). To evaluate the involvement of SREBPs in ACLY-silencing induced up-regulation of these genes we examined the protein expression of mature nuclear form of SREBP-1 and 2 that indicates activation of these proteins. We observed that ACLY-silencing resulted in increase in mature nuclear form of SREBP-1 and 2 proteins in PC3M, HepG2 and HOP-62 derived cell lines **(Figure 6c)**.

Acyl-CoA synthetase short-chain family member 2 (ACSS2) another enzyme that is known to be up-regulated in ACLY-deficient hepatocytes (36) is also regulated by SREBPs (37). We examined the expression of ACSS2 in cancer cells in which ACLY was knocked down. It was observed that upon ACLY-silencing ACSS2 expression was also significantly increased by 1.4-fold in PC3M- shACLY-17 cells and by 2.8-folds in HepG2 and HOP62-derived cells **(Figure 6d)**.

In ACLY-deficient cancer cells ACSS2 may compensate for the loss of ACLY

The enhanced expression of ACSS2 upon ACLY-silencing suggests that in case of ACLY-deficiency ACSS2 becomes more relevant. It has been recently reported that in the absence of physiologic ACLY-levels, incubation of cells with supraphysiologic levels of acetate, the substrate used by ACSS2, rescues

cells from proliferation arrest or decreased histone-acetylation caused by ACLY-knockdown (28, 31). We observed that acetate supplementation clearly reverses the growth arrest induced by shRNA-dependent ACLY-silencing in PC3M-derived cells (**figure 7a-b**).

To further evaluate the dependence of cancer cells on either of these two pathways we studied the effect of silencing of ACLY, ACSS2 or both on cell viability. In contrast to ACLY silencing that caused significant induction of apoptosis in PC3M cells, ACSS2-knockdown by two independent siRNAs had less of an effect on cell viability. However, co-inhibition of ACLY and ACSS2 caused a dramatic increase in the number of cells in early apoptotic cell fraction (**figure 7b)**. It shows that co-inhibition of the two pathways known to be involved in cytosolic acetyl CoA production in cancer cells (5, 27, 38, 39) is highly cytotoxic for the cells.

To confirm the effect of ACLY-KD on acetate-dependent lipid biosynthesis, cells were exposed to stable isotope labeled acetate and its incorporation into cellular lipids was measured. As expected acetate-dependent lipid synthesis was significantly increased in PC3M upon ACLY-silencing **(Figure 7c)**.

Discussion

The fact that ACLY is up-regulated in several types of cancer cells and that upon inhibition of ACLY, cancer cells undergo proliferation arrest indicate that this enzyme plays an important role in cancer cell progression (5, 18). ACLY converts citrate into acetyl-CoA (6-8) that is an essential substrate for fatty acids synthesis pathway and mevalonate pathway that leads to cholesterol biosynthesis and isoprenylation of various proteins. Hence, ACLY affects several pathways and consequently its suppression will also influence multiple downstream pathways.

In the present study we investigated the effects of shRNA mediated ACLY-silencing in different cell lines derived from different types of tumors. These cell lines were selected on the basis of previous reports that indicated over-expression or increased-activity of ACLY in these cell lines (5, 18) and also on our preliminary studies using ACLY-siRNA transfections. We generated IPTG-inducible ACLY-shRNA clones from the selected cell lines and examined the effects of ACLY-silencing in these clones. Our data clearly indicates that ACLY-knockdown induces growth arrest in PC3M (prostate cancer-derived), HepG2 (liver cancer-derived) and HOP62 (lung cancer-derived) cell lines cultivated in lipid reduced (LR)-growth conditions. However, this proliferation-arrest induced by ACLY-suppression was not observed when these cells were grown under normal growth conditions. This may reflect the importance of exogenous lipids for cancer cell growth. It can be speculated that in LR-growth conditions the cancer cells are more reliant on *de novo* lipid synthesis. Hence, silencing of ACLY that catalyses generation of acetyl CoA, a precursor for lipid-synthesis pathways, has anti-proliferative effects in LR-growth conditions. Additionally, we observed that ACLY-knockdown induced proliferation arrest is mediated by defective cell cycle progression and induction of apoptosis. This effect extends the previous findings by other research groups (5, 18).

ACLY is involved in several pathways hence, the tumoricidal effects that occur after ACLY-blockade may relate to the cellular starvation of the end-products of any of these aforementioned pathways. In one of the previous studies it was reported that fatty acid supplementation does not rescue lung cancer-derived A549 cells from the proliferation-arrest mediated by ACLY knockdown (18). We observed that exposure to exogenous oleic acid, a monosaturated fatty acid, completely rescues HepG2 cells from ACLY-silencing mediated growth arrest. However, in PC3M and HOP62 cells oleic acid supplementation does not completely reverses but partially suppress these anti-proliferative effects induced by ACLY deficiency.

Recently, it was reported that the anti-tumor effects of ACLY inhibition are dramatically enhanced in combination with statins, the cholesterol lowering drugs that inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) (31). It was speculated that this effect might be due to cholesterol starvation that may interfere with cell growth *via* the impairment of cell membrane synthesis. To study

whether the anti-proliferative effects of ACLY-suppression were mediated by cholesterol starvation we supplemented the cells undergoing growth arrest after ACLY-silencing with cholesterol. Cholesterol supplementation markedly rescued PC3M and HOP62 cells from ACLY-knockdown mediated growth arrest. Whereas, HepG2 cells showed only slight effect on cholesterol administration. It shows that multiple downstream pathways could be affected by ACLY-suppression in different cell lines. However, the extent to which certain pathway is affected varies from one cell line to another. The underlying mechanisms which regulate these effects require further elucidation.

Blockade of *de novo* fatty acid synthesis by inhibiting ACACA, which is one of the key players in this pathway, induces apoptosis in cancer cells. But this effect could be rescued completely by fatty acid supplementation (32). We also observed that cell death induced by soraphen A that is a highly potent inhibitor of ACACA could be rescued by oleic acid supplementation. Cholesterol supplementation has no effect on the cells undergoing apoptosis due to ACACA-blockade. On the other hand inhibition of mevalonate pathway by simvastatin could be rescued by cholesterol but not by fatty acid supplementation. However, as we discussed earlier the anti-proliferative effects of ACLY could be reverted back by cholesterol and/or oleic acid supplementation depending on the cell line. Indicating that these effects are mediated by FA-synthesis or/and mevalonate pathways.

Perturbation at different steps of the lipogenic pathway could bring about major changes in expression profile of other metabolic genes (36). Here, we found that the expression of FASN, a key player in fatty acid pathways, was up- regulated upon ACLY-silencing in different cancer cell lines. Additionally, the expression of HMGCR that is the rate limiting enzyme in mevalonate pathway is also significantly enhanced after ACLY-knockdown. These transcriptional effects indicate that ACLY-deficiency compels the cells to up-regulate the expression of downstream genes in FA- or cholesterol synthesis pathways to compensate for the loss of ACLY.

Fatty acid and cholesterol biosynthesis pathways are influenced by a single family of transcription factors namely SREBPs (33). Specific analyses of individual isoforms suggest that SREBP-1 may be selectively involved in activation of genes involved in fatty acid synthesis such as FASN and ACACA (34). SREBP-2 is more selective for genes involved in cholesterol homeostasis such as HMGCR (34, 35). SREBP proteins (125 kDa) are anchored to the endoplasmic reticulum (ER) membrane (33). Through proteolytic cleavage, the activated amino-terminus (68 kDa) of SREBP translocates into the nucleus to bind SRE (sterol regulatory element) *cis*-acting elements and trigger gene expression (40). SRE is found in the promoter regions of genes encoding enzymes for fatty acid and cholesterol biosynthesis (41). We observed that upon ACLY knockdown there was an increase in the expression of mature nuclear form of SREBP-1 and 2 proteins in PC3M, HepG2 and HOP62 derived cells. This indicates that ACLY-induced upregulation of FASN and HMGCR may also be controlled by SREBPs.

The up-regulation of downstream metabolic genes after ACLY-silencing may partially compensate for the loss of acetyl CoA production by ACLY. However, in order to keep fatty acid and mevalonate synthesis pathways running it is essential to have at least some supply of the precursor acetyl CoA. Mammalian cells also express another enzyme namely acyl-CoA synthetase short-chain family member 2 (ACSS2) that catalyses synthesis of acetyl-CoA from acetate (**figure 1)**. However, mammalian cells mainly use glucose as their major carbon source and have exposure to only low-concentration of extracellular acetate. Moreover, conversion of acetate to acetyl-CoA is an energy dependent process, while ACLY-dependent production of acetyl-CoA from glucose is an energy producing reaction. It makes ACLY-dependent acetyl CoA production pathway more preferable in mammalian cells but, the absence of ACLY may derive the cells towards ACSS2-dependent pathway. Our data clearly shows that the expression of ACSS2 is highly elevated upon ACLY-suppression in all the cell lines tested. We further clarified the importance of ACSS2 upon ACLY silencing by exposing the ACLY-deficient cells to acetate. As expected the anti-proliferative effects of ACLY-knockdown were partially recovered by acetate supplementation. The effects ACLY-silencing on the activity of acetate-dependent *de novo* lipid synthesis was evaluated by determination of ^{14}C-acetate into newly synthesized cellular lipids. As expected upon ACLY-silencing PC3M cells showed up-regulated acetate-dependent *de novo* lipid synthesis.

In the present study we demonstrated that ACLY-silencing clearly induces proliferation arrest and apoptosis in variety of cancer cell lines by affecting multiple downstream pathways. Our data clearly shows that ACLY-knockdown also influences the expression of other lipogenic genes. This may indicate the attempts made by the cancer cells to counterbalance ACLY-deficiency. Additionally, this work also shows the importance of ACSS2 in the absence of ACLY. The present study provides a new outlook on the role of ACLY in cancer cell growth. Lipogenesis is an extremely synchronized mechanism and perturbation at different steps of this pathway cause alterations in expression and activity of related enzymes. Our study indicates that therapeutic targeting of ACLY may not be enough for cancer treatment. However, combinatorial approaches targeting several metabolic enzymes simultaneously may have more beneficial effects. Low-lipid diet may have also have synergistic effects together with treatments targeting lipogenesis.

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Figure Legends:

Figure 1: ATP-citrate lyase at the crossroads of several pathways. Citrate is converted by the action of ATP-citrate lyase (ACLY) to acetyl-CoA that is used as a precursor in fatty acids synthesis and mevalonate pathways. Under conditions of low cellular glucose uptake, an alternative pathway for the generation of cytosolic acetyl-CoA works through the activation of acetate by acetyl-CoA synthetase 2 (ACSS2).

Figure 2: ACLY-silencing induces decreased growth rates in PC3M cells in lipid-reduced growth conditions. Two independent IPTG-inducible expression vectors were constructed for conditional expression of shRNA targeting ACLY and stably integrated into the genome of PC3M cells. After transduction the independent clones (shACLY-17 and 86) as well as the clone transfected with mock shRNA were cultivated ± IPTG (0.5mM). **(a)** ACLY expression at mRNA level was analyzed by RTQ-PCR and normalized to PGK1 (mean ± S.D) (**b)** ACLY expression at protein level was analyzed by Western blot analysis **(c)** Proliferation curves for PC3M- shMock, shACLY-17 and 86 clones. Cells were cultivated ± IPTG (0.5mM) and cell proliferation was monitored by *Incucyte real-time imaging.*

Figure 3: ACLY-silencing induces decreased growth rates in HepG2 and HOP62 cells in lipid-reduced growth conditions. Proliferation curves for HOP62 and HepG2-derived shACLY-17 cell lines. Cells were cultivated ± IPTG (0.5mM) and cell proliferation was monitored.

Figure 4: ACLY-silencing induces cell cycle arrest and apoptosis in PC3M cells in lipid-reduced growth conditions. (a) Induction of apoptosis in PC3M-derived cells after 72 hours of ACLY-silencing (n=3) *P<0.01. Symbol of statistical significance/Non-significance (*/N.S) refers to the comparison of Late apoptotic/Dead cells. The viable cell population was also significantly different between IPTG-induced/un-induced PC3M- shACLY clones. **(b)** Cell-cycle phase distribution in PC3M-derived cells after 24 hours of ACLY-silencing (n=3) *P<0.01. Symbol of statistical significance/Non-significance (*/N.S) refers to the comparison of S phases. G0/G1 phase distribution was also significantly different between IPTG-induced/un-induced PC3M-shACLY clones.

Figure 5: Effect of oleic acid, cholesterol or isoprenoid intermediates availability on anti-proliferative effects of ACLY-silencing. Cells were cultivated ± IPTG (0.5 mM) in medium supplemented with or without oleic acid (100 µM), cholesterol (1X) or farnesyl-pyrophosphate (FPP) (20 µM). **(a)** Proliferation was monitored for shACLY-17 clones derived from PC3M, HePG2 and HOP62.

(b) Induction of apoptosis was determined in PC3M-shACLY-17 cells. (n=3) *P<0.01. Symbol of statistical significance or Non-significance (*/N.S) refers to the comparison of Late apoptotic/Dead cells **(c)** Cell Cycle Regulation was examined in PC3M-shACLY-17 cells. (n=3) *P<0.01. Symbol of statistical significance or Non-significance (*/N.S) refers to the comparison of S phases. **(d)** PC3M-shACLY-17 cells were treated with soraphen (100 nM) or DMSO with or without oleic acid (100 μM). **(e)** PC3M-shACLY-17 cells were treated with simvastatin (0.5 μM) or DMSO with or without cholesterol (1X).

Figure 6: ACLY-silencing increases mRNA expression of FASN, HMGCR and ACSS2 in different cell lines. Gene expression of **(a)** FASN **(b)** HMGCR after IPTG induction (48 hr) was analyzed by RTQ-PCR and normalized to TFRC (mean ± S.D). **(c)** SREBP-1/SREBP-2expression at protein level was analyzed by Western blot analysis. **(d)** Gene expression of ACSS2 after IPTG induction (48 hr) was analyzed by RTQ-PCR and normalized to TFRC (mean ± S.D).

Figure 7: In absence of ACLY the ACSS2-dependent pathway may compensate for the loss of ACLY. (a) PC3M-shACLY-17 cells with or without IPTG-induced ACLY-knockdown were supplemented with sodium acetate (1 mM). Proliferation rate was compared with the untreated cells. (b) Induction of apoptosis was determined in PC3M- shACLY-17 cells after silencing of ACLY, ACSS2 or co-silencing of both ACLY and ACSS2. ACLY silencing was achieved by IPTG induction. ACSS2 was silenced by using either of the two independent siRNAs. (c) PC3M-shACLY-17 cells with or without IPTG-induced ACLY-knockdown were incubated with ^{14}C-acetate, cellular lipids were extracted and radioactivity was determined by liquid scintillation counting. (n=3) *P<0.01.

Figure 1

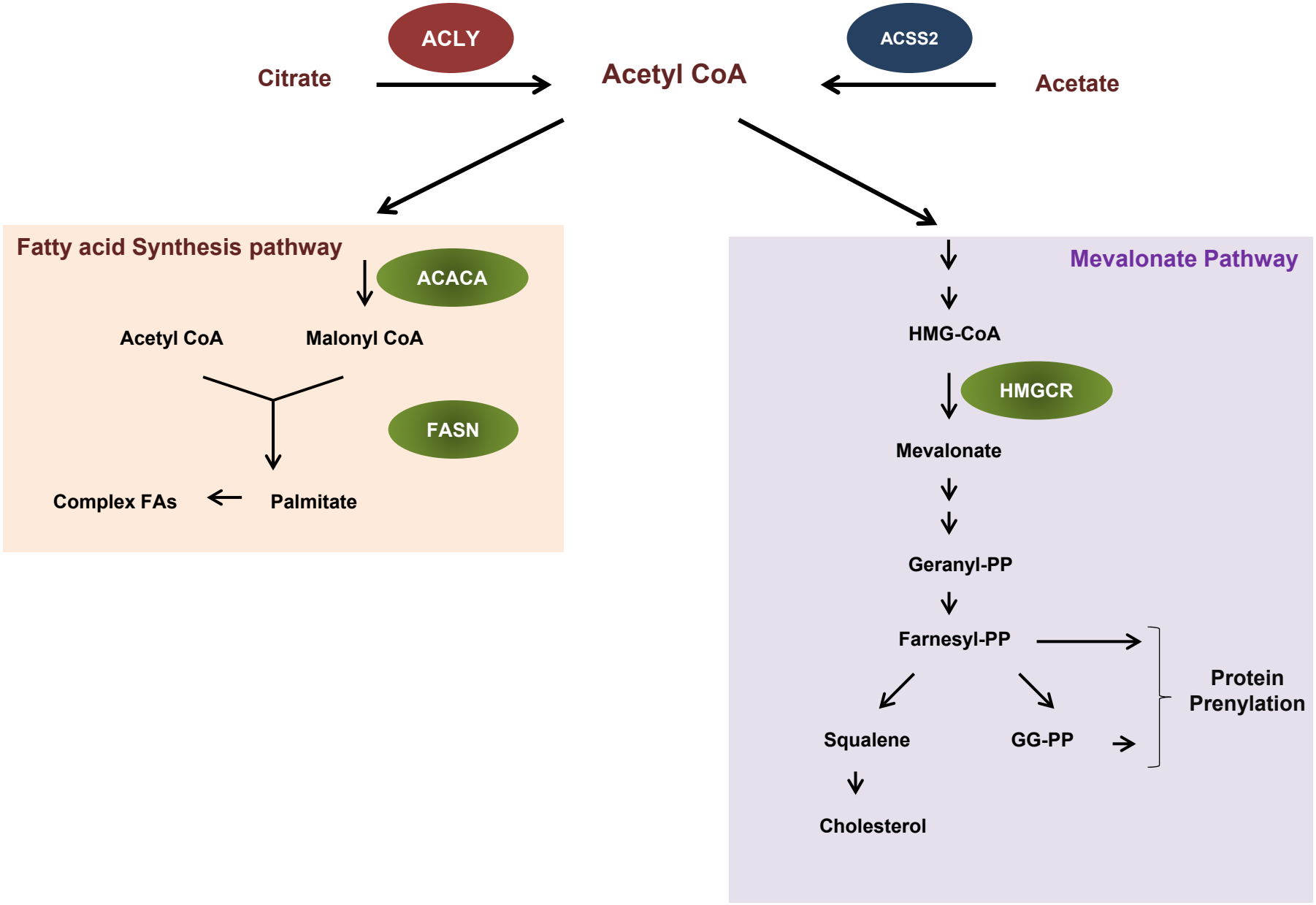
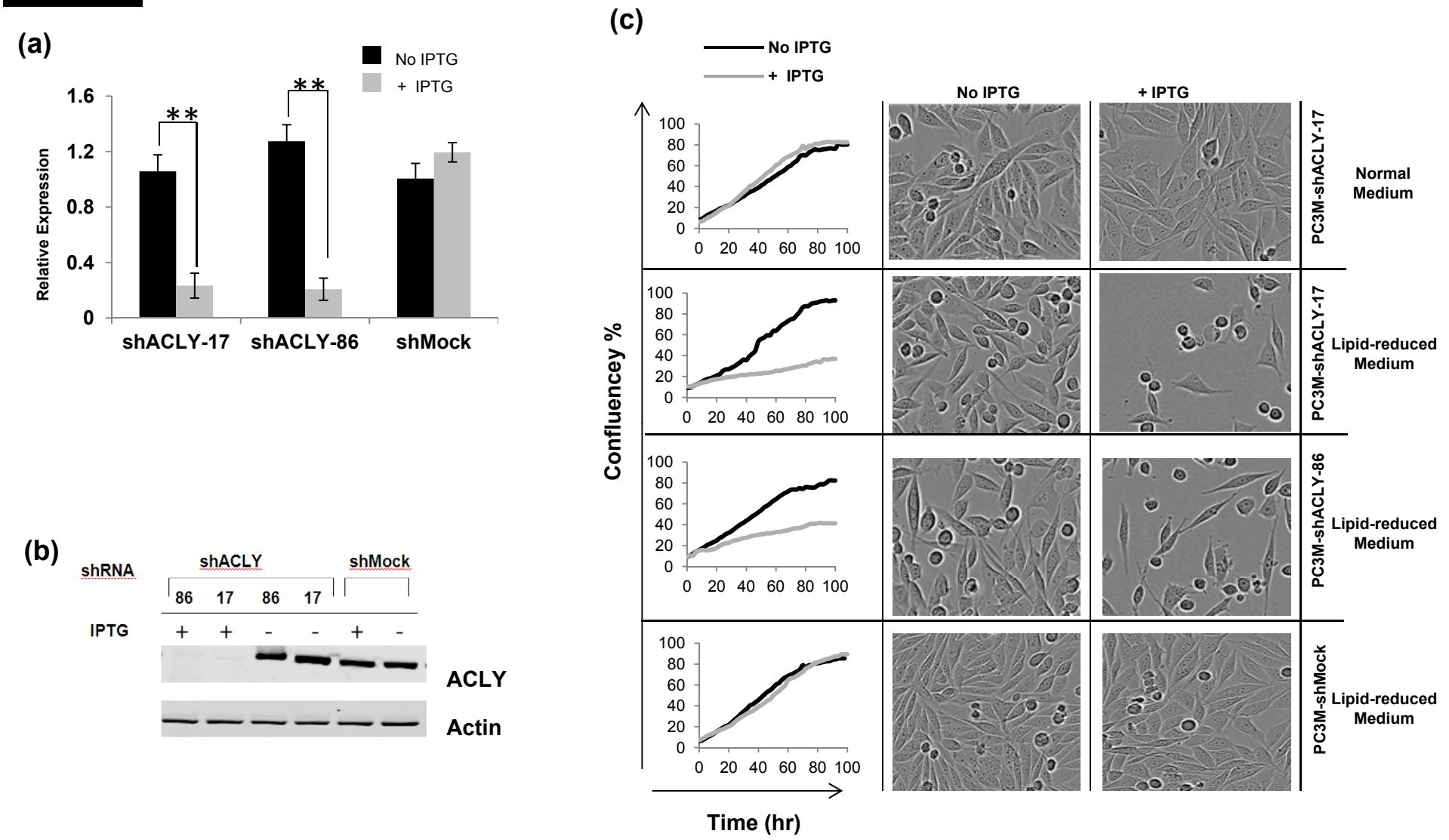


Figure 2



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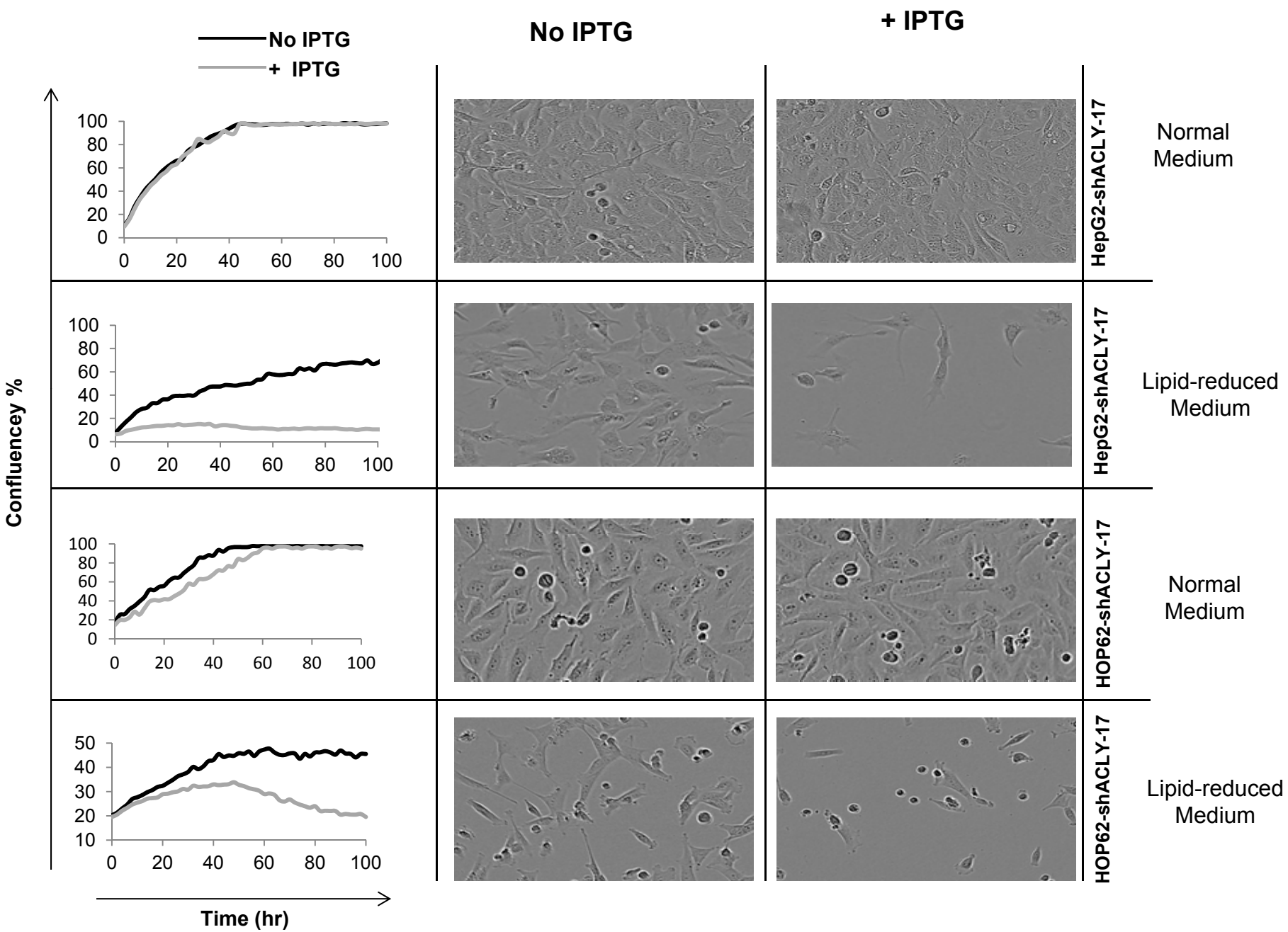

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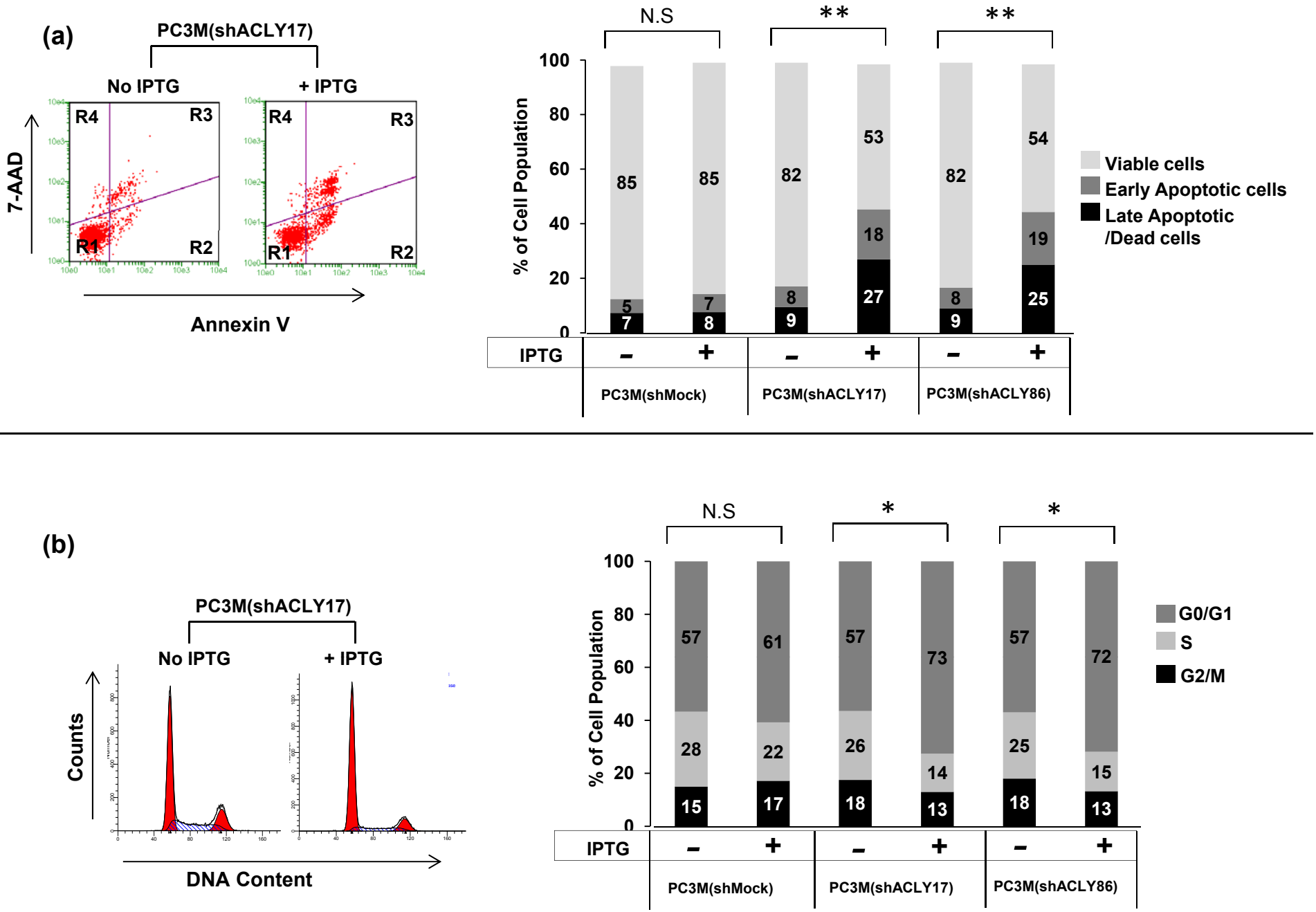

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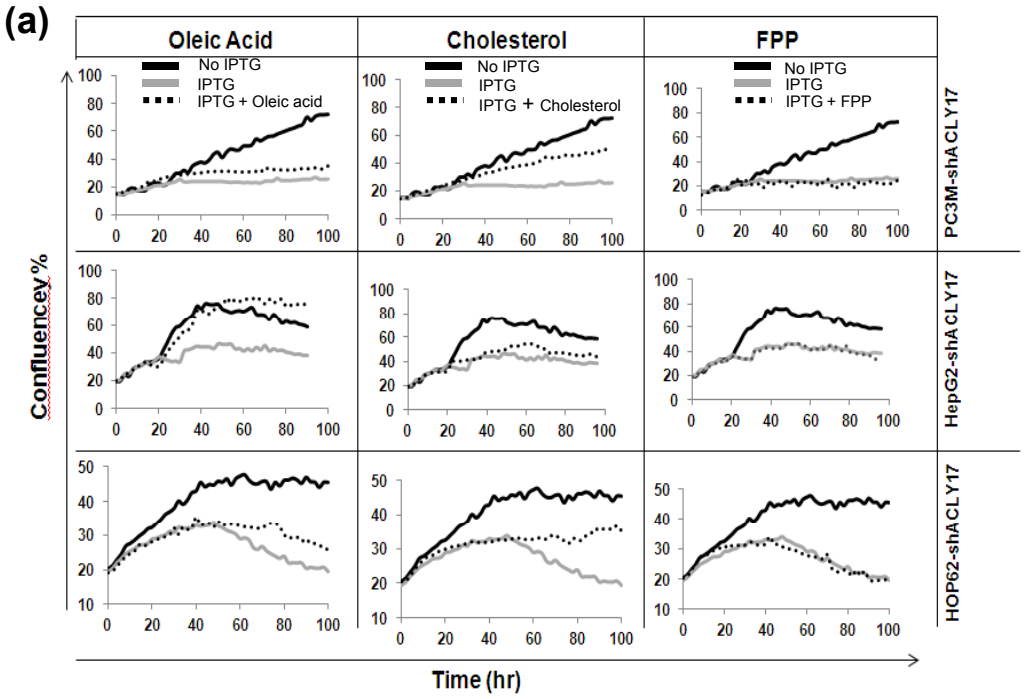
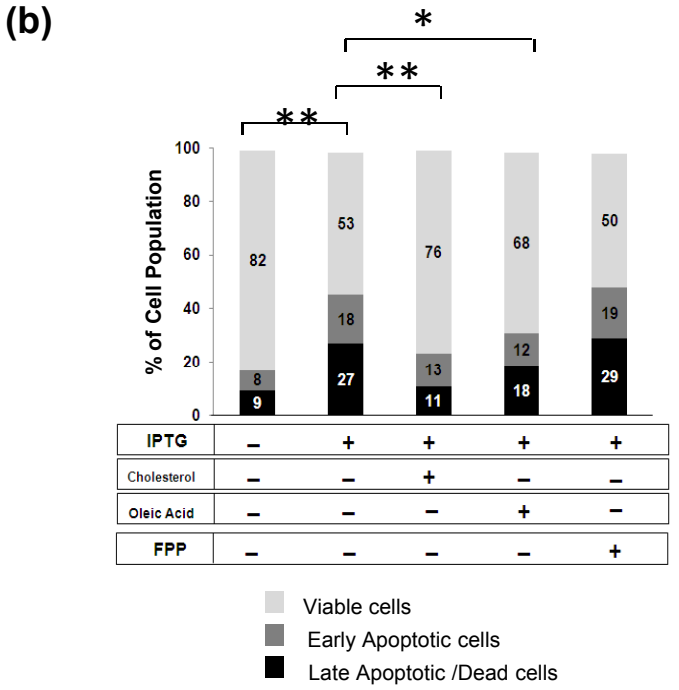
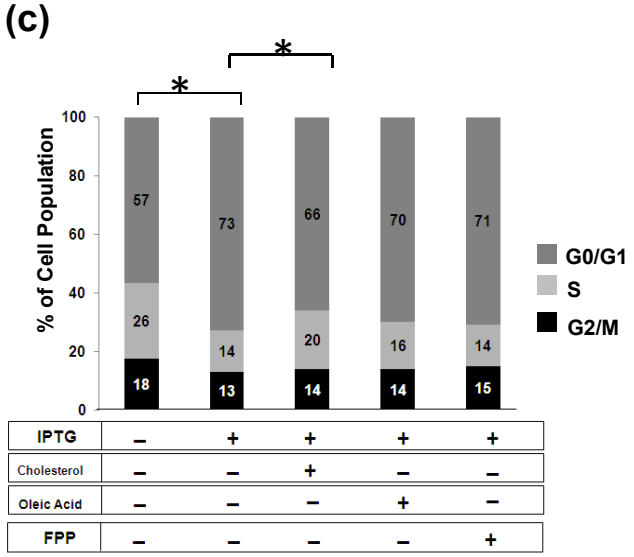
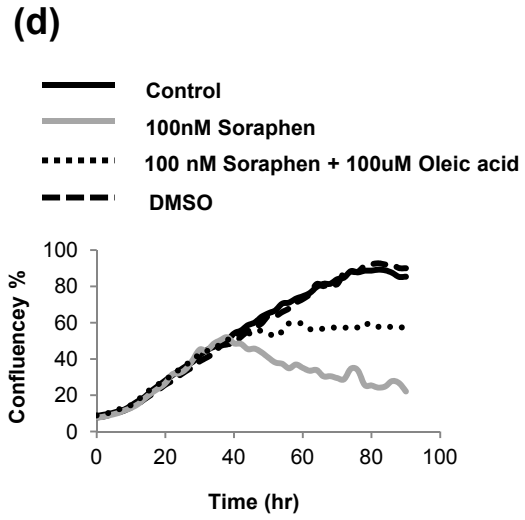








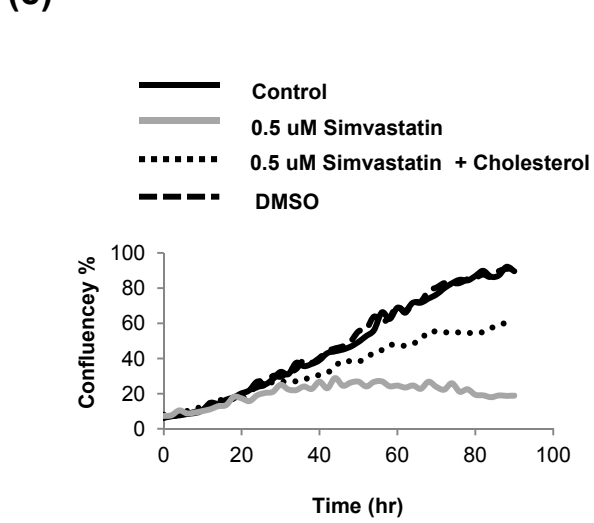
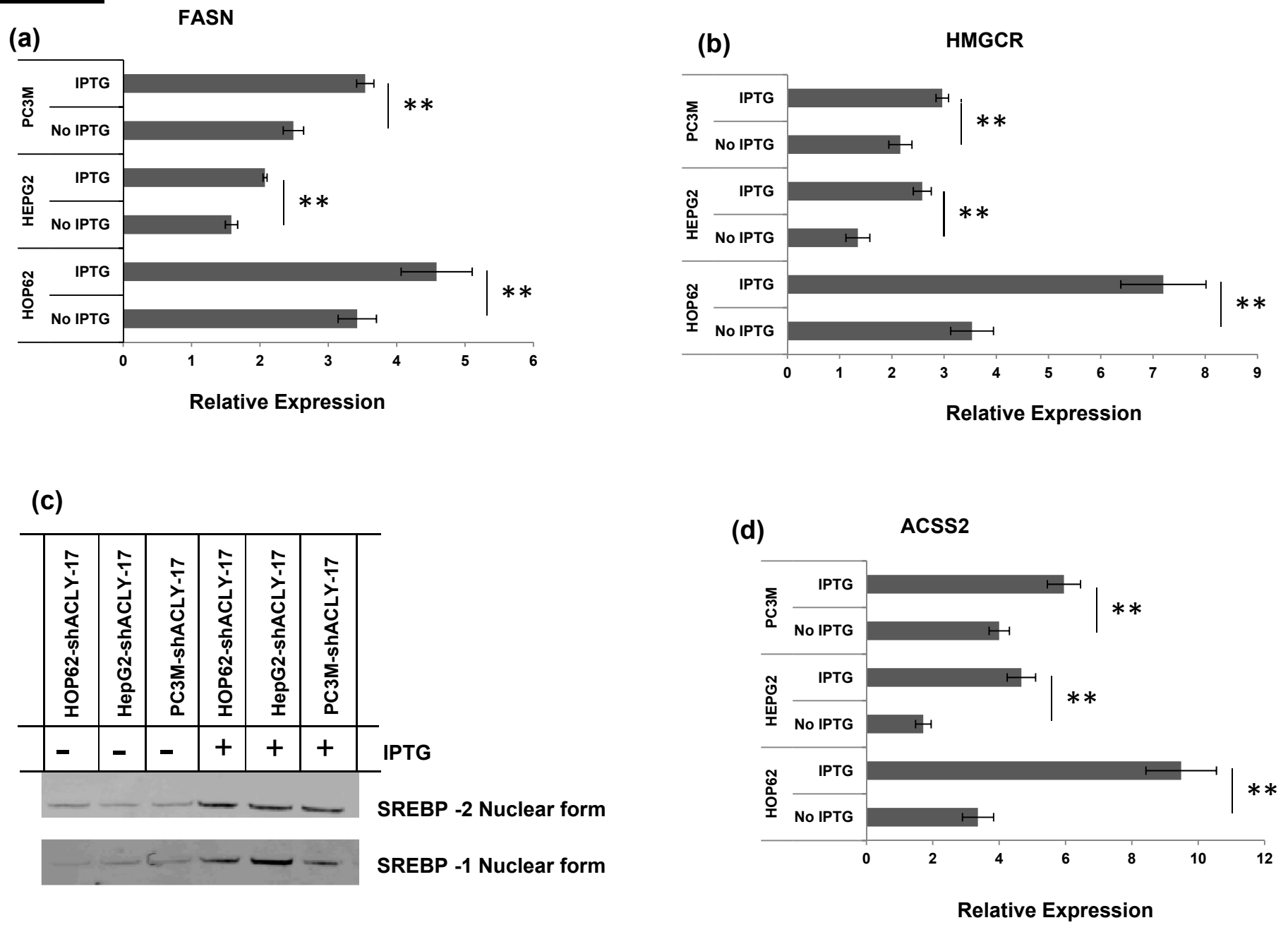


Figure 6



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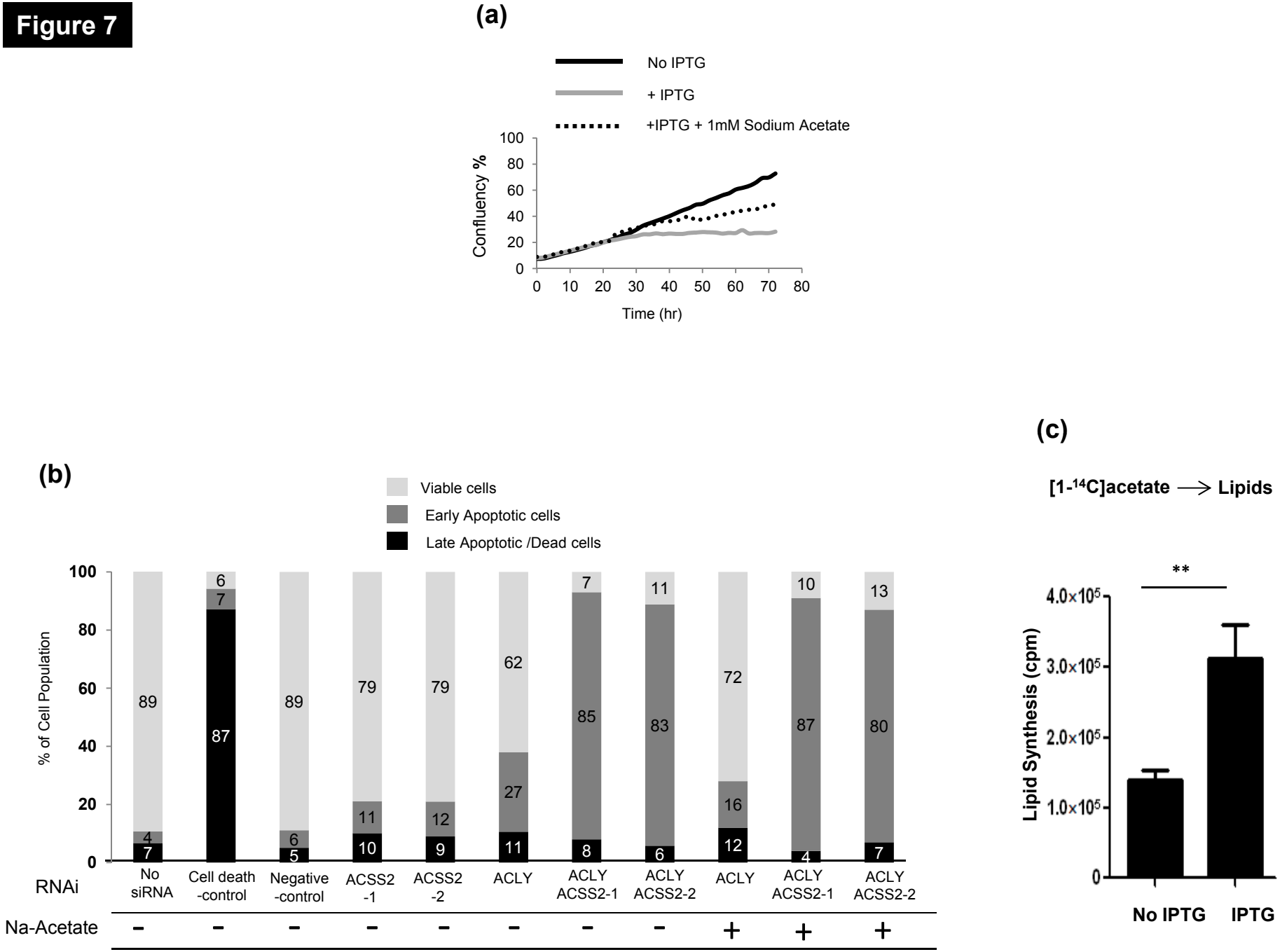

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