Enhanced acyl-CoA: cholesterol acyltransferase activity increases cholesterol levels on the lipid droplet surface and impairs adipocyte function

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Running title: Cholesterol esterification in adipocytes

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ABSTRACT

Cholesterol plays essential structural and signaling roles in mammalian cells, but too much cholesterol can cause cytotoxicity. Acyl-CoA: cholesterol acyltransferases 1 and 2 (ACAT1/2) convert cholesterol into its storage form, cholesteryl esters, regulating a key step in cellular cholesterol homeostasis. Adipose tissue can store > 50% of whole-body cholesterol. Interestingly, however, almost no ACAT activity is present in adipose tissue and most adipose cholesterol is stored in its free form. We therefore hypothesized that increased cholesterol esterification may have detrimental effects on adipose tissue function. Here, using several approaches, including protein overexpression, quantitative RT-PCR, immunofluorescence, and various biochemical assays, we found that ACAT1 expression is significantly increased in the adipose tissue of the ob/ob mice. We further demonstrated that ACAT1/2 overexpression partially inhibited the differentiation of 3T3-L1 preadipocytes. In mature adipocytes, increased ACAT activity reduced the size of lipid droplets (LDs) and inhibited lipolysis and insulin signaling. Paradoxically, the amount of free cholesterol increased on the surface of LDs in ACAT1/2 overexpressing adipocytes, accompanied by increased LD localization of caveolin-1. Moreover, cholesterol depletion in adipocytes by treating the cells with cholesterol-deficient media or β-cyclodextrins induced changes in cholesterol distribution that were similar to those caused by ACAT1/2 overexpression. Our results suggest that ACAT1/2 overexpression increases the level of free cholesterol on the LD surface, thereby impeding adipocyte function. These findings provide detailed insights into the role of free cholesterol in LD and adipocyte function and suggest that ACAT inhibitors have potential utility for managing disorders associated with extreme obesity.
Free cholesterol is a key component of mammalian cell membranes and plays essential structural and signaling roles in mammalian cells. However, too much free cholesterol can cause cellular toxicity (1). Thus, an elaborate system exists to maintain cellular cholesterol homeostasis. Key to this regulation is the SREBP/SCAP machinery, which senses excess cellular free cholesterol and suppresses the expression of the enzymes for cholesterol synthesis, including 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR), as well as the low-density lipoprotein receptor for cholesterol uptake (2-5). Excess cholesterol can also trigger the degradation of enzymes for cholesterol synthesis (6,7). Another crucial biochemical reaction that maintains a proper level of free cholesterol in mammalian cells is cholesterol esterification, that is the synthesis of cholesteryl esters through the formation of an ester linkage between the 3β-OH moiety in free cholesterol and the carboxyl group of a long chain fatty acyl coenzyme A. This reaction is mediated by the enzyme acyl-CoA: cholesterol acyltransferase (ACAT) (also known as sterol O-acyltransferase; SOAT) (8). When in excess, free cholesterol is converted into cholesteryl esters and stored in lipid droplets (LDs), which are dynamic cellular organelles comprising a neutral lipid core enclosed by a phospholipid monolayer (9,10). Triacylglycerols (TAGs) and cholesteryl esters are two major storage neutral lipids that make up the core of LDs (8,11). Also, cholesteryl esters can be packaged as part of the neutral lipid core within plasma lipoproteins for lipid transport purposes (12).

There are two acyl-CoA: cholesterol acyltransferases (ACAT1 and 2) in mammals, encoded by the ACAT1 and ACAT2 genes, respectively. ACAT1 and 2 are closely related enzymes which are responsible for all intracellular cholesterol esterification (13-15). Both enzymes are membrane-spanning proteins located in the endoplasmic reticulum (ER) (8). ACAT1 is ubiquitously expressed in many cell types, while ACAT2 is only expressed in the cells secreting apolipoprotein B (apoB)-containing lipoproteins, such as the liver and intestine (16-18). It has been shown that cholesterol esterification catalyzed by ACAT2 is essential to cholesterol absorption in the intestine (19). Adipose tissue is the major storage site of excess calories in the form of TAGs in mammals (20). It also contains the largest pool of free cholesterol in the body (21,22). In obese subjects, over 50% of total body cholesterol resides in the adipose tissue (21). We have recently demonstrated a major contribution of adipose tissue to the storage of whole-body cholesterol (23). Due to a lack of ACAT enzyme activity in white adipose tissue, nearly all cholesterol (> 95%) exists in its free form (24). Most free cholesterol in adipocytes is believed to localize to the plasma membrane, where there are abundant caveolae, small flask-shaped invaginations that are highly enriched in free cholesterol (25,26). The activity of de novo cholesterol biosynthesis in adipocytes is fairly low (21). Therefore, much of the adipocyte cholesterol is obtained through the uptake of circulating lipoproteins.

Given that a large quantity of cholesterol is stored in adipose tissue, the absence of ACAT expression and activity in adipose tissue is intriguing. As mentioned above, excess free cholesterol is toxic and the best way to handle excess cholesterol is to convert it into cholesteryl esters, which can be stored in the core of LDs. What are the benefits, then, for adipocytes to maintain low ACAT expression and activity? In this study, we first detected significantly increased level of ACAT1 in the adipose tissue/adipocytes of...
ob/ob mice. We further investigated the effects of increased cholesterol esterification on adipocyte function and LD dynamics by overexpressing ACAT1/2 in pre- and mature adipocytes. Our data demonstrated that increased ACAT activity partially blocked adipogenesis, caused dramatic enrichment of free cholesterol on the LD surface, and impaired key functions of adipocytes including lipolysis and insulin signalling.

Results

ACAT1/2 overexpression in 3T3-L1 stable cell lines

Since normal adipose tissue maintains very low ACAT expression and activity, we suspect that increased ACAT expression/activity may exert adverse effects on adipose function and may also be associated with dysfunctional adipose tissue. Indeed, we found that the protein level of ACAT1 was ~7-fold higher in the adipose tissue of the ob/ob mice than that of the wild-type (WT) mice (Fig. 1A and B). Given that ob/ob adipose tissue has very high levels of macrophages and that macrophages are known to express ACAT1 (27), the detected ACAT1 on whole adipose tissue could be derived from adipose tissue macrophages. We therefore isolated adipocytes: ACAT1 was detected in isolated adipocytes from the ob/ob, but not WT mice (Fig. S1A). By contrast, the macrophage marker CD11b was detected only in the stromal vascular fraction, but not in isolated adipocytes. Thus, the increase in adipose tissue ACAT1 is at least in part due to increased ACAT1 in adipocytes of the ob/ob mice. Thus, it is important to determine if and how increased ACAT activity may impact adipocyte function. For this purpose, we generated stable cell lines (3T3-L1) overexpressing Flag-tagged ACAT1/2, an ACAT2 stabilizing mutant (ACAT2-C277A) (28), and their catalytic dead mutants (ACAT1-H460A, ACAT2-H360A, and ACAT2-H360A-C277A). For both WT and mutants of ACAT1 and ACAT2, the mRNA levels of ACAT1 or ACAT2 were increased by at least three orders of magnitude above endogenous levels in the empty vector (EV) control group (Fig. 1C). Accordingly, protein levels of ACAT1 and ACAT2 were also much higher in these overexpressing cell lines (Fig. 1D and E), with the level of the ACAT2 stable mutant, ACAT2-C277A, about 5-fold higher than the WT (Fig. S1B and C). In both 3T3-L1 preadipocytes (Fig. 1F) and differentiated 3T3-L1 adipocytes (Fig. 1G), total cholesteryl esters were significantly higher than that of control/EV cells when overexpressing ACAT1, ACAT2, and ACAT2-C277A, but not the catalytic dead mutants. The proportion of total cholesterol that was converted to cholesteryl esters was shown in Fig. 1H and I. Finally, the enzymatic activity of the ACATs was determined by a cholesterol esterification assay in which those stable cells were pulse-labelled with [14C]-oleic acid conjugated to bovine serum albumin (BSA). While WT ACAT1/2 and the ACAT2 stabilizing mutant (ACAT2-C277A) demonstrated significantly higher activities, the catalytic dead mutants appeared to be inactive (Fig. 1J and K). Therefore, our 3T3-L1 stable cell lines were shown to overexpress functional ACAT1/2 with the catalytic dead mutants serving as useful controls.

Increased ACAT activity impairs the differentiation of 3T3-L1 preadipocytes

The effects of ACAT1/2 on adipogenesis were investigated by differentiating 3T3-L1 preadipocytes into mature adipocytes using our stable cell lines. The mRNA levels of adipose-specific genes, such as aP2, PPARγ, and C/EBPα, are up-regulated in normal adipocytes while Prefl is
mainly expressed in preadipocytes (29-31). Thus, the expression of \(aP2\), \(PPAR_\gamma\), and \(C/EBP_\alpha\) was induced during differentiation (day 8 vs day 0), whereas that of \(Pref1\) was down-regulated (Fig. 2A). However, after 8 days of differentiation, overexpression of functional ACAT1/2 blunted the changes in these differentiation markers. Overexpression of ACAT2 and ACAT2-C277A decreased the extent of differentiation by \(~70\%\) (Fig. 2A and B), while the catalytic dead mutants had little or much weaker effects on differentiation (Fig. 2C). These results were reflected by reduced triglyceride accumulation as assessed by Oil Red O staining (Fig. 2D), with catalytic dead mutants serving as controls (Fig. 2E). Together, these data suggest that ACAT expression negatively impacts the differentiation of 3T3-L1 cells.

**ACAT1/2 overexpression impairs lipid droplet morphology in adipocytes**

Lipid droplets form during adipocyte differentiation/adipogenesis. We next examined the morphology of lipid droplets in mature adipocytes. ACAT1/2 overexpression, especially ACAT2, substantially reduced the LD size in adipocytes on day 8 after differentiation (Fig. 3A and C, S2A). The size of LDs also decreased in adipocytes on day 0 and 2, as well as in HeLa cell lines upon ACAT1/2 overexpression (Fig. S2B-D). Catalytic dead mutants of ACAT1 and ACAT2 did not affect the LD size (Fig. 3B and C). In order to exclude the possibility that the impaired LD size was related to the differentiation status, we additionally investigated the lipid droplet growth in mature adipocytes transduced by lentivirus carrying the \(ACAT1\) or \(ACAT2\) genes, respectively (Fig. 3D and E), and obtained consistent results that the presence of ACATs in mature adipocytes, especially ACAT2, reduced LD growth (Fig. 3F and G). Importantly, this effect can be reversed by inhibiting ACAT1 with Sandoz 58-035 and ACAT2 with Pyripyropene A (PPPA), respectively (Fig. H and I).

CIDE/Csp27 is a well-known LD-associated protein. Although very low in preadipocytes, the expression of CIDE/C is highly induced during differentiation to regulate lipid droplet dynamics in adipocytes (32-34). Hence, we examined the expression of CIDE/C in mature adipocytes when overexpressing ACAT1/2. Overexpression of ACAT1/2 dramatically decreased the CIDE/C expression in total cell lysates and LD fractions in mature adipocytes (Fig. 3J and K), which implied that increased ACAT activity in adipocytes may influence LD-associated proteins and disturb LD dynamics.

LDs are composed of a core of neutral lipids, including cholesteryl esters produced by ACAT1/2, and TAGs by DGAT1 and DGAT2 (11,35,36). The impaired LD size when overexpressing ACATs may also be related to TAG synthesis. Therefore, we examined TAG synthesis in adipocytes in the presence of ACAT1/2. The mRNA level of \(Dgat1\) or \(Dgat2\) was significantly reduced on day 8 of differentiation (Fig. 4A), with concomitant dramatically reduced TAG production (Fig. 4B). Also, we found consistent results in mature adipocytes transiently overexpressing ACAT1 or ACAT2 (Fig. 4C-E). Taken together, these data suggest that ACAT1/2 overexpression impairs LD expansion in adipocytes, possibly due to less LD fusion mediated by CIDE/C and down-regulated TAG synthesis.

**ACAT1/2 overexpression impairs lipolysis and insulin signaling in adipocytes**

The dramatic changes in LD morphology in adipocytes upon ACAT1/2 overexpression may impact adipocyte functions. First, we investigated lipolysis in
mature adipocytes transiently overexpressing ACAT1/2. ACAT1/2 overexpression almost abolished hormone-stimulated lipolysis in mature adipocytes (Fig. 5A). Perilipin is a key regulator for lipolysis and regulates lipases such as HSL and ATGL (37,38). Phosphorylated forms of HSL were clearly reduced in mature adipocytes transiently overexpressing ACAT1/2 (Fig. 5B). In addition to perilipin 1, perilipin 2 expression was also significantly reduced in mature adipocytes upon overexpressing ACAT2 (Fig. 5C and D). This effect was much stronger in stable cell lines, where ACAT1 overexpression also dramatically reduced perilipin 2 expression (Fig. 5E and F). No changes were detected in the expression of ATGL (Fig. 5C-F).

Insulin triggers the uptake of glucose, fatty acids, and amino acids in the liver, adipose tissue, and muscle, and promotes the storage of these nutrients in the form of glycogen, lipids, and protein respectively (39,40). Protein kinase B (AKT), a downstream target of insulin, is one of the important regulators during this process. ACAT1/2 overexpression substantially decreased the level of p-AKT (S473), with ACAT2 overexpression displaying a stronger effect (Fig. 5G and H). All these results strongly indicate that increased ACAT activity severely disrupts the metabolic functions of mature adipocytes.

**ACAT1/2 overexpression promotes free cholesterol accumulation on lipid droplets in adipocytes**

Next, we wanted to understand the molecular basis underlying the impact of ACAT expression on adipocytes. We focused on cholesterol, the substrate of ACAT enzymes and a key component of mammalian membranes. Strikingly, as revealed by filipin staining, there was appreciable free cholesterol accumulating on the LD surface instead of the plasma membrane when overexpressing ACAT1/2 in adipocytes on day 8 after differentiation, and ACAT2 overexpression showed stronger effects (Fig. 6A and B). Moreover, this phenotype was also seen in mature adipocytes transiently overexpressing ACAT1 or ACAT2, respectively (Fig. 6C and D). The biosynthesis of cholesterol is sensitive to the level of free sterols, the feedback of which elaborately regulates the endogenous synthesis and exogenous uptake of cholesterol. ACAT1/2 in adipocytes consumes free cholesterol, which may reduce the cholesterol sensing “pool” in the ER, thereby triggering the ER to keep producing cholesterol, leading to free cholesterol accumulation on lipid droplets. To test this hypothesis, two other approaches of depleting cholesterol were carried out in mature adipocytes: using hydroxypropyl-β-cyclodextrin (HPCD) and/or starvation medium (DMEM + 1% lipoprotein-deficient serum (LPDS)). The depletion of cholesterol enhanced the accumulation of free cholesterol on the LD surface (Fig. 7). Striking accumulation of free cholesterol on the LD surface could be observed even in control cells when depleting cholesterol using HPCD (Fig. 7A and C) or shutting down cholesterol uptake (LPDS medium) (Fig. 7B and C).

Caveolin-1 is a free cholesterol-binding protein and required for cholesterol homeostasis, maintenance, and distribution (41-45). Caveolin-1 colocalized with free cholesterol on the LD surface in mature adipocytes upon overexpression of ACAT1 and especially ACAT2 (Fig. 8A). The protein level of caveolin-1 in the presence of ACAT1/2 was increased either in total cell lysates or LD fractions isolated from mature adipocytes (Fig. 8B and C). In summary, our results showed that the overexpression of ACAT1/2 in 3T3-L1 adipocytes perturbs...
cholesterol transport and localization, promoting cholesterol accumulation on the 
LD surface.

**ACAT1/2 overexpression impairs cholesterol homeostasis in adipocytes**

We next sought to investigate cholesterol status and homeostasis in mature 
adipocytes when ACAT1/2 are overexpressed. The free cholesterol level was 
significantly increased in mature adipocytes that were differentiated from 3T3-L1 
preadipocytes stably overexpressing ACAT1/2 (Fig. 9A), as well as mature 
adipocytes transiently overexpressing ACAT1/2 (Fig. 9C) compared with control 
cells. Accordingly, increased free cholesterol was associated with the down-regulation of 
*Hmgcr* (Fig. 9B and D), an SREBP-target gene that is sensitive to changes in cellular 
cholesterol status. Consistent results were also found in preadipocytes stably 
overexpressing ACAT1/2 (Fig. 9E and F). While overexpression of WT ACAT1/2 or 
ACAT2 stable mutant increased free cholesterol levels, the catalytic dead mutants 
of ACAT1/2 had no effects (Fig. 9E and F). Furthermore, the increased free cholesterol 
could be reversed through inhibiting HMGCR activity by statin treatment (Fig. 9G 
and H). Together, these results indicate that ACAT overexpression perturbs cholesterol 
homeostasis in adipocytes.

**Discussion**

Adipose tissue can store more than 50% of whole-body cholesterol. Our 
previous study demonstrated that mice deficient in both LDLR and adipose tissue 
had ~five-fold higher plasma cholesterol than *ldlr*−/− mice (23). Thus, adipose tissue is just 
as important as the LDLR pathway for whole body cholesterol homeostasis. Cholesterol is 
usually stored in cells in the form of cholesterol esters through the actions of 
ACAT1/2. Yet, ACAT1/2 expression and activity are extremely low in normal 
adipocytes, which is highly intriguing. We hypothesize that increased ACAT 
expression/activity may exert adverse effects on adipose function and may also be 
associated with dysfunctional adipose tissue. Indeed, we show here for the first time that 
ACAT1 is dramatically upregulated in the adipose tissue and adipocytes of the *ob/ob* 
mice (Fig. 1A, 1B, and S1A). Thus, it is important to determine whether and how 
increased ACAT activity may negatively impact adipocyte function. In this work, we 
demonstrated that the overexpression of ACAT1/2 impaired adipogenesis and key 
metabolic functions of mature adipocytes. We further demonstrate that overexpressing 
ACAT1/2 caused striking accumulation of free cholesterol on the surface of LDs, as well 
as an overall increase in cellular free cholesterol. Thus, the adverse metabolic 
effects on adipocytes may arise from dysregulated cellular cholesterol homeostasis 
upon increased ACAT activity.

Adipose tissue stores a large amount of free cholesterol due to a relative deficiency 
of ACAT enzymes. However, what little cholesterol esterifying activity remains in 
adipocytes still contributes to adipocyte function, since ACAT inhibition and *ACAT* 
deficiency have been reported to suppress lipogenesis and reduce the intracellular 
cholesterol pool (46). Here we found that ACAT overexpression reduced TAG 
synthesis and yet increased intracellular cholesterol, thus transgressing the strong 
correlation usually observed between adipocyte cholesterol content and TAG load 
(22). ACAT overexpression led to smaller LDs (Fig. 3C and G), and hence too much 
ACAT could be expected to inhibit the formation of the adipocyte’s characteristic 
unilocular LDs, which helps to maximize energy storage in minimal space.
ACAT2 appeared to display stronger effects than that of ACAT1, which may help to explain its far more restricted tissue expression. ACAT2 expression is mostly limited to the intestine and liver where it helps to package cholesteryl esters into apoB-containing lipoproteins (47-49). Accordingly, endogenous ACAT2 was down-regulated during adipocyte differentiation unlike ACAT1 (Fig. S3 and Table S4). Moreover, we did not detect any difference in the protein level of ACAT2 between the adipose tissue of normal and ob/ob mice (data not shown), further confirming ACAT2's tissue specificity.

Cys277 of ACAT2 can be ubiquitinated for degradation when the level of certain lipids is low. Free cholesterol and saturated free fatty acids can protect the protein from degradation through inducing cellular reactive oxygen species to oxidize Cys277 (28). This may be another reason for the absence of ACAT2 in adipose tissue which stores the most lipids, including free cholesterol and free fatty acids. Cysteine ubiquitination of ACAT2 is believed to be an important mechanism of maintaining lipid homeostasis by sensing lipid overload. A stable mutant of ACAT2 (C277A) caused higher insulin sensitivity in the liver of ACAT2 KO mice (28). In addition to ACAT2, the stability of HMGCR is also sensitively regulated by lipid levels (6). The sterol induced degradation of both ACAT2 and HMGCR is accomplished through the same gp78-Insigs complex. Gp78 is a membrane-anchored ubiquitin ligase and associates with Insig1/2. Both ACAT2 and HMGCR are substrates of gp78 (50-53). When the cellular cholesterol is low, gp78-Insig complex would release and stabilize HMGCR to increase cholesterol synthesis, and at the same time, ubiquitinate Cys277 of ACAT2 to reduce the esterification. This may be related to the finding in Fig. 9. In ACAT2 overexpressing cells, the continuous consumption of free cholesterol may inhibit HMGCR degradation and increase cholesterol synthesis, which in time reversibly inhibits HMGCR transcription.

ACAT1/2 expression in adipocytes perturbed cholesterol homeostasis and resulted in an aberrant accumulation of free cholesterol on lipid droplets (Fig. 6 and 9). Cholesterol is synthesized in the ER and quickly transferred to other organelles especially the plasma membrane. However, the molecular mechanism of this transport is still elusive despite some discoveries (54-56). Our lab recently demonstrated that ORP2 is a co-exchanger of cholesterol and PI(4,5)P2 on the plasma membrane (57). Also, ORP2 might regulate cholesterol on the LDs (58). This implies ORP2 might be involved in the cholesterol transport in adipocytes when overexpressing ACAT1/2, which may be tested in future studies. In addition, cholesterol depletion by methyl-β-cyclodextrin (similar to HPCD used in this study) increased caveolin-1 content in lipid droplets and caveolin-1 KO mice showed reduced free cholesterol level on LDs (43). The association of caveolin-1 and cholesterol in adipocyte lipid droplets implies caveolin-1 may play an important role in the aberrant accumulation of free cholesterol on lipid droplets when expressing ACAT1/2 (Fig. 8). Indeed, the redistribution of caveolin-1 to LDs has been proposed to trap free cholesterol from other intracellular pools (43).

Perilipin and CIDEC/Fsp27 are two key LD-associated proteins in adipocytes for regulating lipolysis and lipid dynamics, respectively. Cholesterol accumulating on LDs when ACAT1/2 are overexpressed may interfere with the localization of perilipin on LDs, thereby affecting lipolysis. Also, it may affect the functions of other lipid droplet-associated proteins such as CIDEC (Fig. 3J),
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DGAT2 (Fig. 4A and D) and HSL (Fig. 5). All the perturbations in adipocytes caused by increased esterification seem to be associated with the proteins coating the LD surface. Hence, this work strongly implies that free cholesterol localizing to the surface of LDs interferes with the functions of these metabolic proteins.

In summary, our current work sheds light on why adipocytes, the cellular fat reservoirs, store so little esterified cholesterol and further highlights the importance of adipocyte cholesterol on lipid droplet dynamics. Our work uncovers a unique aspect in the regulation of cholesterol homeostasis in adipocytes and suggests that increased ACAT proteins and activity may underpin certain adverse metabolic changes under pathological conditions such as extreme obesity. Therefore, ACAT inhibitors may be tested in treating disease conditions associated with extreme obesity.

Experimental procedures

Mice

C57BL/6J ob/ob mice and their age-matched wild type control mice were maintained at 22±1°C with a 12 h light/dark cycle and ad libitum access to standard rodent chow and water. Epididymal adipose tissue was dissected from male mice (18-20 weeks old) and immediately snap frozen for later analysis. Ovarian adipose tissue was dissected from female animals (19-32 weeks old) and immediately used for adipocyte isolation. All procedures were approved by the Garvan Institute/St. Vincent’s Hospital Animal Experimentation Ethics Committee and followed guidelines issued by the National Health and Medical Research Council of Australia.

Adipocyte isolation

Ovarian fat pads dissected from mice were minced in 6 cm dish. Digestion solution was freshly prepared by adding collagenase D (Sigma Aldrich) (0.75 mg/mL), 0.901 mM CaCl₂, and 0.493 mM MgCl₂ in Dulbecco’s phosphate buffered saline (PBS) buffer. Minced fat pads were digested using 2 mL digestion solution in 37°C shaking water bath for 30 mins. 10 mL pre-warmed Dulbecco’s Modified Eagle Medium (DMEM) (Life Technologies) medium was added to suspensions which were filtered through a 100 μm cell strainer (Falcon). After centrifugation at 700 x g for 5 mins, mature adipocytes and stromal vascular fraction pellets were collected, respectively, for protein extraction.

cDNA constructs

Flag-tagged human ACAT1 and ACAT2 were subcloned into retroviral vector pBABE-puro using standard procedures. Primers were designed in-frame according to the multiple cloning site of pBABE-puro and are listed in Table S1. The catalytic dead ACAT1/2 (ACAT1-H460A and ACAT2-H360A), and ACAT2 stable mutants (ACAT2-C277A and ACAT2-H360A-C277A) were generated by a two-step site-direct mutagenesis (SDM) (59). SDM primers were designed containing mutations of the corresponding amino acid change and are listed in Table S2. All constructs were verified by Sanger sequencing.

Mammalian cell culture and transfection

HEK293FT and Phoenix Eco cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum (FBS) (Life Technologies) and 1% penicillin/streptomycin/glutamine (PSG) (Life Technologies). 3T3-L1 cells were cultured in high glucose DMEM medium supplemented with 10% newborn calf serum (NCS) (Life Technologies) and 1% PSG.
Cells were incubated at 37°C with 5% CO₂, and the medium was changed every two days. DNA transfection was performed using Lipofectamine™ LTX and Plus Reagent (Life Technologies) according to the manufacturer’s instruction.

Viral transduction
To generate stable cell lines or to transfect mature adipocytes, viral overexpressing constructs were transduced to target cells. Briefly, 2 x 10⁶ Phoenix Eco cells, for retroviral overexpression, or HEK293FT cells, for lentiviral overexpression, were seeded in 10 cm dish 24 h prior to transfection. For retrovirus production, 6 µg of pBABE-puro plasmids, or for lentivirus production, 10 µg of RRL-PGK plasmids together with 3 µg of pRSV (Rev), 2 µg pMD.G (VSV), and 4 µg of pMDLg/pRRE (Gag/Pol) were transfected into target cells for 48 h. The viral medium was filtered through a 0.45 µm filter (Life Technologies) and collected into 15 mL falcon tubes. 8 µg/mL polybrene (Sigma Aldrich) was added to the filtered viral medium, which was then added to the target cells and incubated for 24-48 h. For mature adipocytes transduced with lentivirus, the cells were split and re-seeded into different dishes depending on the experimental purposes. For the generation of stable cell lines transduced with retrovirus, the viral medium was refreshed to DMEM/10% NCS/1% PSG containing 4 µg/mL puromycin (Life Technologies) to select positively infected cells. Cells were selected for 72 h followed by a further 48 h.

Cell treatments
Cells were grown to desired cell confluency using DMEM supplemented with serum and PSG depending on cell types. Insulin (10 nM) (Sigma Aldrich), isoproterenol (10 µM) (Sigma Aldrich), mevastatin (10 µM) (Sigma Aldrich), hydroxypropyl-β-cyclodextrin (HPCD, 1% w/v) (Sigma Aldrich), sandoz 58-035 (10 µM) (Sigma Aldrich), and pyripyropene (PPPA) (10 µM) (Cayman Chemical) (60,61) were used in this study. Incubation times for each drug treatment are stated in the figure legends. To induce lipid droplet formation, 200 µM oleate (Sigma Aldrich) was added to preadipocytes for the indicated time.

RNA extraction and quantitative real-time PCR
Total RNA was extracted using TRIzol™ reagent (Sigma-Aldrich). Mammalian cells were grown in 6-well plates. Cells were washed with PBS once then lysed by addition of 1 mL TRIzol™ regent and incubation for 5 min at room temperature. 200 µL chloroform (Sigma-Aldrich) was added to the lysates, shaking for 30 times violently, following by incubation for 5 min at room temperature. The mixture was then centrifuged at 12,000 x g for 15 min at 4°C. 350 µL upper aqueous phase was carefully removed to a fresh tube and the same volume of isopropanol (Sigma-Aldrich) was added and mixed well by the vortex for 5 sec. The mixture was centrifuged at 12,000 x g for 10 min at 4°C. After removing the upper aqueous phase, the pellet was washed twice with 1 mL 75% ethanol (Sigma-Aldrich). The centrifugation of 7500 x g, 5 min at 4°C was carried out between each wash. The RNA pellet was dried in fume hood after removing ethanol, then dissolved in RNase free water (Life Technologies). RNA concentration and purity were determined using Nanodrop spectrophotometer (Thermo Fischer Scientific). 1 µg of RNA was adopted for cDNA synthesis using the High Capacity cDNA Reverse Transcription kit (Thermal Fisher Scientific). Quantitative RT-PCR (qRT-PCR) was performed with a Rotor-
Gene 6000 real-time PCR machine (Qiagen) using KAPA SYBR® Green mix (KAPA Biosystems). The mRNA levels were normalized against the housekeeping gene and compared to control samples. All qRT-PCR primers used in this study are listed in Table S3.

**Antibodies**

We obtained rabbit polyclonal to HSL, p-HSL (Ser660), ATGL, perilipin2, AKT, β-Actin, GAPDH, and rabbit monoclonal to p-AKT (S473) from Cell Signaling Technology (CST), goat polyclonal to perilipin-1 and rabbit polyclonal to ACAT1 (Abcam), mouse monoclonal to p-perilipin1 (Ser522) (Vala Biosciences), rabbit polyclonal to ACAT2 (Cayman Chemical), caveolin-1 (Sigma Aldrich), CD11b (Novus), Flag (Clontech), and CIDE C (gift from Prof. Peng Li). All antibodies were diluted in 1:1000 except for ACAT1/2 at 1:500. For immunoblotting, we obtained horseradish peroxidase-conjugated secondary antibodies from Jackson ImmunoResearch. For immunofluorescence, we obtained Alexa-Flour conjugated secondary antibodies from Molecular Probes/Life Technologies.

**Immunoblot Analysis**

Samples were mixed with 2 × laemmli buffer, incubated for 10-15 min at 70°C, and then subjected to 10% SDS-PAGE. After electrophoresis, the proteins were transferred to Hybond-C nitrocellulose filters (GE Healthcare). Incubations with primary antibodies were performed at 4°C overnight. Secondary antibodies were peroxidase-conjugated AffiniPure donkey anti-rabbit, donkey anti-mouse or donkey anti-goat IgG (H+L; Jackson ImmunoResearch Laboratories) used at a 1:1000 dilution. The bound antibodies were detected by ECL Western blotting detection reagent (GE Healthcare or Merck Millipore) and visualized with Molecular Imager® ChemiDocTM XRS+ (Bio-Rad Laboratories).

**Filipin staining**

The filipin complex (Sigma Aldrich) was dissolved in DMSO (Sigma Aldrich) and a working solution of 0.05 mg/mL in PBS containing 10% FBS was used. Cells grown on coverslips were rinsed with PBS 3 times and then fixed with 4% paraformaldehyde (PFA) (Electron microscopy science) for 15 min. After washing three times with PBS, cells were incubated with 1 mL of glycine (Sigma Aldrich) in PBS (1.5 mg/mL) for 10 min at room temperature to quench PFA. Then cells were stained with 1 mL filipin working solution for 2 h in the dark at room temperature. When lipid droplets staining was required, 1 µg/mL BODIPY 493/503 (Life Technologies) or HCS LipidTOX™ deep red neutral lipid stain (1:500) (Life Technologies) was supplemented to the filipin working solution at the last 15 or 45 min depending on the dye used. Cells were mounted on the slides after rinsing with PBS for 3 times.

**Immunofluorescence**

Cells grown on coverslips were fixed with 4% PFA for 15 min and then permeabilized using 0.1% TritonX-100 (Sigma Aldrich) in PBS for 30 min. Cells were washed with PBS for 3 times and then blocked using 1% BSA (w/v) (Sigma Aldrich) diluted in PBS for 1 h at 37°C. After blocking, cells were incubated with primary antibody at 4°C overnight. The primary antibody was diluted in PBS at the ratio of 1:100. Cells were then washed 3 times in PBS before the incubation with the appropriate secondary Alexa Fluor antibody (Life Technologies) at 37°C for 1 h. The secondary antibody was diluted in PBS at the ratio of...
Coverslips were washed 3 times, 5 min each, in PBS and then mounted onto slides using ProLong® Antifade Gold reagent (Life Technologies) and sealed with nail polish. When filipin staining was required after immunofluorescence, cells were permeabilized using 0.1% saponin (Sigma Aldrich). All antibodies were diluted in 0.05% saponin. Confocal microscopy was performed using an Olympus FV1200 laser scanning confocal microscope (Olympus, Tokyo, Japan). A 100×/1.4 oil immersion objective was used for all imaging except for special specifications. The diameters of the LDs were measured using ImageJ software (National Institutes of Health).

**Oil Red O staining**

3T3-L1 adipocytes grown in 6-well plates were washed with PBS once before fixed with 2 mL 4% PFA for 1 h at room temperature. After fixing, 2 mL 100% isopropanol (Ajax FineChem) was added to fixed cells. The plate was swirled to mix well. The mixture was removed and washed with 2 mL 60% isopropanol (Ajax FineChem). After the removal of isopropanol, plates were left to dry. 2 mL Oil Red O (Sigma Aldrich) solution was added and left to incubate for 10 min. Cells were then immediately washed with MilliQ H₂O 4 times until all residual Oil Red O solution was removed. The plates were dried completely before imaging by a scanner.

**Lipolysis assay**

3T3-L1 mature adipocytes overexpressing ACATs were split into 96-well plates. The lipolysis assay was carried out using the Glycerol release assay kit (Biovision) according to the manufacturer’s protocol with minor changes. To induce lipolysis, 75 µL of lipolysis assay medium was added to cells for indicated time points with 10 µM isoproterenol supplement. Glycerol standard was made using dilutions of glycerol in glycerol assay buffer. 50 µL of lipolysis assay medium was used to determine the glycerol released in samples. The reaction mixture was added to glycerol standards and samples, incubated for 30 min at room temperature protected from light. Reaction results were determined using a plate reader at 570 nm. Glycerol released from samples was normalized to the protein concentration of each sample.

**Neutral lipid extraction**

After washing cells once with PBS, the neutral lipids were extracted by 2 mL mixture of hexane (Ajax FineChem) and isopropanol (Ajax FineChem) (3:2) for 30 min in the fume hood. The solvent was then transferred into 2 mL glass vials. Another 1 mL fresh hexane and isopropanol was used to collect the lipid residues in the dish and then transferred to glass vials together with the previous 2 mL solvent. The lipids were dried using a speed vacuum centrifuge. The cells were lysed with 0.1 M NaOH (Ajax FineChem) for 15 min at room temperature after the dish was dried. The protein concentrations were determined by bicinchoninic acid (BCA) (Thermo Fisher Scientific) assay.

**Thin-layer chromatography (TLC)**

Neutral lipids were reconstituted in 60 µL hexane. The samples were then loaded on and separated using a Silica Gel 60 plate (Millipore), and developed in a solvent system consisting of heptane/diethyl ether/glacial acetic acid (90:30:1) (Ajax FineChem). Separated lipids were stained with iodine for about 15 min. The TLC plate was scanned using the Epson Perfection 4490 Photo, and TAG bands were quantified using ImageJ software and normalized to the protein concentration.
**Cholesterol esterification in adipocytes**

**Cholesterol and cholesteryl ester measurement**

Cholesterol was extracted using the same method as the neutral lipid extraction. Amplex™ Red cholesterol assay kit (Life Technologies) was used to check the levels of free cholesterol and cholesteryl esters according to the manufacturer’s protocol. Cholesterol-containing samples were diluted in an appropriate volume of 1 x reaction buffer. Free cholesterol was measured by the enzyme-coupled reaction without cholesterol esterase. The cholesteryl esters were hydrolyzed into free cholesterol by cholesterol esterase and were then detected by subtracting free cholesterol from total cholesterol. The reactions were incubated for 30 min at 37°C protected from light before measuring fluorescence using a microplate reader.

**Cholesterol esterification assay**

3T3-L1 preadipocytes stably overexpressing ACAT1/2 were seeded in 6 cm dishes and grown to about 60-70% confluency. Cells were washed once using PBS, incubated with 2 mL cholesterol-free medium (LPDS medium) overnight. The starvation medium was replaced by DMEM medium supplemented with 20% FBS for 5 h. ^14^C-oleic acid (1 μCi) conjugated to BSA was then directly added to the medium and cells were chased for further 2 h. Cells were washed twice using buffer A (50 mM Tris-HCl, 150 mM NaCl, 0.2% (w/v) BSA, pH 7.4) and once with buffer B (50 mM Tris-HCl, 150 mM NaCl, pH 7.4). The liquid residues were removed completely. Lipid extraction and TLC were carried out as described above. The TLC plate was exposed to a BAS-MS imaging sheet (Fujifilm, Tokyo, Japan) for 5-7 days in an enclosed cassette at room temperature before visualizing the cholesteryl ester band using the FLA-5100 phosphoimager (Fujifilm). The relative intensities of bands corresponding to cholesteryl esters were quantified using ImageJ.

**Statistical analysis**

All data were expressed as mean ± S.D. or mean ± S.E. Comparisons between two groups were analyzed using two-tailed student’s t-test or one-way ANOVA using GraphPad Prism 6.0 software. Differences at values of P < 0.05 were considered as significant.
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Author contributions: YX, XD, and HY conceived the project and designed the experiments. YX performed the experiments and analyses with advice from XD, NT, A JB, and HY. YX, A JB, and HY wrote the manuscript with input from XD.
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FIGURE LEGENDS:

Figure 1. Characterization of ACAT1/2 overexpression in 3T3-L1 stable cells. (A) The protein level of ACAT1 in adipose tissue of WT and ob/ob mice (three mice for each genotype, male, 18-20 weeks) as detected by western blot analyses. (B) Quantification of ACAT1 levels in (A). (C) The mRNA levels of ACAT1, ACAT2, and related mutants stably expressed in 3T3-L1 adipocytes. (D-E) The protein levels of ACAT1, ACAT2, and related mutants stably expressed in 3T3-L1 adipocytes. (F-G) The levels of cholesteryl esters (CEs) in preadipocytes and adipocytes. (H-I) The proportion of esterified cholesterol over total cholesterol. (J-K) Cholesterol esterification by ACAT1 and ACAT2 in preadipocytes. Non-specific lipids (*) were used as references for normalization. 3T3-L1 preadipocytes were transduced with pBABE-puro empty vector (EV) or Flag-tagged ACAT1/2 or ACAT1/2 catalytic dead mutants followed by the selection of puromycin to generate stable cell lines. Two-tailed Student’s t test was used: mean ± S.D.; n = 3, *P < 0.05, **P < 0.01, ****P < 0.0001.

Figure 2. ACAT1/2 overexpression impaired 3T3-L1 differentiation. (A-C) The mRNA levels of aP2, PPARγ, C/EBPa, and Pref1 during differentiation. The status of differentiation was assayed by examining the mRNA levels of adipose-specific genes when overexpressing (A) WT ACAT1 and ACAT2, (B) stable mutant of ACAT2 (ACAT2-C277A), and (C) catalytic dead mutants of ACAT1/2. (D-E) Oil red O staining of adipocytes on day 8 of differentiation. Two-tailed Student’s t test was used: mean ± S.D.; n = 3, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns, no significance.

Figure 3. ACAT1/2 overexpression impaired LD expansion in adipocytes. (A) The effect of WT ACAT1/2 overexpression on LD sizes on day 8 of differentiation. Immunofluorescence was carried out with anti-Flag primary antibody on cells expressing Flag-tagged ACAT1/2, but not on EV control cells. LDs were stained using BODIPY. Bars = 10 µm. (B) The effect of overexpressing catalytic dead mutants of ACAT1/2 on LD size on day 8 of differentiation. LDs were stained using BODIPY. Bars = 10 µm. (C) Quantification of LD sizes in adipocytes stably expressing ACAT1/2. LDs from ~15 cells/cell type were used. (D) Protein level of ACAT1 in mature adipocytes transiently overexpressing ACAT1. (E) Protein level of ACAT2 in mature adipocytes transiently overexpressing ACAT2. (F) The effect of transient overexpression of ACAT1/2 on LD size in mature adipocytes. Immunofluorescence was carried out with anti-Flag primary antibody on cells expressing Flag-tagged ACAT1/2, but not on EV control cells. LDs
were stained using LipidTOX. Bars = 10 µm. (G) Quantification of LD sizes in mature adipocytes overexpressing ACAT1/2. LDs from ~15 cells/cell type were used. (H) The effects of ACAT1 and ACAT2 inhibitors on LD size in mature adipocytes. Bars = 10 µm. LDs were stained by BODIPY. Sandoz 58-035 (ACAT1 inhibitor) and Pyripyropene A (PPPA, ACAT2 inhibitor) were dissolved in DMSO. 10 µM of inhibitor was added to respective cells for 10 h. (I) Quantification of diameters of 2 largest LDs in each cell type as shown in (H). Two-tailed Student’s t test was used: mean ± S.E.; n = 20 LDs from 10 cells for each cell type, **P < 0.01, ***P < 0.01, ns, no significance. (J) Immunoblot analysis of CIDEC in cell lysates and LD fractions isolated from mature adipocytes transiently overexpressing ACAT1/2. LD, lipid droplets; CL, cell lysates. (K) Quantification of CIDEC level in (J). Two-tailed Student’s t test was used: mean ± S.D.; n = 3, *P < 0.05, **P < 0.01.

**Figure 4. ACAT1/2 overexpression impaired TAG synthesis in adipocytes.** (A) The mRNA level of Dgat1/2 during differentiation. (B) The amount of TAG in adipocytes on day 8 of differentiation. (C-D) The mRNA level of Dgat1/2 in mature adipocytes transiently overexpressing ACAT1 and ACAT2. (E) The amount of TAG in mature adipocytes overexpressing ACAT1 and ACAT2. Two-tailed Student’s t test was used: mean ± S.D.; n = 3, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

**Figure 5. ACAT1/2 overexpression impaired metabolic functions in adipocytes.** (A) Lipolysis assay in mature adipocytes overexpressing ACAT1/2. Isoproterenol, 100 nM. Two-tailed Student’s t test was used: mean ± S.D.; n = 3, **P < 0.01, ***P < 0.001, ****P < 0.0001. (B) Immunoblot analysis of p-perilipin 1 (S522) and p-HSL (S660) in mature adipocytes transiently overexpressing ACAT1/2. ISO, isoproterenol, 10 µM, 2 h. (C) Immunoblot analysis of perilipin 2 and ATGL in mature adipocytes overexpressing ACAT1/2. (D) Quantification of perilipin2 and ATGL levels in (C). Two-tailed Student’s t test was used: mean ± S.D.; n = 3, *P < 0.05, ns, no significance. (E) Immunoblot analysis of perilipin 2 and ATGL in mature adipocytes stably overexpressing ACAT1/2. (F) Quantification of perilipin2 and ATGL levels in (E). Two-tailed Student’s t test was used: mean ± S.D.; n = 3, *P < 0.05, ns, no significance. (G) Immunoblot analysis of p-AKT (S473) in mature adipocytes stably overexpressing ACAT1/2. Insulin, 10 nM, 15 min. (H) Quantification of p-AKT level in (G). Two-tailed Student’s t test was used: mean ± S.D.; n = 3, **P < 0.01, ***P < 0.001.
Figure 6. ACAT1/2 overexpression resulted in accumulation of free cholesterol on the LD surface in adipocytes. (A) The localization of free cholesterol in adipocytes on day 8 of differentiation upon ACAT1/2 overexpression. Bars = 10 µm. Inlay, Bars = 5 µm. (B) Quantification of free cholesterol-LD colocalization in (A). Two-tailed Student’s t test was used: min to max, line at median; n = 15 cells. **P < 0.01, ***P < 0.001. (C) The localization of free cholesterol in mature adipocytes transiently overexpressing ACAT1/2. Immunofluorescence was carried out with anti-Flag primary antibody on cells expressing Flag-tagged ACAT1/2, but not on EV control cells. Bars = 10 µm. Inlay, Bars = 1 µm. (D) Quantification of free cholesterol-LD colocalization in (C). Two-tailed Student’s t test was used: min to max, line at median; n = 10 cells. **P < 0.01.

Figure 7. Cholesterol depletion enhanced free cholesterol accumulation on LD surface in adipocytes. Cholesterol depletion by (A) HPCD in FBS medium and (B) HPCD in LPDS medium promoted free cholesterol accumulation on the LD surface in mature adipocytes stably overexpressing ACAT1/2. Bars = 10 µm. Inlay, Bars = 1 µm. (C) Quantification of free cholesterol-LD colocalization in (A) and (B). One-way ANOVA was used: min to max, line at median; n = 10 cells. **P < 0.01, ****P < 0.0001.

Figure 8. ACAT1/2 overexpression promoted caveolin-1 localization to the LDs in adipocytes. (A) Caveolin-1 colocalized with free cholesterol on the LD surface in mature adipocytes upon ACAT1/2 overexpression. Bars = 10 µm. Inlay, Bars = 3 µm. CAV-1, caveolin-1. (B) Immunoblot analysis of caveolin-1 in LD fractions isolated from mature adipocytes transiently overexpressing ACAT1/2. LD, lipid droplets; CL, cell lysates. (C) Quantification of caveolin-1 level in (B, CL). Two-tailed Student’s t test was used: mean ± S.D.; n = 3, *P < 0.05.

Figure 9. ACAT1/2 overexpression impaired cholesterol homeostasis in adipocytes. (A) The level of free cholesterol and (B) the mRNA level of Hmgcr in mature adipocytes stably overexpressing ACAT1/2. (C) The free cholesterol level and (D) the mRNA level of Hmgcr in mature adipocytes transiently overexpressing ACAT1/2. (E) The free cholesterol level and (F) the mRNA level of Hmgcr in preadipocytes stably overexpressing ACAT1 and ACAT2. 200 µM oleate was added to cells for 20 h before harvest. (G-H) HMGCR inhibition restored the free cholesterol level in preadipocytes and adipocytes stably overexpressing ACAT1 and ACAT2. Before harvest, cells were washed once with PBS.
followed by the treatment of LPDS media for 20 h. for preadipocytes, 200 µM oleate was added to cells with LPDS media to induce LDs for 20 h. Two-tailed Student’s *t* test was used: mean ± S.D.; n = 3, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns, no significance.
Fig 2

A

WT

aP2 mRNA

Fold change

EV
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT

**

***

PPARγ mRNA

Fold change

EV
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT

**

***

C/EBPα mRNA

Fold change

EV
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT

*

Pref1 mRNA

Fold change

EV
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT

*

B

ACAT2 stable mutant

aP2 mRNA

Fold change

EV
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT

**

***

PPARγ mRNA

Fold change

EV
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT

**

***

C/EBPα mRNA

Fold change

EV
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT

*

Pref1 mRNA

Fold change

EV
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT
Off Flag-ACAT

*

C

Catalytic dead mutants

aP2 mRNA

Fold change

EV
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A

ns

PPARγ mRNA

Fold change

EV
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A

*

C/EBPα mRNA

Fold change

EV
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A

ns

Pref1 mRNA

Fold change

EV
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A
Off Flag-ACAT-H66A

ns

D

EV
ACAT1
ACAT2
ACAT2-C277A

E

EV
ACAT1-H460A
ACAT2-H360A
ACAT2-H360A-C277A
Fig 6

A

| EV       | OE ACAT1 | OE ACAT2 |
|----------|----------|----------|
| Filipin  | BODIPY   | Merge    |

B

Day 8

![Graph](image)

C

| EV       | OE ACAT1 | OE ACAT2 |
|----------|----------|----------|
| Flag     | Filipin  | LipidTOX |

D

Mature adipocytes

![Graph](image)
Fig 8

A

B

C

CAV-1 protein

Fold change

0.5
1.0
1.5
2.0
2.5

EV
ACAT1
ACAT2

LD
CL

Western Blot Analysis

Protein Levels

GAPDH
Enhanced acyl-CoA: cholesterol acyltransferase activity increases cholesterol levels on the lipid dropletsurface and impairs adipocyte function

Yanqing Xu, Ximing Du, Nigel Turner, Andrew J. Brown and Hongyuan Yang

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