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## **4 $\beta$ -hydroxycholesterol is a pro-lipogenic factor that promotes SREBP1c expression and activity through Liver X-receptor.**

Ofer Moldavski<sup>1,2</sup>, Peter-James H. Zushin<sup>3</sup>, Charles A. Berdan<sup>3</sup>, Robert J. Van Eijkeren<sup>1,2</sup>, Xuntian Jiang<sup>4</sup>, Mingxing Qian<sup>5</sup>, Daniel S. Ory<sup>4</sup>, Douglas F. Covey<sup>5</sup>, Daniel K. Nomura<sup>3</sup>, Andreas Stahl<sup>3</sup>, Ethan J. Weiss<sup>6</sup>, Roberto Zoncu<sup>1,2\*</sup>

<sup>1</sup>Department of Molecular and Cell Biology, University of California at Berkeley, Berkeley, CA 94720, USA

<sup>2</sup>The Paul F. Glenn Center for Aging Research at the University of California, Berkeley, Berkeley, CA 94720, USA

<sup>3</sup>Department of Nutritional Sciences and Toxicology, University of California at Berkeley, Berkeley, CA 94720, USA

<sup>4</sup>Diabetic Cardiovascular Disease Center, Washington University School of Medicine, St Louis, MO 63110, USA

<sup>5</sup>Department of Developmental Biology, Washington University School of Medicine, St Louis, MO 63110, USA

<sup>6</sup>Cardiovascular Research Institute, UCSF, San Francisco, California, USA

\*Corresponding author: rzoncu@berkeley.edu

**Oxysterols are oxidized derivatives of cholesterol that play signaling roles in lipid biosynthesis and homeostasis. Here we show that 4 $\beta$ -hydroxycholesterol (4 $\beta$ -HC), a liver and serum abundant oxysterol of poorly defined function, is a potent and selective inducer of the master lipogenic transcription factor, Sterol Regulatory Element Binding Protein 1c (SREBP1c), but not the related steroidogenic transcription factor SREBP2. Mechanistically, 4 $\beta$ -HC acts as a putative agonist for Liver X receptor (LXR), a sterol sensor and transcriptional regulator previously linked to SREBP1c activation. Unique among the oxysterol agonists of LXR, 4 $\beta$ -HC induced expression of the lipogenic program downstream of SREBP1c, and triggered *de novo* lipogenesis both in primary hepatocytes and in mouse liver. 4 $\beta$ -HC acted in parallel to insulin-PI3K-dependent signaling to stimulate triglyceride synthesis and lipid droplet accumulation. Thus, 4 $\beta$ -HC is an endogenous regulator of *de novo* lipogenesis through the LXR-SREBP1c axis.**

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## INTRODUCTION

All cells must achieve and maintain a balanced composition of their internal membranes in order to grow, proliferate or adapt to sudden changes in external conditions and nutrient availability (Nohturfft and Zhang, 2009). Dedicated biosynthetic pathways mediate the synthesis of fatty acids, sterols, phospholipids and sphingolipids, but how these pathways communicate with each other to coordinate their respective activities and respond to changing metabolic needs is poorly understood (Thelen and Zoncu, 2017; van Meer *et al.*, 2008).

Liver-X-Receptor (LXR)  $\alpha$  and  $\beta$  are transcription factors belonging to the nuclear receptor superfamily that play key roles in maintaining lipid homeostasis in multiple cells and organs (Janowski *et al.*, 1996; Kalaany and Mangelsdorf, 2006; Repa *et al.*, 2000; Yoshikawa *et al.*, 2001). LXR  $\alpha$  and  $\beta$  dimerize with the retinoic X-receptor (RXR) and activate target genes that mediate cholesterol efflux from cells, including ABC-family transporters, as well as genes that mediate conversion of cholesterol into bile acids in the liver to facilitate cholesterol elimination from the body, such as cytochrome p450 7 $\alpha$ -hydroxylase (CYP7A1) (Costet *et al.*, 2000; Peet *et al.*, 1998; Svensson *et al.*, 2003). Accordingly, mice lacking LXR $\alpha$  exhibit impaired bile acid metabolism and defective cholesterol elimination (Peet *et al.*, 1998), along with enhanced inflammation and formation of atherosclerotic plaques (Hong *et al.*, 2012). Conversely, synthetic LXR $\alpha$  agonists have shown promise in reducing atherosclerosis and preventing cardiovascular disease in animal models (Calkin and Tontonoz, 2010; Joseph *et al.*, 2002; Terasaka *et al.*, 2003).

Another key mediator of lipid homeostasis is the helix-loop-helix-leucine zipper transcription factor, Sterol Regulatory Element Binding Protein (SREBP) 1c. SREBP1c is a master regulator of biosynthesis of fatty acids and triglycerides (collectively referred to as *de-novo* lipogenesis, DNL) that is subject to tight transcriptional and post-translational regulation. Along with its paralogue, the master steroidogenic transcription factor SREBP2, SREBP1c resides at the endoplasmic reticulum (ER) membrane, to which it is anchored via a single transmembrane helix. When cholesterol concentration in the ER membrane is low, SREBP1c and SREBP2 are transported to the Golgi apparatus via interaction with SREBP cleavage-activating protein (SCAP), a cholesterol-sensing chaperone that favors their loading into COPII vesicles. At the Golgi membrane, resident proteases cleave the DNA-binding portion of SREBP1c and SREBP2 from the transmembrane portion, enabling their translocation to the nucleus and activation of downstream programs for *de-novo* lipogenesis and steroidogenesis, respectively.

In addition to their homeostatic regulation by cholesterol levels, the SREBPs lie downstream of metabolic hormone signaling. For example in the liver, both the expression and proteolytic activation of SREBP1c are stimulated by the insulin-phosphatidylinositol 3-kinase (PI3K)-mechanistic Target of

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Rapamycin (mTOR) pathway, as part of a mechanism that converts excess of glucose into lipids, which are required for energy storage (Azzout-Marniche et al., 2000; Horton et al., 2002; Ricoult and Manning, 2013). However, the range of regulatory inputs to SREBP1c and their respective interplay remain to be fully elucidated.

LXR $\alpha$  and LXR $\beta$  were shown to directly bind to the promoter of the SREBP1c gene and trigger activation of its downstream lipogenic genes (Repa et al., 2000). Accordingly, synthetic LXR ligands strongly promote *de novo* lipogenesis and increased plasma triglyceride levels (Grefhorst et al., 2002; Joseph et al., 2002; Schultz et al., 2000), providing evidence for cross-talk between LXR- and SREBP1c-dependent programs.

While the physiological significance of LXR-dependent regulation of DNL through SREBP1c remains unclear, this cross-talk has important clinical implications. In particular, LXR-dependent upregulation of SREBP1c potentially limits the usefulness of LXR agonists to improve cholesterol metabolism, as the resulting induction of lipogenic programs could lead to undesirable effects, such as non alcoholic fatty liver disease (NAFLD), a condition that has risen to epidemic proportions in recent years (Cai et al., 2018). Thus, understanding how LXR-dependent activation of SREBP1c occurs, and its functional interaction with other pathways controlling lipid homeostasis such as PI3K-mTOR signaling are key open questions.

Oxysterols are a family of metabolites that originate from an oxygenation reaction of cholesterol. Some Oxysterols are signaling molecules involved in a wide range of physiological processes controlling cholesterol, glucose and lipid metabolism (Mutemberezi et al., 2016). Levels of oxysterols are known to change in pathological situations like obesity, atherosclerosis and Alzheimer disease (Guillemot-Legris et al., 2016b; Poli et al., 2013). A subset of oxysterols function as endogenous LXR ligands and were shown to activate LXR $\alpha$ -dependent gene expression *in vitro*, including those bearing hydroxyl groups in position 4, 7, 20, 22, 24, 25 and 27 on the cholesterol backbone (Janowski et al., 1999; Janowski et al., 1996; Nury et al., 2014). Interestingly, although these oxysterols are considered *bona fide* LXR activators, none is known to activate SREBP1c and its downstream lipogenic programs, whereas several oxysterols have been shown to promote LXR-dependent cholesterol efflux. In contrast, synthetic LXR ligands including T0901317 and GW3965 can induce both cholesterol efflux and SREBP1c-dependent DNL (Grefhorst et al., 2002; Schultz et al., 2000). This leads to the question of whether *de novo* lipogenesis is a physiologically relevant LXR-dependent response, and if so, the identity of the endogenous ligand that triggers LXR-dependent SREBP1c expression.

Here we identify 4 $\beta$ -hydroxycholesterol (4 $\beta$ -HC) as an LXR activator that selectively triggers SREBP1c activation and *de novo* fatty acid and triglyceride synthesis. 4 $\beta$ -HC promoted the expression and

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proteolytic processing of SREBP1c but not of the related steroidogenic factor SREBP2, thus triggering de novo synthesis of fatty acids but not cholesterol. In primary mouse hepatocytes, 4 $\beta$ -HC additively enhance insulin action in promoting SREBP1c expression and activation, leading to increased triglyceride synthesis and storage. Thus, 4 $\beta$ -HC may be a novel lipogenic factor that can shift lipid homeostasis towards triglycerides accumulation via regulation on SREBP1c.

## RESULTS

### 4 $\beta$ -HC is a unique oxysterol that drives SREBP1c gene expression

To identify oxysterol ligands that could promote SREBP1c expression, we treated liver carcinoma-derived Huh7 cells with a panel of oxysterols selected among the most abundant in the bloodstream, including 4 $\beta$ -, 7 $\beta$ -, 19-, 20-, 24(S)-, 25- and 27-hydroxycholesterol (HC). By quantitative PCR (qPCR), several oxysterols previously identified as LXR activators, including 4 $\beta$ -HC, 7 $\beta$ -HC, 24(S)-HC and 25-HC, induced the expression of a canonical LXR target gene, ATP binding cassette subfamily A member 1 (ABCA1), with variable potency (Fig. 1A). In contrast, 4 $\beta$ -HC was the only oxysterol to induce significant upregulation of the SREBP1c transcript (Fig. 1B). A dose-response comparison between 4 $\beta$ -HC and 24(S)-HC showed that 24(S)-HC is a more potent activator than 4 $\beta$ -HC toward ABCA1 (Fig. 1C) and another canonical LXR gene target, ABCG1 (Fig. 1D). Conversely, 4 $\beta$ -HC activated SREBP1c, more potently than 24(S)-HC (Fig. 1E). 4 $\beta$ -HC-mediated induction of the SREBP1c gene was enantioselective, as the non-natural enantiomer of 4 $\beta$ -HC (ent-4HC) was unable to induce SREBP1c mRNA expression even at the highest concentration used (20 $\mu$ M) (Fig. 1F). This data suggest that SREBP1c induction depends on unique structural features of 4 $\beta$ -HC.

### 4 $\beta$ -HC induces expression and activation of SREBP1 but not SREBP2

Oxysterols such as 25- and 27-hydroxycholesterol suppress SREBP1 and SREBP2 activation by blocking their trafficking to the Golgi, where proteolytic processing of the SREBPs to the mature nuclear form occurs (Adams et al., 2004; Radhakrishnan et al., 2007).

In contrast to these oxysterols, 4 $\beta$ -HC significantly increased SREBP1c mRNA levels (Fig 2A) as well as protein levels in a cycloheximide-sensitive manner (Fig 2B). However, 4 $\beta$ -HC did not increase either mRNA or protein levels of SREBP2 (Fig 2A-2B). In keeping with the increased total levels of SREBP1c, 4 $\beta$ -HC increases both cytosolic and nuclear forms of SREBP1 in a dose-dependent manner, whereas levels of cytoplasmic or nuclear SREBP2 protein levels did not change (Fig 2C). Consistent with previous reports (Adams et al., 2004; Radhakrishnan et al., 2007) and in contrast to 4 $\beta$ -HC, 25-HC reduced

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the nuclear forms of both SREBP1 and SREBP2, thereby causing the accumulation of the unprocessed cytoplasmic form of both proteins but without transcriptional upregulation (Fig. 1B).

This data suggest that, unlike other oxysterols that function as inhibitors of both SREBP1c and SREBP2, 4 $\beta$ -HC is a specific inducer of SREBP1c expression and activation.

### **4 $\beta$ -HC induce lipogenic programs through the LXRs**

Along with other oxysterols, 4 $\beta$ -HC was previously shown to activate LXR $\alpha$ -dependent transcription in luciferase assays *in vitro*, supporting its role as a putative LXR ligand (Janowski et al., 1996; Nury et al., 2013). In turn, LXR transcriptionally activates SREBP1c by directly binding to its promoter region (Repa et al., 2000). Combining these observations, we thus hypothesized that 4 $\beta$ -HC may transcriptionally activate SREBP1c and its downstream lipogenic programs via LXR. Consistent with this possibility, co-treating cells with 4 $\beta$ -HC together with an LXR antagonist (GSK-2033) abolished 4 $\beta$ -HC-dependent induction of SREBP1c gene expression (Fig. 3A).

The effect of 4 $\beta$ -HC on SREBP1c induction was additive with an RXR ligand, 9-cis Retinoic acid (9-RA). Moreover, co-incubation of 4 $\beta$ -HC with the LXR agonist, GW3965, used at concentrations that activate LXR maximally, caused no additional increase in SREBP1c expression over GW3965 alone (Fig. 3A). siRNA-mediated knock down of either LXR $\alpha$  or LXR $\beta$  (both of which are expressed in Huh7 cells) largely abolished 4 $\beta$ -HC-dependent SREBP1c mRNA expression (Fig. 3B). Interestingly, we noticed that 4 $\beta$ -HC treatment increased LXR $\alpha$  protein levels, a stabilizing effect observed for other established LXR ligands (Ignatova et al., 2013) (Fig. 3C). Together, and combined with previous reports these data support the hypothesis that 4 $\beta$ -HC induces SREBP1c gene expression by acting as an LXR agonist.

We next compared the ability of 4 $\beta$ -HC to induce SREBP1c-dependent lipogenic programs with that of the LXR agonist, GW3965. Fatty acid synthase (FASN), Stearoyl-CoA desaturase (SCD1) and Lipin1 (LPIN1) are validated SREBP1c downstream targets in Huh7 cells (Ishimoto et al., 2009; Shimomura et al., 1998). Treatment with either 4 $\beta$ -HC or GW3965 significantly increases the expression of these genes (Fig. 3D). In contrast, 24-HC, another putative LXR ligand that failed to induce SREBP1c in our hands (Fig. 1B and 1E), had minimal or no effect on these SREBP1c target genes (Fig. 3D).

Previous work had shown that GW3965 induces FASN to a greater extent than the 1.6-fold we observed in Huh7 (Peng et al., 2011). Huh7, a hepatocellular carcinoma line, are known to hyperactivated DNL to supply membranal lipids required for rapid division and growth (Calvisi et al., 2011; Li et al., 2016). We speculate that the modest increase in FASN by GW3965 or 4 $\beta$ -HC is due to already elevated baseline expression that cannot be increased much further. To further substantiate the pro-lipogenic effect of 4 $\beta$ -HC, we directly measured DNL by C13 incorporation into triglycerides using liquid chromatography coupled to

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mass spectrometry (LC/MS). Similarly to lipogenic gene induction, both GW3965 and 4 $\beta$ -HC had a modest but statistically significant 1.5-fold increase in C13-labeled C16:C16:C16 TAG, or trending toward significance for C16:C18:C16 TAG, whereas 24-HC caused no significant change (Fig 3E)- Combined, these data suggest that the pro-lipogenic action of 4 $\beta$ -HC is comparable, in mechanism and potency, to known LXR agonists.

#### **4 $\beta$ -HC induces lipid droplet formation and triglyceride accumulation.**

In keeping with the ability of 4 $\beta$ -HC to upregulate fatty acid biosynthetic genes via SREBP1c, treating Huh7 cells with 4 $\beta$ -HC (but not with its unnatural enantiomer, *ent*-4HC) for 72 hours resulted in marked accumulation of lipid droplets (LDs), as revealed by staining with the lipophilic dye BODIPY 493/503 (Fig. 4A and 4B). LD accumulation induced by 4 $\beta$ -HC was suppressed by simultaneous treatment with a FASN inhibitor, TVB-3166, or with the LXR inhibitor GSK-2033. Measurement of triglyceride content in cell extracts confirmed the ability of 4 $\beta$ -HC to induce triglyceride accumulation, albeit with lower potency than the LXR agonist GW3965, whereas cholesterol levels remained unchanged (Fig. 4C). Consistent with the BODIPY staining, both LXR and FASN inhibitors hindered 4 $\beta$ -HC-induced triglyceride accumulation (Fig. 4C). Moreover, as seen with SREBP1c induction, the enantiomer of 4 $\beta$ -HC (*ent*-4HC) failed to induce triglyceride accumulation (Fig. 4C). Thus, 4 $\beta$ -HC is sufficient to induce the formation of triglyceride-containing lipid droplets in an LXR- and FASN-dependent manner in cell culture.

Next, we tested the effect of 4 $\beta$ -HC on *in-vivo* lipogenesis by feeding mice a normal diet supplemented with either 4 $\beta$ -HC or GW3965. After 7 days, livers were harvested, lipid droplets were assessed by BODIPY staining, and liver lipid content (normalized to protein mass) was measured. Consistent with the results in Huh7 cells, 4 $\beta$ -HC significantly increased the size and number of lipid droplets in liver sections (Fig 4D) and liver triglyceride content (Fig 4E), albeit with lower potency than the synthetic LXR agonist, GW3965. Collectively, these data suggest that 4 $\beta$ -HC is a pro-lipogenic factor that can increase liver lipid content *in vivo*.

#### **4 $\beta$ -HC acts in parallel to insulin-PI3K signaling to drive SREBP1c expression**

Insulin is a key hormone that drives SREBP1c transcription, proteolytic processing and DNL in the postprandial state. Insulin regulates SREBP1c transcription via poorly understood mechanisms, which include AKT-dependent transcriptional downregulation of Insig-2a, the ER-retention factor that blocks translocation of SCAP-SREBP1c to the Golgi (Yabe et al., 2003; Yecies et al., 2011). LXR was shown to be required for insulin-dependent activation on SREBP1c (Chen et al., 2004), but whether and how insulin activates LXR is not understood.

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To interrogate the relationship between 4 $\beta$ -HC and insulin signaling in driving SREBP1c transcription and processing, we used an insulin-responsive primary mouse hepatocytes (Foretz et al., 1999). In these cells, stimulation with either 4 $\beta$ -HC or insulin alone increased the mRNA levels of SREBP1c, while combined 4 $\beta$ -HC and insulin increased SREBP1c mRNA levels additively [as previously shown for LXR agonists (Chen et al., 2004)] (Fig. 5A). Interestingly, treatment with PI3K or mTORC1 inhibitors abolished SREBP1c induction by both Insulin and 4 $\beta$ -HC (Fig. 5A), raising the possibility that 4 $\beta$ -HC may act in a common pathway with insulin-PI3K-mTORC1 signaling.

To further elucidate the relationship between 4 $\beta$ -HC and insulin, we compared their effects on AKT phosphorylation and SREBP1 protein levels. While insulin strongly increased AKT phosphorylation in primary hepatocytes, we observed a mild increase in SREBP1 expression and processing (Fig 5B). In contrast, 4 $\beta$ -HC stimulation alone was able to induce SREBP1 expression and processing without any change to AKT phosphorylation, suggesting AKT activation is not required for 4 $\beta$ -HC action (Fig 5B). Combined stimulation with insulin and 4 $\beta$ -HC had an additive effect on the levels of both cytoplasmic and nuclear (processed) forms of SREBP1c, supporting our transcriptional data.

Consistent with previous reports, we also detected a marked decrease in Insig-2a mRNA in insulin-stimulated hepatocytes (Fig. 5C). In contrast, 4 $\beta$ -HC caused a mild increase of Insig-2a mRNA levels, and combined insulin and 4 $\beta$ -HC was similar to insulin alone, suggesting Insig-2a downregulation is not required for 4 $\beta$ -HC-dependent SREBP1c activation (Fig. 5C).

To further probe possible connections between insulin-PI3K and 4 $\beta$ -HC-LXR signaling, we tested whether insulin signaling promotes 4 $\beta$ -HC synthesis. Previous reports had shown that in humans 4 $\beta$ -HC has a very slow kinetics, with an extremely long half-life in plasma (60 hours) (Bodin et al., 2002). Pharmacological induction of the main 4 $\beta$ -HC-synthesizing enzyme, cytochrome P450 3A (CYP3A), double 4 $\beta$ -HC concentration in human plasma in 8 days (Kasichayanula et al., 2014), a very different pattern from insulin, which peaks within 1-2 hours following a meals and drops in between. On the other hand, *in vitro* work in primary rat hepatocytes led to the hypothesis that insulin signaling may produce an unknown LXR ligand that, in turn, induces SREBP1c (Chen et al., 2004). To test the possibility of insulin-dependent 4 $\beta$ -HC production, we compared 4 $\beta$ -HC levels in the liver of mice that were either fasted or refed. While mice that were refed showed significant induction of SREBP1c transcription, consistent with SREBP1c regulation by insulin (Fig 5D), the levels of 4 $\beta$ -HC did not increase accordingly (Fig 5E). Collectively, these data suggest that insulin does not induce 4 $\beta$ -HC production according to fasting/feeding cycles, and that 4 $\beta$ -HC most likely acts in parallel to insulin-PI3K signaling in driving SREBP1c transcription and SREBP1c-dependent DNL (Fig 5F).

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## Discussion

Here we identify 4 $\beta$ -HC as a unique oxysterol that activates SREBP1c expression and promotes lipogenic gene programs, resulting in induction of fatty acid biosynthesis and cellular accumulation of triglycerides in lipid droplets both in cell culture and *in vivo*. Our results are most consistent with a model in which 4 $\beta$ -HC acts in parallel to insulin-PI3K-mTOR signaling, and the two pathways have additive effects on SREBP1c activation. A simple mechanism that explains the additive effect is that the SREBP1c promoter contains both an LXR binding element (LXRE) and an SREBP binding element (SRE), and transcription can be initiated by the two transcription factors independently and without synergism (Herschlag and Johnson, 1993). Collectively, 4 $\beta$ -HC kinetics suggest that it stimulate SREBP1c expression in a chronic manner, setting the baseline expression, while insulin acts acutely in the postprandial state.

The physiological contexts under which 4 $\beta$ -HC-mediated lipogenesis occurs remain to be determined. Interestingly, several groups using different animal models (mice, rats, rabbits and swine) had all observed that 4 $\beta$ -HC levels increase when animals are fed a high cholesterol diet (Kim et al., 2014; Serviddio et al., 2016; Shimabukuro et al., 2016; Wooten et al., 2014), while a high-fat but with low-cholesterol diet reduces 4 $\beta$ -HC levels in mice (Guillemot-Legris et al., 2016a). Dietary cholesterol was shown to increase SREBP1c expression in an LXR-dependent manner (Peet et al., 1998; Repa et al., 2000). Furthermore, genetically disrupting hepatic cholesterol synthesis through SREBP2 knockout also causes SREBP1c down regulation, which can be rescued by an LXR agonist (Rong et al., 2017). This study also determined that 4 $\beta$ -HC levels are decreases in young SREBP2-null mice, defining a correlation between SREBP2-dependent cholesterol synthesis, 4 $\beta$ -HC levels and SREBP1c expression. Together with this published literature, our results strongly suggest that 4 $\beta$ -HC may be the cholesterol-derived molecule that induces SREBP1c activation via LXR.

An important question is why 4 $\beta$ -HC is the sole oxysterol ligand of LXRs to activate SREBP1c expression in our hands. Several possibilities can be envisioned. The LXR-RXR heterodimer can recruit co-activators (PGC-1 $\alpha$ , TRRAP, ACS-2, p300, SRC-1) as well as co-repressors (NCoR, SMRT) to the promoters of target genes in a ligand-dependent manner (Hu et al., 2003; Huuskonen et al., 2004; Oberkofler et al., 2003; Wagner et al., 2003; Zhang et al., 2004), but whether all LXR ligands are equally effective in recruiting specific combinations of cofactors is unclear. Supporting this model was an observation in macrophages that the ability of LXR to Recruit RNA polymerase II to SREBP1c promoter requires a specific LXR ligand, while recruitment of RNA polymerase II to the ABCA1 promoter is more promiscuous (Ignatova et al., 2013). Thus, 4 $\beta$ -HC may be able to direct a unique set of co-activators and RNA polymerase II to the SREBP1c promoter, resulting in its activation.



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Consistent with previous reports, the synthetic LXR agonist GW3965 was also able to trigger SREBP1 expression (Grefhorst et al., 2002; Schultz et al., 2000). Synthetic LXR agonists are generally more potent than natural LXR ligands, possibly reflecting higher affinity for the ligand-binding site of LXR. By analogy, 4 $\beta$ -HC may bind to LXR with higher affinity than other oxysterol ligands. In turn, higher affinity may translate into longer residence time on the SREBP1c promoter DNA, a possible prerequisite for its efficient activation.

Our data points to the importance of the enzyme that produces 4 $\beta$ -HC, Cyp3A4 (Cyp3A11 in mice) (Bodin et al., 2001), as a crucial regulator of lipogenesis. Consistent with that, several groups have reported that increased Cyp3A4 expression by over expressing its activator, Pregnane X Receptor (PXR), correlated with increases lipogenic gene expression as well as liver triglyceride levels (Huang et al., 2016; Zhou et al., 2006). Conversely, decreased Cyp3A4 expression (He et al., 2013) or its pharmacological inhibition (Chudnovskiy et al., 2014) were associated with lower lipogenic gene expression and liver triglyceride levels. Taken together, these data suggest that Cyp3A4 and 4 $\beta$ -HC may regulate diet-induced lipogenic genes and liver triglyceride levels.

From a more clinical perspective, 4 $\beta$ -HC might have an aggravating effect on the development of Non-Alcoholic Fatty Liver disease (NAFLD). NAFLD is characterized by elevated liver triglycerides not due to alcohol consumption or any other known causes (Neuschwander-Tetri and Caldwell, 2003). Elevated triglyceride levels are associated with LXR and SREBP1c upregulation in NAFLD (Higuchi et al., 2008). NAFLD patients show a significant increase in 4 $\beta$ -HC plasma levels compared to healthy patients (Ikegami et al., 2012). Thus, it is plausible that elevated 4 $\beta$ -HC levels could be an unrecognized driver of triglyceride accumulation in NAFLD. It would be interesting to determine the effect of pharmacologic Cyp3A4 inhibition on disease progression in NAFLD patients

In conclusion, this work highlights a role for 4 $\beta$ -HC, which was long viewed as an ‘orphan’ oxysterol, in regulating lipid metabolism in the liver together with insulin. Future work, dissecting the role of 4 $\beta$ -HC in other organs and in different pathological settings will provide a full picture on the function and significance of this highly abundant oxysterol.

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## REFERENCES

- Adams, C.M., Reitz, J., De Brabander, J.K., Feramisco, J.D., Li, L., Brown, M.S., and Goldstein, J.L. (2004). Cholesterol and 25-hydroxycholesterol inhibit activation of SREBPs by different mechanisms, both involving SCAP and Insigs. *J Biol Chem* 279, 52772-52780.
- Azzout-Marniche, D., Becard, D., Guichard, C., Foretz, M., Ferre, P., and Foufelle, F. (2000). Insulin effects on sterol regulatory-element-binding protein-1c (SREBP-1c) transcriptional activity in rat hepatocytes. *Biochem J* 350 Pt 2, 389-393.
- Bodin, K., Andersson, U., Rystedt, E., Ellis, E., Norlin, M., Pikuleva, I., Eggertsen, G., Bjorkhem, I., and Diczfalusy, U. (2002). Metabolism of 4 beta -hydroxycholesterol in humans. *J Biol Chem* 277, 31534-31540.
- Bodin, K., Bretillon, L., Aden, Y., Bertilsson, L., Broome, U., Einarsson, C., and Diczfalusy, U. (2001). Antiepileptic drugs increase plasma levels of 4beta-hydroxycholesterol in humans: evidence for involvement of cytochrome p450 3A4. *J Biol Chem* 276, 38685-38689.
- Cai, J., Zhang, X.J., and Li, H. (2018). Progress and challenges in the prevention and control of nonalcoholic fatty liver disease. *Med Res Rev*.
- Calkin, A.C., and Tontonoz, P. (2010). Liver x receptor signaling pathways and atherosclerosis. *Arterioscler Thromb Vasc Biol* 30, 1513-1518.
- Calvisi, D.F., Wang, C., Ho, C., Ladu, S., Lee, S.A., Mattu, S., Destefanis, G., Delogu, S., Zimmermann, A., Ericsson, J., *et al*. (2011). Increased lipogenesis, induced by AKT-mTORC1-RPS6 signaling, promotes development of human hepatocellular carcinoma. *Gastroenterology* 140, 1071-1083.
- Chen, G., Liang, G., Ou, J., Goldstein, J.L., and Brown, M.S. (2004). Central role for liver X receptor in insulin-mediated activation of Srebp-1c transcription and stimulation of fatty acid synthesis in liver. *Proc Natl Acad Sci U S A* 101, 11245-11250.
- Chudnovskiy, R., Thompson, A., Tharp, K., Hellerstein, M., Napoli, J.L., and Stahl, A. (2014). Consumption of clarified grapefruit juice ameliorates high-fat diet induced insulin resistance and weight gain in mice. *PLoS One* 9, e108408.
- Costet, P., Luo, Y., Wang, N., and Tall, A.R. (2000). Sterol-dependent transactivation of the ABC1 promoter by the liver X receptor/retinoid X receptor. *J Biol Chem* 275, 28240-28245.
- Duvel, K., Yecies, J.L., Menon, S., Raman, P., Lipovsky, A.I., Souza, A.L., Triantafellow, E., Ma, Q., Gorski, R., Cleaver, S., *et al*. (2010). Activation of a metabolic gene regulatory network downstream of mTOR complex 1. *Mol Cell* 39, 171-183.
- Foretz, M., Pacot, C., Dugail, I., Lemarchand, P., Guichard, C., Le Liepvre, X., Berthelie-Lubrano, C., Spiegelman, B., Kim, J.B., Ferre, P., *et al*. (1999). ADD1/SREBP-1c is required in the activation of hepatic lipogenic gene expression by glucose. *Mol Cell Biol* 19, 3760-3768.
- Grefhorst, A., Elzinga, B.M., Voshol, P.J., Plosch, T., Kok, T., Bloks, V.W., van der Sluijs, F.H., Havekes, L.M., Romijn, J.A., Verkade, H.J., *et al*. (2002). Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. *J Biol Chem* 277, 34182-34190.
- Guillemot-Legris, O., Mutemberezi, V., Cani, P.D., and Muccioli, G.G. (2016a). Obesity is associated with changes in oxysterol metabolism and levels in mice liver, hypothalamus, adipose tissue and plasma. *Sci Rep* 6, 19694.
- Guillemot-Legris, O., Mutemberezi, V., and Muccioli, G.G. (2016b). Oxysterols in Metabolic Syndrome: From Bystander Molecules to Bioactive Lipids. *Trends Mol Med* 22, 594-614.
- He, J., Gao, J., Xu, M., Ren, S., Stefanovic-Racic, M., O'Doherty, R.M., and Xie, W. (2013). PXR ablation alleviates diet-induced and genetic obesity and insulin resistance in mice. *Diabetes* 62, 1876-1887.
- Herschlag, D., and Johnson, F.B. (1993). Synergism in transcriptional activation: a kinetic view. *Genes Dev* 7, 173-179.

Moldavski, O. *et al*

- Higuchi, N., Kato, M., Shundo, Y., Tajiri, H., Tanaka, M., Yamashita, N., Kohjima, M., Kotoh, K., Nakamuta, M., Takayanagi, R., *et al.* (2008). Liver X receptor in cooperation with SREBP-1c is a major lipid synthesis regulator in nonalcoholic fatty liver disease. *Hepatology* 38, 1122-1129.
- Hong, C., Bradley, M.N., Rong, X., Wang, X., Wagner, A., Grijalva, V., Castellani, L.W., Salazar, J., Realegeno, S., Boyadjian, R., *et al.* (2012). LXRalpha is uniquely required for maximal reverse cholesterol transport and atheroprotection in ApoE-deficient mice. *J Lipid Res* 53, 1126-1133.
- Horton, J.D., Goldstein, J.L., and Brown, M.S. (2002). SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* 109, 1125-1131.
- Hu, X., Li, S., Wu, J., Xia, C., and Lala, D.S. (2003). Liver X receptors interact with corepressors to regulate gene expression. *Mol Endocrinol* 17, 1019-1026.
- Huang, J.H., Zhang, C., Zhang, D.G., Li, L., Chen, X., and Xu, D.X. (2016). Rifampicin-Induced Hepatic Lipid Accumulation: Association with Up-Regulation of Peroxisome Proliferator-Activated Receptor gamma in Mouse Liver. *PLoS One* 11, e0165787.
- Huuskonen, J., Fielding, P.E., and Fielding, C.J. (2004). Role of p160 coactivator complex in the activation of liver X receptor. *Arterioscler Thromb Vasc Biol* 24, 703-708.
- Ignatova, I.D., Angdisen, J., Moran, E., and Schulman, I.G. (2013). Differential regulation of gene expression by LXRs in response to macrophage cholesterol loading. *Mol Endocrinol* 27, 1036-1047.
- Ikegami, T., Hyogo, H., Honda, A., Miyazaki, T., Tokushige, K., Hashimoto, E., Inui, K., Matsuzaki, Y., and Tazuma, S. (2012). Increased serum liver X receptor ligand oxysterols in patients with non-alcoholic fatty liver disease. *J Gastroenterol* 47, 1257-1266.
- Ishimoto, K., Nakamura, H., Tachibana, K., Yamasaki, D., Ota, A., Hirano, K., Tanaka, T., Hamakubo, T., Sakai, J., Kodama, T., *et al.* (2009). Sterol-mediated regulation of human lipin 1 gene expression in hepatoblastoma cells. *J Biol Chem* 284, 22195-22205.
- Janowski, B.A., Grogan, M.J., Jones, S.A., Wisely, G.B., Kliewer, S.A., Corey, E.J., and Mangelsdorf, D.J. (1999). Structural requirements of ligands for the oxysterol liver X receptors LXRalpha and LXRbeta. *Proc Natl Acad Sci U S A* 96, 266-271.
- Janowski, B.A., Willy, P.J., Devi, T.R., Falck, J.R., and Mangelsdorf, D.J. (1996). An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature* 383, 728-731.
- Joseph, S.B., McKilligin, E., Pei, L., Watson, M.A., Collins, A.R., Laffitte, B.A., Chen, M., Noh, G., Goodman, J., Hagger, G.N., *et al.* (2002). Synthetic LXR ligand inhibits the development of atherosclerosis in mice. *Proc Natl Acad Sci U S A* 99, 7604-7609.
- Kalaany, N.Y., and Mangelsdorf, D.J. (2006). LXRS and FXR: the yin and yang of cholesterol and fat metabolism. *Annu Rev Physiol* 68, 159-191.
- Kasichayanula, S., Boulton, D.W., Luo, W.L., Rodrigues, A.D., Yang, Z., Goodenough, A., Lee, M., Jemal, M., and LaCreta, F. (2014). Validation of 4beta-hydroxycholesterol and evaluation of other endogenous biomarkers for the assessment of CYP3A activity in healthy subjects. *Br J Clin Pharmacol* 78, 1122-1134.
- Kim, E.J., Kim, B.H., Seo, H.S., Lee, Y.J., Kim, H.H., Son, H.H., and Choi, M.H. (2014). Cholesterol-induced non-alcoholic fatty liver disease and atherosclerosis aggravated by systemic inflammation. *PLoS One* 9, e97841.
- Li, L., Che, L., Tharp, K.M., Park, H.M., Pilo, M.G., Cao, D., Cigliano, A., Latte, G., Xu, Z., Ribback, S., *et al.* (2016). Differential requirement for de novo lipogenesis in cholangiocarcinoma and hepatocellular carcinoma of mice and humans. *Hepatology* 63, 1900-1913.
- Mutemberezi, V., Guillemot-Legris, O., and Muccioli, G.G. (2016). Oxysterols: From cholesterol metabolites to key mediators. *Prog Lipid Res* 64, 152-169.
- Neuschwander-Tetri, B.A., and Caldwell, S.H. (2003). Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. *Hepatology* 37, 1202-1219.
- Nohturfft, A., and Zhang, S.C. (2009). Coordination of lipid metabolism in membrane biogenesis. *Annu Rev Cell Dev Biol* 25, 539-566.

Moldavski, O. *et al*

- Nury, T., Samadi, M., Varin, A., Lopez, T., Zarrouk, A., Boumhras, M., Riedinger, J.M., Masson, D., Vejux, A., and Lizard, G. (2013). Biological activities of the LXRA and beta agonist, 4beta-hydroxycholesterol, and of its isomer, 4alpha-hydroxycholesterol, on oligodendrocytes: effects on cell growth and viability, oxidative and inflammatory status. *Biochimie* 95, 518-530.
- Nury, T., Zarrouk, A., Vejux, A., Doria, M., Riedinger, J.M., Delage-Mourroux, R., and Lizard, G. (2014). Induction of oxiaoptophagy, a mixed mode of cell death associated with oxidative stress, apoptosis and autophagy, on 7-ketocholesterol-treated 158N murine oligodendrocytes: impairment by alpha-tocopherol. *Biochem Biophys Res Commun* 446, 714-719.
- Oberkofler, H., Schraml, E., Krempler, F., and Patsch, W. (2003). Potentiation of liver X receptor transcriptional activity by peroxisome-proliferator-activated receptor gamma co-activator 1 alpha. *Biochem J* 371, 89-96.
- Peet, D.J., Turley, S.D., Ma, W., Janowski, B.A., Lobaccaro, J.M., Hammer, R.E., and Mangelsdorf, D.J. (1998). Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. *Cell* 93, 693-704.
- Peng, D., Hiipakka, R.A., Xie, J.T., Dai, Q., Kokontis, J.M., Reardon, C.A., Getz, G.S., and Liao, S. (2011). A novel potent synthetic steroidal liver X receptor agonist lowers plasma cholesterol and triglycerides and reduces atherosclerosis in LDLR(-/-) mice. *Br J Pharmacol* 162, 1792-1804.
- Poli, G., Biasi, F., and Leonarduzzi, G. (2013). Oxysterols in the pathogenesis of major chronic diseases. *Redox Biol* 1, 125-130.
- Radhakrishnan, A., Ikeda, Y., Kwon, H.J., Brown, M.S., and Goldstein, J.L. (2007). Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: oxysterols block transport by binding to Insig. *Proc Natl Acad Sci U S A* 104, 6511-6518.
- Repa, J.J., Liang, G., Ou, J., Bashmakov, Y., Lobaccaro, J.M., Shimomura, I., Shan, B., Brown, M.S., Goldstein, J.L., and Mangelsdorf, D.J. (2000). Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRA and LXRbeta. *Genes Dev* 14, 2819-2830.
- Ricoult, S.J., and Manning, B.D. (2013). The multifaceted role of mTORC1 in the control of lipid metabolism. *EMBO Rep* 14, 242-251.
- Rong, S., Cortes, V.A., Rashid, S., Anderson, N.N., McDonald, J.G., Liang, G., Moon, Y.A., Hammer, R.E., and Horton, J.D. (2017). Expression of SREBP-1c Requires SREBP-2-mediated Generation of a Sterol Ligand for LXR in Livers of Mice. *Elife* 6.
- Schultz, J.R., Tu, H., Luk, A., Repa, J.J., Medina, J.C., Li, L., Schwendner, S., Wang, S., Thoolen, M., Mangelsdorf, D.J., *et al.* (2000). Role of LXRs in control of lipogenesis. *Genes Dev* 14, 2831-2838.
- Serviddio, G., Bellanti, F., Villani, R., Tamborra, R., Zerbinati, C., Blonda, M., Ciacciarelli, M., Poli, G., Vendemiale, G., and Iuliano, L. (2016). Effects of dietary fatty acids and cholesterol excess on liver injury: A lipidomic approach. *Redox Biol* 9, 296-305.
- Shimabukuro, M., Okawa, C., Yamada, H., Yanagi, S., Uematsu, E., Sugasawa, N., Kurobe, H., Hirata, Y., Kim-Kaneyama, J.R., Lei, X.F., *et al.* (2016). The pathophysiological role of oxidized cholesterol in epicardial fat accumulation and cardiac dysfunction: a study in swine fed a high caloric diet with an inhibitor of intestinal cholesterol absorption, ezetimibe. *J Nutr Biochem* 35, 66-73.
- Shimomura, I., Shimano, H., Korn, B.S., Bashmakov, Y., and Horton, J.D. (1998). Nuclear sterol regulatory element-binding proteins activate genes responsible for the entire program of unsaturated fatty acid biosynthesis in transgenic mouse liver. *J Biol Chem* 273, 35299-35306.
- Svensson, S., Ostberg, T., Jacobsson, M., Norstrom, C., Stefansson, K., Hallen, D., Johansson, I.C., Zachrisson, K., Ogg, D., and Jendeborg, L. (2003). Crystal structure of the heterodimeric complex of LXRA and RXRbeta ligand-binding domains in a fully agonistic conformation. *EMBO J* 22, 4625-4633.
- Terasaka, N., Hiroshima, A., Koieyama, T., Ubukata, N., Morikawa, Y., Nakai, D., and Inaba, T. (2003). T-0901317, a synthetic liver X receptor ligand, inhibits development of atherosclerosis in LDL receptor-deficient mice. *FEBS Lett* 536, 6-11.

Moldavski, O. *et al*

Thelen, A.M., and Zoncu, R. (2017). Emerging Roles for the Lysosome in Lipid Metabolism. *Trends Cell Biol* 27, 833-850.

van Meer, G., Voelker, D.R., and Feigenson, G.W. (2008). Membrane lipids: where they are and how they behave. *Nat Rev Mol Cell Biol* 9, 112-124.

Wagner, B.L., Valledor, A.F., Shao, G., Daige, C.L., Bischoff, E.D., Petrowski, M., Jepsen, K., Baek, S.H., Heyman, R.A., Rosenfeld, M.G., *et al.* (2003). Promoter-specific roles for liver X receptor/corepressor complexes in the regulation of ABCA1 and SREBP1 gene expression. *Mol Cell Biol* 23, 5780-5789.

Wooten, J.S., Wu, H., Raya, J., Perrard, X.D., Gaubatz, J., and Hoogeveen, R.C. (2014). The Influence of an Obesogenic Diet on Oxysterol Metabolism in C57BL/6J Mice. *Cholesterol* 2014, 843468.

Yabe, D., Komuro, R., Liang, G., Goldstein, J.L., and Brown, M.S. (2003). Liver-specific mRNA for Insig-2 down-regulated by insulin: implications for fatty acid synthesis. *Proc Natl Acad Sci U S A* 100, 3155-3160.

Yecies, J.L., Zhang, H.H., Menon, S., Liu, S., Yecies, D., Lipovsky, A.I., Gorgun, C., Kwiatkowski, D.J., Hotamisligil, G.S., Lee, C.H., *et al.* (2011). Akt stimulates hepatic SREBP1c and lipogenesis through parallel mTORC1-dependent and independent pathways. *Cell Metab* 14, 21-32.

Yoshikawa, T., Shimano, H., Amemiya-Kudo, M., Yahagi, N., Hasty, A.H., Matsuzaka, T., Okazaki, H., Tamura, Y., Iizuka, Y., Ohashi, K., *et al.* (2001). Identification of liver X receptor-retinoid X receptor as an activator of the sterol regulatory element-binding protein 1c gene promoter. *Mol Cell Biol* 21, 2991-3000.

Zhang, Y., Castellani, L.W., Sinal, C.J., Gonzalez, F.J., and Edwards, P.A. (2004). Peroxisome proliferator-activated receptor-gamma coactivator 1alpha (PGC-1alpha) regulates triglyceride metabolism by activation of the nuclear receptor FXR. *Genes Dev* 18, 157-169.

Zhou, J., Zhai, Y., Mu, Y., Gong, H., Uppal, H., Toma, D., Ren, S., Evans, R.M., and Xie, W. (2006). A novel pregnane X receptor-mediated and sterol regulatory element-binding protein-independent lipogenic pathway. *J Biol Chem* 281, 15013-15020.

## Material and methods

### Material

Reagents were purchased from the following sources. Antibodies used are: SREBP1 (2A4, Santa cruz), SREBP2 (30682, Abcam), LXR $\alpha$  (PP-PPZ0412-00, R&D systems), LXR $\beta$  (K8917, R&D systems), phospho-T308 AKT (C31E5E), AKT (11E7) Cell signaling technologies.

Drugs used are: Cycloheximide (Cell signaling Technology) was used at 10 $\mu$ g/ml. 9-cis-Retinoic acid (Sigma) was used at 50 $\mu$ M. LXR antagonist GSK-2033 (Axon Medchem) was used at 500nM. LXR agonist GW3965 (Fisher Scientific) was used at 500nM. PI3K inhibitor, LY294002 (Cell Signaling Technologies), was used at 10 $\mu$ M. Rapamycin was used at 100nM and received as a gift from David Sabatini. Methyl-beta-cyclodextrin was purchased from Sigma. All Sterols except for custom-synthesized ent-4 $\beta$ -HC (see below) were purchased from Steraloids. C13-glucose was purchased from Cambridge Isotope laboratories.

### Sterol:MbCD pre-complexing

All Sterols were made to 50mM stocks in ethanol. To deliver the sterols to cells, 1.25mM Sterol was complexed with 25mM Methyl-beta-cyclodextrin (MbCD) and vortexed until the solution is clear. Sterols was added media in indicated concentration and incubation time. Control samples were treated by adding the same volume of ethanol to MbCD, which then was delivered to cells in the same corresponding volume.

### Cell culture

Huh7 Cells were maintained on Dulbecco modified Eagle medium (DMEM, 5g/l Glucose. + Glutamine, Gibco) supplemented with 10% FBS (VMR) and p/s (Gibco). Lipid depleted serum (LDS) was made as described in (Goldstein et al., 1983). For assays, on day one; 10<sup>5</sup> cells were plates in 6cm plates. On day two, media was change to 1% LDS, 1g/l glucose DMEM. On Day three; plates were spiked with pre-complexed sterols for indicated times, concentrations and additional compounds.

Primary mouse hepatocytes were purchased from UCSF liver center. Isolation protocol is based on (Li et al., 2010) and adjusted in the following manner. Mice were fasted over night prior to isolation. Hepatocytes were isolated by perfusion protocol (Seglen, 1972) and plated at density of 7x10<sup>5</sup>/well on a 6-well collagen coated plates (Corning) in DMEM supplemented with 10% FBS. Once cells adhere, media was replaced to Medium 199 (GIBCO) containing 100nM Dexamethasone (Sigma), 3,3,5-triiodo-L-tyronine (T3, Sigma) and Insulin-Transferrin-Selenium (Gibco). Next day the same media was used without Insulin-transferrin-Selenium to assay Insulin, 4 $\beta$ -HC and inhibitors at indicated times and concentrations.

## **Real Time PCR analysis for gene expression**

RNA was extracted using RNAeasy kit (Qiagen). 1µg of RNA was reverse transcribed using Super Script III (Invitrogen). Quantitative PCR was performed using SSO advance (BioRad) in Step one Plus (ABI). List of primers is in table 1

## **Protein extraction and western blot**

Cells were harvested with RIPA buffer supplemented with Phosphatase inhibitor and protease inhibitor (10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 10mM Na-PPi, 10mM Na-Beta-glycerophosphate), sonicated with Bioruptor (Diagenode) and normalized using BCA kit (Thermo Scientific).

## **Knock down using siRNA**

siRNA ON-TARGET plus smart pool against LXR $\alpha$  (cat# L-003413-00-0005), LXR $\beta$  (cat# L-003412-02-0005) or non-targeted siRNA ON-TARGETplus Non-targeting Pool (cat# D-001810-10-05) were purchased from Dharmcon. 5µM siRNA was mixed with 5µl Lipofectamine RNAiMAX (Life Technologies) in Opti-MEM (Gibco). siRNA is added to Pre-plated Huh7 (10<sup>5</sup> cells/6cm plate) in regular media w/o penicillin streptomycin for 5 hours followed by replacement to regular media for 72 hours.

## **Lipid droplet microscopy**

Huh7 were plated on a coverslip coated with Fibronectin (Corning) and treated as indicated with sterols and drugs. Cells were fixed with paraformaldehyde and stained with 1µg/ml BODIPY 493/503 for 1 hour. Coverslips were mounted with Vectashield with DAPI (Vector Laboratories) and imaged on a spinning disk confocal system (Andor Revolution on a Nikon Eclipse Ti microscope). BODIPY signal was measured using ImageJ and normalized by the number of nuclei.

## **Triglyceride and Cholesterol measurements**

Liver samples were powdered with pestle and mortar and lysed in RIPA buffer. Huh7 cells were also harvested in RIPA buffer. 5µl of Samples was used to measure Triglyceride using Triglyceride Infinity (Thermo Fisher) or cholesterol using Amplex red cholesterol measuring kit (Invitrogen) in a clear 96-well sample. BCA kit (Thermo scientific) was used for normalization to protein level. Absorbance and fluorescence was measured by Perkin-Elmer Envision Multi label plate reader.

## **C13 incorporation into Triglycerides**



Huh7 Cells were seeded at 200K per 6cm plates. Next day, DMEM media with glutamine, containing 5mM C13 glucose (Cambridge Isotope laboratories) and 1% LDS including oxysterols and LXR agonist were added for 24 hours. C12 glucose treated plates were used as reference. Cells were washed twice with ice cold PBS, scraped and pellets were snap-frozen and kept in -80 for later analysis. Lipid extraction and analysis by LC/MS was preformed as described in (Benjamin et al., 2015).

### **Husbandry and Diets**

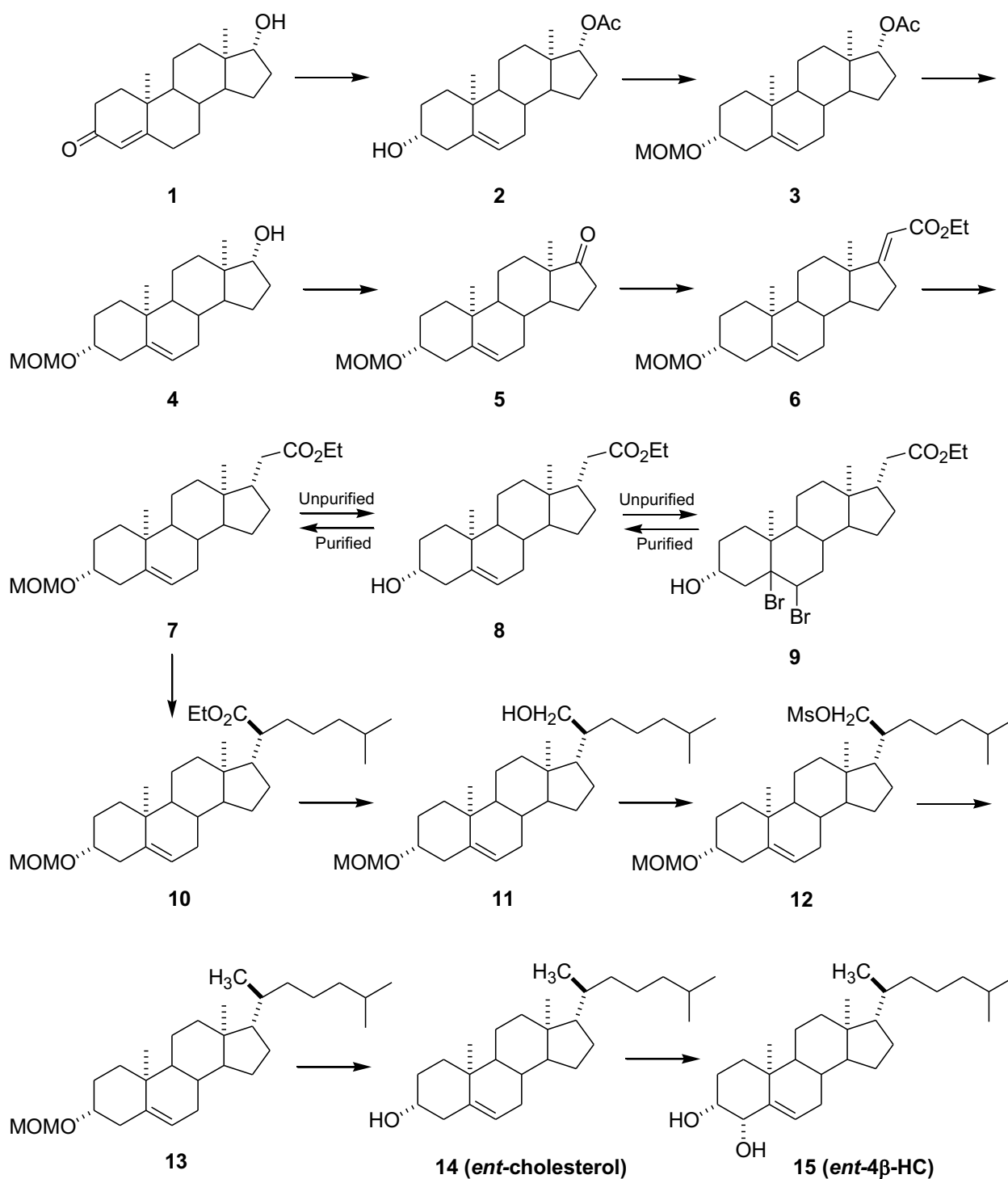
All mouse procedures were performed and approved under the University of California, Berkeley Animal Care and Use Committee. 10-week-old C57BL/6J male mice were purchased from the Jackson Laboratory and housed for one week in our facility under standard conditions before experiments were performed. Free access to water and chow (Lab Diets, #3038) was provided throughout this acclimation period. Afterwards, mice were placed on a diet with 50mg/kg/day 4 $\beta$ -HC or LXR agonist GW3965 10mg/kg/day for 7 days. Powdered 10% by kCal fat diet (Research Diets Inc. #D12450J) was used as the base of each treatment food, forming pellets that were dried overnight at room temperature in a laminar flow hood. After seven days, mice were euthanized using CO<sub>2</sub> and cervical dislocation.

### **Cryosectioning and Fluorescent Histochemistry**

Liver samples were fixed using 4% (v/v) paraformaldehyde overnight at 4°C. The next day, samples were cryopreserved using sterile-filtered 30% sucrose (w/v) in DPBS (Gibco, 14190-144). After three days, each sample was placed in a 1:1 30% sucrose:Neg-50 (Richard-Allen Scientific) solution and incubated overnight at 4°C. The samples were then frozen on dry ice using undiluted -50°C and stored at -80°C until sectioning. Sequential 20 $\mu$ M thick sections were obtained from each sample using a Leica CM3050S cryostat.

For nuclei and lipid droplet labeling, sectioned tissue was washed three times at room temperature in DPBS for 5 minutes each. Afterward, DPBS containing 10 $\mu$ M BODIPY (Invitrogen, #D3922) was placed on the samples and incubated for 30 minutes at room temperature in the dark. Next, the slides were washed with DPBS twice before incubating in DPBS containing 5 $\mu$ g/mL DAPI (Invitrogen, D1306) for 10 mins at room temperature in the dark. Following DAPI staining, the slides were washed three times in DPBS for 5 minutes each before being mounted using SlowFade Diamond antifade (Invitrogen, #S36972), and sealing with nail polish overnight. Slides were imaged immediately using a Zeiss LSM710 confocal microscope. Images were developed using the IMARIS (Bitplane) image analysis software suite.

### **Synthesis of *ent*-4 $\beta$ -HC**



### *ent*-Steroid 2

*ent*-Testosterone (**1**) was prepared as described previously (Covey, D.F., *Polish J. Chem.*, 2006, **80**, 511-522; see also references therein). To a solution of *ent*-testosterone (**1**, 3.8 g, 13.2 mmol) in acetic anhydride (80 mL) was added NaI (7.92 g, 52 mmol) and trimethylsilyl chloride (5.8 mL, 52 mmol) at 0 °C under N<sub>2</sub>. After addition, the reaction was allowed to warm to room temperature for 2 h. The reaction was added to Et<sub>3</sub>N (40 mL) in diethyl ether (100 mL). The ether solution was washed with brine (50 mL x 4), aqueous NaHCO<sub>3</sub> (50 mL x 2) and dried over Na<sub>2</sub>SO<sub>4</sub>. After filtration, the solvent was removed under reduced pressure and the residue was purified by flash column chromatography (silica gel eluted with 25% EtOAc in hexanes) to give *ent*-steroid **2** (3.05 g, 70%): <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 5.33-5.32 (m, 1H), 4.60 (t, *J* = 8.3 Hz, 1H), 3.52-3.47 (m, 1H), 2.30-0.90 (m), 2.02 (s, 3H), 1.00 (s, 3H), 0.79 (s, 3H); <sup>13</sup>C NMR (100 MHz,

CDCl<sub>3</sub>)  $\delta$  171.2, 140.9, 121.1, 82.7, 71.5, 51.0, 50.0, 42.3, 42.2, 37.2, 36.7, 36.5, 31.6, 31.5, 31.4, 27.4, 23.5, 21.1, 20.5, 19.3, 11.8.

### ***ent*-Steroid 3**

*ent*-Steroid **2** (3.05 g, 4.04 mmol) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (50 mL) and cooled to 0 °C. (*i*-Pr)<sub>2</sub>EtN (3.0 mL) and ClCH<sub>2</sub>OMe (1.35 ml, 18.0 mmol) were added and the reaction was stirred at room temperature for 16 h. The reaction was made basic by adding aqueous NaHCO<sub>3</sub> solution and the product was extracted into CH<sub>2</sub>Cl<sub>2</sub>. The combined extracts were washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and solvent removed to give a viscous liquid which was purified by flash column chromatography (silica gel eluted with 10% EtOAc in hexanes) to give *ent*-steroid **3** as a colorless liquid (2.65 g, 77%): <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  5.33-5.32 (m, 1H), 4.65 (s, 2H), 4.59 (t, *J* = 8.2 Hz, 1H), 3.39-3.35 (m, 1H), 3.34 (s, 3H), 2.35-0.89 (m), 2.01 (s, 3H), 0.99 (s, 3H), 0.78 (s, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  171.0, 140.7, 121.2, 94.6, 82.6, 76.7, 55.0, 50.9, 50.0, 42.3, 39.4, 37.1, 36.7, 31.6, 31.4, 28.8, 27.4, 23.5, 21.0, 20.4, 19.3, 11.8.

### ***ent*-Steroid 4**

To a solution of *ent*-steroid **3** (2.65 g, 7.05 mmol) in methanol (60 mL) was added K<sub>2</sub>CO<sub>3</sub> (4.0 g) at room temperature. The mixture was refluxed for 16 h. Methanol was removed under reduced pressure and the residue was purified by flash column chromatography (silica gel eluted with 25% EtOAc in hexanes) to give *ent*-steroid **4** (2.31 g, 99%): <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  5.32-5.30 (m, 1H), 4.64 (s, 2H), 3.61 (t, *J* = 8.6 Hz, 1H), 3.40-3.34 (m, 1H), 3.33 (s, 3H), 2.31-0.87 (m), 0.95 (s, 3H), 0.72 (s, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  140.7, 121.3, 94.5, 81.6, 76.7, 55.0, 51.2, 50.2, 42.6, 39.4, 37.2, 36.7, 36.5, 31.8, 31.4, 30.3, 28.8, 23.3, 20.5, 19.3, 10.9.

### ***ent*-Steroid 5**

To a solution of *ent*-steroid **4** (1.5 g, 4.54 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (60 mL) was added Dess–Martin periodinane (2.5 g, 6 mmol) at room temperature. After 1 h, water (50 mL) was added, the product was extracted into CH<sub>2</sub>Cl<sub>2</sub> (150 mL x 3) and the combined extracts were washed with brine (50 mL x 2). The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and the solvents removed. The residue was purified by flash column chromatography (silica gel eluted with 10% EtOAc in hexanes) to give *ent*-steroid **5** (1.5 g, 100%): <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  5.39-5.38 (m, 1H), 4.68 (s, 2H), 3.45-3.38 (m, 1H), 3.37 (s, 3H), 2.49-0.98 (m), 1.03 (s, 3H), 0.88 (s, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  221.0, 140.9, 120.9, 94.7, 76.7, 55.1, 51.7, 50.2, 47.5, 39.5, 37.1, 36.8, 35.8, 31.4, 31.3, 30.8, 28.8, 21.8, 20.3, 19.3, 13.5.

### ***ent*-Steroid 6**

A solution of freshly prepared sodium ethoxide (sodium 0.4 g, 15 mmol dissolved in ethanol 15 mL) was added dropwise slowly to a solution of *ent*-steroid **5** (1.5 g, 4.54 mmol) and triethyl phosphonoacetate (3.44 g, 15 mmol) in anhydrous ethanol (25 mL) under N<sub>2</sub> while stirring at 35-40 °C. After addition, the reaction was refluxed for 16 h. After cooling to room temperature, the ethanol was removed and the residue was dissolved in ether which was washed with water, dried over Na<sub>2</sub>SO<sub>4</sub> and filtered. Solvent was removed and the residue was purified by flash column chromatography (silica gel eluted with 10% EtOAc in hexanes) to give *ent*-steroid **6** (1.68 g, 87%): <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 5.52 (s, 1H), 5.35-5.34 (m, 1H), 4.66 (s, 2H), 4.15-4.09 (m, 2H), 3.43-3.33 (m, 1H), 3.35 (s, 3H), 2.84-2.79 (m, 2H), 2.36-0.93 (m), 1.01 (s, 3H), 0.82 (s, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 176.1, 167.3, 140.7, 121.3, 108.6, 94.6, 76.7, 59.4, 55.1, 53.7, 50.2, 46.0, 39.5, 37.2, 36.8, 35.1, 31.6, 31.5, 30.4, 28.8, 24.4, 20.9, 19.3, 18.2, 14.3.

The reaction sequence reported below that converts *ent*-steroid **6** into *ent*-steroid **16** (*ent*-VP1-001) is based on that reported previously for the preparation of the natural stereoisomer of *ent*-steroid **16** (Wicha, J.; Bal, K. *J. C. S. Perkin I*, **1978**, 1282-1288).

### Unpurified *ent*-Steroid **7**

To a solution of *ent*-steroid **6** (1.4 g, 3.48 mmol) in EtOAc (150 mL) was added PtO<sub>2</sub> (15 mg) at room temperature. Hydrogenation was carried out under 20 psi for 6 h. Solvent was removed and the residue was purified by flash column chromatography (silica gel eluted with 10% EtOAc in hexanes) to give unpurified *ent*-steroid **7** (1.4 g, 100%): <sup>1</sup>H NMR δ 4.63-4.60 (m, 1H), 4.08-4.03 (m, 2H), 3.48-3.32 (m, 1H), 3.31 (s, 3H), 2.34-0.57 (m), 0.76 (s, 3H), 0.54 (s, 3H); <sup>13</sup>C NMR δ 176.1, 140.7, 121.3, 94.4, 76.2, 60.0, 55.3, 55.0, 54.5, 46.9, 44.9, 42.1, 37.4, 37.0, 35.6, 35.5, 35.3, 35.2, 32.1, 28.7, 28.1, 24.4, 20.9, 14.2, 12.5.

Unpurified *ent*-steroid **7** contains minor amounts of the *ent*-steroid in which the Δ<sup>5</sup> double bond has been hydrogenated. This saturated *ent*-steroid could not be removed easily by chromatography on silica gel. To separate the two compounds chromatographically, *ent*-steroid **7** was converted first into *ent*-steroid **8** and then into *ent*-steroid **9** which is easily purified. *ent*-Steroid **9** was then converted back via *ent*-steroid **8** into *ent*-steroid **7** and then subsequently into *ent*-steroid **10**.

### Unpurified *ent*-Steroid 8

Acetyl chloride (2 mL) was slowly added to unpurified hydrogenation product *ent*-steroid 7 (1.4 g, 3.48 mmol) in ethanol (30 mL) at room temperature. After 2 h, water was added and the product was extracted into CH<sub>2</sub>Cl<sub>2</sub> (100 mL x 2). The combined extracts were dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and the solvent was removed under reduced pressure. The residue was purified by flash column chromatography (silica gel eluted with 25% EtOAc in hexanes) to give unpurified *ent*-steroid 8 (1.2 g): <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 5.35-5.34 (m, 1H), 4.13-4.07 (m, 2H), 3.55-3.47 (m, 1H), 2.38-0.81 (m), 1.10 (s, 3H), 0.61 (s, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 173.9, 140.8, 121.5, 71.6, 60.1, 55.5, 50.3, 46.8, 42.2, 41.9, 37.3, 37.2, 36.5, 35.2, 31.9, 31.8, 31.6, 28.1, 24.5, 20.8, 19.4, 14.2, 12.4.

### *ent*-Steroid 9

To a solution of unpurified *ent*-steroid 8 (1.2 g, 3.33 mmol) in diethyl ether (100 mL) and acetic acid (5 mL) was slowly added Br<sub>2</sub> in HOAc (3 mL) until a brown color persisted. After 5 min, aqueous Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> was added and the reaction became colorless. EtOAc (100 mL) was added and the EtOAc solution was washed with aqueous NaHCO<sub>3</sub> (50 mL x 2), brine (50 mL) and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration, the solvent was removed under reduced pressure and the residue was purified by flash column chromatography (silica gel eluted with 20% EtOAc in hexanes) to give *ent*-steroid 9 (1.4 g, 81%): <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 4.82-4.81 (m, 1H), 4.44-4.37 (m, 1H), 4.12-4.06 (m, 2H), 2.72-1.08 (m), 1.43 (s, 3H), 0.62 (s, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 173.8, 89.6, 68.9, 60.1, 56.0, 54.0, 47.6, 46.6, 45.6, 42.2, 42.0, 37.2, 37.0, 36.7, 35.2, 30.9, 30.1, 28.0, 24.2, 21.0, 20.3, 14.2, 12.7.

### Purified *ent*-Steroid 8

Zinc dust (6.0 g) was added to a solution of *ent*-steroid 9 (1.4 g, 2.7 mmol) in HOAc (20 mL) and EtOAc (30 mL) at room temperature. After 16 h, the mixture was filtered through Celite and washed with EtOAc (200 mL). Solvent was removed under reduced pressure and the residue was purified by flash column chromatography (silica gel eluted with 25% EtOAc in hexanes) to give purified *ent*-steroid 8 (925 mg, 95%): <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 5.26-5.25 (m, 1H), 4.06-4.01 (m, 2H), 3.85 (s, br, 1H), 3.47-3.40 (m, 1H), 2.31-0.73 (m), 0.93 (s, 3H), 0.54 (s, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 173.8, 140.7, 121.1, 71.2, 60.0, 55.4, 50.1, 46.6, 41.9, 41.7, 37.1, 37.0, 36.3, 35.0, 31.7, 31.7, 31.2, 27.9, 24.3, 20.6, 19.2, 14.0, 12.2.

### Purified *ent*-Steroid 7

Purified *ent*-steroid 8 (925 mg, 2.57 mmol) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (20 mL) and cooled to 0 °C. (*i*-Pr)<sub>2</sub>EtN (1.3 mL, 7.5 mmol) and ClCH<sub>2</sub>OMe (0.45 mL, 6.0 mmol) were added and the reaction was stirred at room

temperature for 16 h. The reaction mixture was made basic by adding aqueous saturated NaHCO<sub>3</sub> solution and the product extracted into CH<sub>2</sub>Cl<sub>2</sub>. The combined extracts were washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and solvent removed to give a viscous liquid which was purified by flash column chromatography (silica gel eluted with 20% EtOAc in hexanes) to give purified *ent*-steroid **7** as a colorless liquid (1.02 g, 98%): <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 5.34-5.33 (m, 1H), 4.67 (s, 2H), 4.12 (q, *J* = 7.0 Hz, 2H), 3.42-3.36 (m, 1H), 3.35 (s, 3H), 2.37-0.80 (m), 1.00 (s, 3H), 0.60 (s, 3H); <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 173.8, 140.7, 121.5, 94.6, 76.8, 60.0, 55.5, 55.1, 50.3, 46.7, 41.9, 39.5, 37.2, 37.1, 36.7, 35.2, 31.9, 31.8, 28.9, 28.1, 24.5, 20.7, 19.3, 14.2, 12.3.

### ***ent*-Steroid 10**

To a solution of the *ent*-steroid **7** (202 mg, 0.5 mmol) in THF (10 mL) was added LDA (0.75 mL, 2.0 M in THF, 1.5 mmol) and HMPA (0.29 mL, 1.65 mmol) at -78 °C. After 1 h, 1-bromo-4-methylpentane (0.44 mL, 3 mmol) was added. After addition, the reaction was warmed to room temperature for 16 h. Aqueous NH<sub>4</sub>Cl was added and extracted with EtOAc (100 mL x 2) and the combined extracts were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Solvent was removed under reduced pressure and the residue was purified by flash column chromatography (silica gel eluted with 20% EtOAc in hexanes) to give *ent*-steroid **10** (236 mg, 97%): <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 5.34-5.33 (m, 1H), 4.67 (s, 2H), 4.13-4.08 (q, *J* = 7.4 Hz, 2H), 3.41-3.37 (m, 1H), 3.35 (s, 3H), 2.35-0.79 (m), 0.98 (s, 3H), 0.70 (s, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 176.2, 140.7, 121.5, 94.6, 76.9, 59.6, 56.0, 55.1, 52.6, 50.1, 47.4, 41.9, 39.5, 38.8, 37.5, 37.2, 36.7, 32.2, 31.8, 31.7, 28.9, 27.8, 27.0, 25.0, 23.8, 22.7, 22.3, 20.8, 19.3, 14.2, 12.0.

### ***ent*-Steroid 11**

To a solution of *ent*-steroid **10** (236 mg, 0.5 mmol) in diethyl ether (20 mL) was added LiAlH<sub>4</sub> (2.0 M in diethyl ether, 4.0 mL, 8.0 mmol) at room temperature. After 2 h, water (0.32 mL), 10 % of NaOH (0.64 mL) and water (0.96 mL) were slowly added sequentially. After stirring for 30 min, the mixture was filtered through Celite and washed with CH<sub>2</sub>Cl<sub>2</sub> (100 mL). Solvent was removed under reduced pressure and the residue was purified by flash column chromatography (silica gel eluted with 25% EtOAc in hexanes) to give *ent*-steroid **11** (212 mg, 98%): <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 5.34-5.33 (m, 1H), 4.66 (s, 2H), 3.71-3.61 (m, 2H), 3.44-3.36 (m, 1H), 3.34 (s, 3H), 2.35-0.88 (m), 0.99 (s, 3H), 0.68 (s, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 140.6, 121.6, 94.6, 76.7, 62.5, 56.6, 55.1, 50.3, 50.1, 42.3, 42.0, 39.5, 39.1, 37.2, 36.6, 31.8, 29.5, 28.8, 27.9, 27.5, 24.1, 24.0, 22.7, 22.5, 21.0, 19.3, 12.1.

### ***ent*-Steroid 12**

To a solution of *ent*-steroid **11** (212 mg, 0.48 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (10 mL) was added mesyl chloride (1 mmol, 0.08 mL) and Et<sub>3</sub>N (0.28 mL, 2 mmol) at 0 °C. After 1 h, aqueous NH<sub>4</sub>Cl was added and the product was extracted into CH<sub>2</sub>Cl<sub>2</sub> (100 mL x 2). The combined extracts were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and the solvents removed. The residue was purified by flash column chromatography (silica gel eluted with 10% EtOAc in hexanes) to give *ent*-steroid **12** (241 mg, 97%): <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 5.33-5.32 (m, 1H), 4.66 (s, 2H), 4.36-4.32 (m, 1H), 4.18-4.09 (m, 1H), 3.42-3.37 (m, 1H), 3.34 (s, 3H), 2.97 (s, 3H), 2.34-0.89 (m), 0.98 (s, 3H), 0.69 (s, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 140.6, 121.4, 94.6, 76.8, 70.0, 56.4, 55.1, 50.0, 49.9, 42.0, 39.7, 39.4, 39.2, 39.0, 37.2, 37.1, 36.6, 31.7, 31.6, 29.4, 28.8, 27.7, 27.4, 24.0, 23.4, 22.6, 22.4, 20.9, 19.3, 12.1.

### *ent*-Steroid **13**

To a solution of *ent*-steroid **12** (241 mg, 0.46 mmol) in diethyl ether (30 mL) was added LiAlH<sub>4</sub> (2.0 M in diethyl ether, 4.0 mL, 8.0 mmol) at room temperature. After 2 h, water (0.32 mL), 10 % of NaOH (0.64 mL) and water (0.96 mL) were slowly added sequentially. After stirring for 30 min, the mixture was filtered through Celite and washed with CH<sub>2</sub>Cl<sub>2</sub> (100 mL). Solvent was removed under reduced pressure and the residue was purified by flash column chromatography (silica gel eluted with 10% EtOAc in hexanes) to give *ent*-steroid **13** (188 mg, 95%): <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 5.35-5.34 (m, 1H), 4.68 (s, 2H), 3.46-3.38 (m, 1H), 3.36 (s, 3H), 2.37-0.86 (m), 1.01 (s, 3H), 0.68 (s, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 140.7, 121.7, 94.6, 76.9, 56.7, 56.1, 55.1, 50.1, 42.3, 39.8, 39.5, 39.4, 37.2, 36.7, 36.2, 35.8, 31.9, 31.8, 28.9, 28.2, 28.0, 24.3, 23.8, 22.8, 22.5, 21.0, 19.3, 18.7, 11.8.

### *ent*-Steroid **14** (*ent*-cholesterol)

To a solution of *ent*-steroid **13** (188 mg, 0.44 mmol) in THF (20 mL) was added 6 N HCl (10 mL) at room temperature. After 4 h, the product was extracted into CH<sub>2</sub>Cl<sub>2</sub> (100 mL x 2) and the combined extracts were washed with aqueous NaHCO<sub>3</sub> (50 ml x 2), dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and filtered. Solvent was removed under reduced pressure and the residue was purified by flash column chromatography (silica gel eluted with 20% EtOAc in hexanes) to give *ent*-steroid **14** (165 mg, 98%): <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 5.36-5.35 (m, 1H), 3.57-3.49 (m, 1H), 2.33-0.86 (m), 1.01 (s, 3H), 0.68 (s, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 140.7, 121.7, 71.8, 56.7, 56.1, 50.1, 42.3, 42.2, 39.8, 39.5, 37.2, 36.5, 36.2, 35.8, 31.9(2C), 31.6, 28.2, 28.0, 24.3, 23.8, 22.8, 22.6, 21.1, 19.4, 18.7, 11.8.

### *ent*-Steroid **15** (*ent*-4β-HC)

A procedure previously reported to convert cholesterol to 4β-hydroxycholesterol was used (Nury, T; Samadi, M; Zarrouk, A; Riedinger, J. M; Lizard, G. *Eur. J. Med. Chem.* **2013**, *70*, 558-567.) to convert *ent*-cholesterol **14** into *ent*-4β-hydroxycholesterol **15**.

To a solution of *ent*-cholesterol **14** (29 mg, 0.0747 mmol) in dioxane (5 mL) and water (2 drops) was added SeO<sub>2</sub> (17 mg, 0.15 mmol) at room temperature. The mixture was heated to 90 °C for 16 h. After cooling to room temperature, solvent was removed under reduced pressure. The residue was purified by flash column chromatography (silica gel eluted with 30% EtOAc in hexanes) to give *ent*-4β-hydroxycholesterol **15** (17 mg, 58%): mp 169-171 °C; [α]<sub>D</sub><sup>20</sup> +41.7 (*c* = 0.12, CHCl<sub>3</sub>); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 5.69-5.68 (m, 1H), 4.15-4.14 (m, 1H), 3.58-3.55 (m, 1H), 2.20-0.78 (m), 1.19 (s, 3H), 0.69 (s, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 142.7, 128.8, 77.3, 72.5, 56.9, 56.1, 50.2, 42.3, 39.7, 39.5, 36.9, 36.2, 36.0, 35.8, 32.1, 31.8, 28.2, 28.0, 25.4, 24.2, 23.8, 22.8, 22.5, 21.0, 20.5, 18.7, 11.8; IR (film, cm<sup>-1</sup>) 3406, 1455, 1366, 1072.



## References

- Benjamin, D.I., Li, D.S., Lowe, W., Heuer, T., Kemble, G., and Nomura, D.K. (2015). Diacylglycerol Metabolism and Signaling Is a Driving Force Underlying FASN Inhibitor Sensitivity in Cancer Cells. *ACS Chem Biol* *10*, 1616-1623.
- Goldstein, J.L., Basu, S.K., and Brown, M.S. (1983). Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. *Methods Enzymol* *98*, 241-260.
- Li, S., Brown, M.S., and Goldstein, J.L. (2010). Bifurcation of insulin signaling pathway in rat liver: mTORC1 required for stimulation of lipogenesis, but not inhibition of gluconeogenesis. *Proc Natl Acad Sci U S A* *107*, 3441-3446.
- Seglen, P.O. (1972). Preparation of rat liver cells. I. Effect of Ca<sup>2+</sup> on enzymatic dispersion of isolated, perfused liver. *Exp Cell Res* *74*, 450-454.

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**Figure 1: The oxysterol 4 $\beta$ -HC selectively upregulates SREBP1c expression**

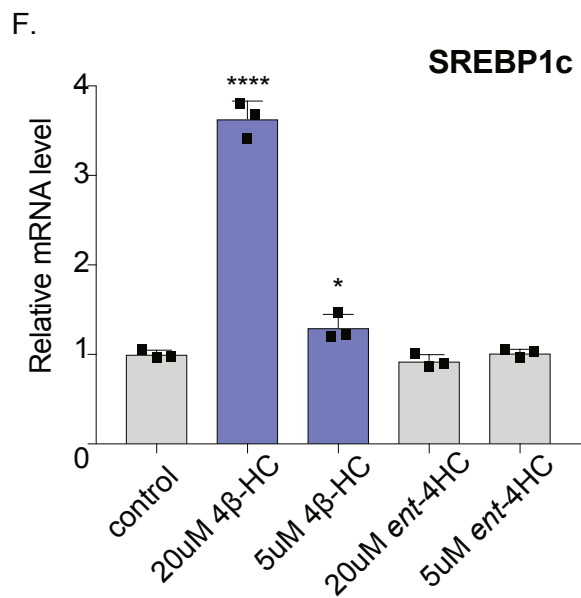
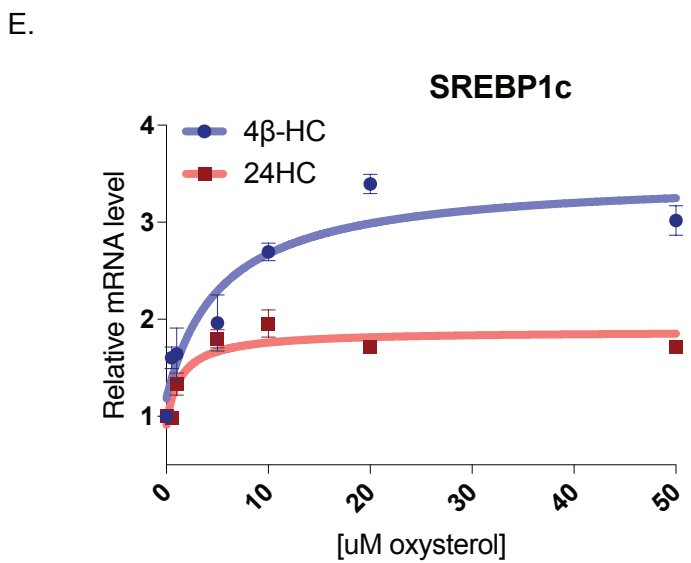
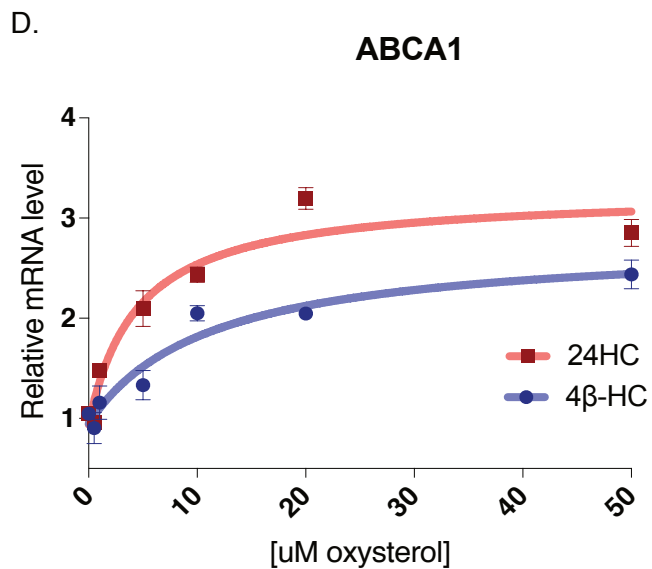
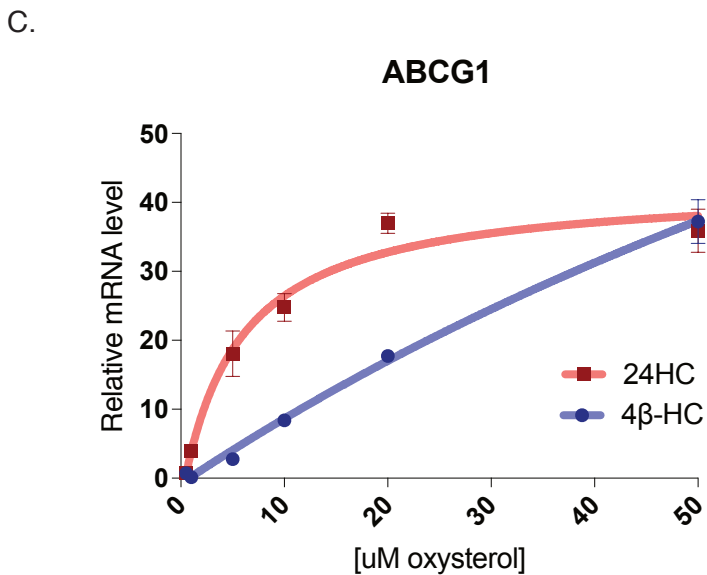
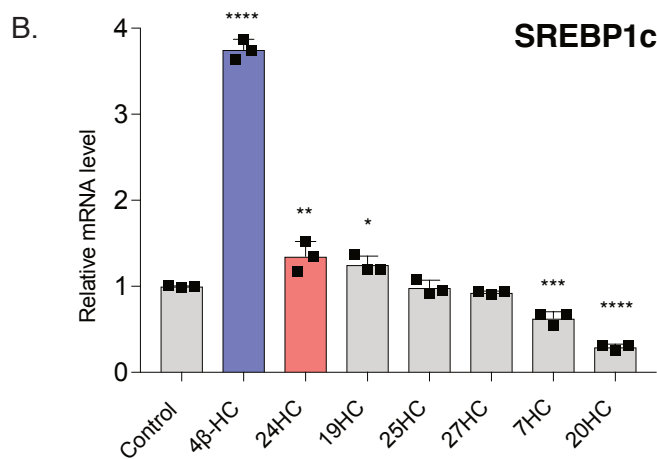
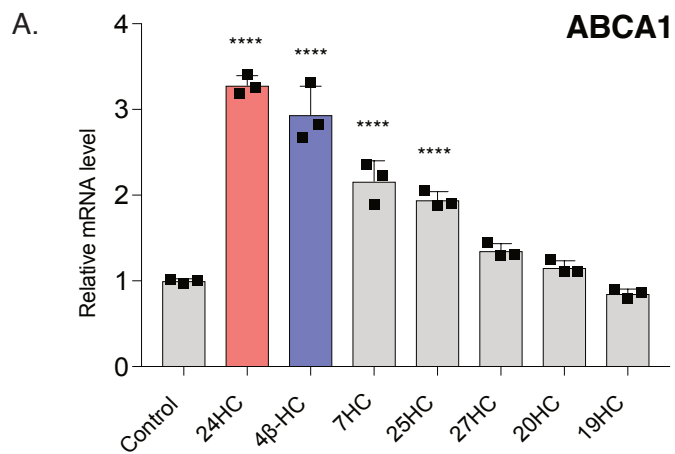
**A.** Oxysterol screen for LXR target gene expression. Huh7 cells were treated with indicated oxysterols (20 $\mu$ M) in 24 hours time course. ABCA1 mRNA levels or **(B)** SREBP1c mRNA level were measured by RT-PCR.

**C.** Dose response curves of 4 $\beta$ -HC and 24HC in Huh7 cells treated for 24 hours. mRNA levels of ABCA1, **(D)** ABCG1 and **(E)** SREBP1c were measured by RT-PCR. Line plotted by non-linear fit.

**E.** SREBP1c induction by 4 $\beta$ -HC is stereo-specific. Huh7 cells were treated with 4 $\beta$ -HC or an enantiomer of 4HC (ent-4HC) for 24 hours in the indicated concentration.

Bars are Mean+SD. Statistical significance calculated by one-way ANOVA. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , NS: Not significant.

Figure 1



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**Figure 2: 4 $\beta$ -HC induces expression and activation of SREBP1 but not SREBP2**

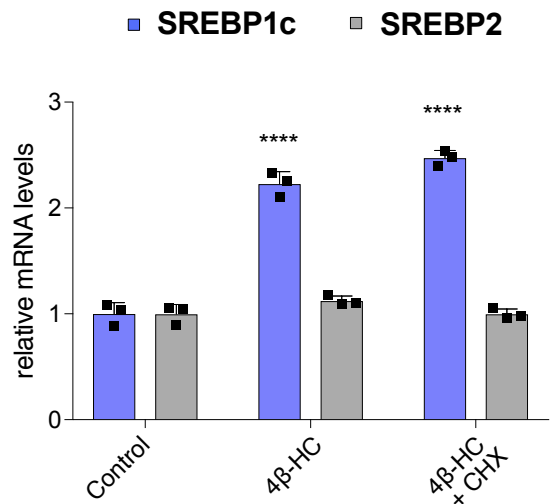
**A.** 4 $\beta$ -HC increases SREBP1 protein expression. Huh7 cells were treated with 20 $\mu$ M 4 $\beta$ -HC and a translation inhibitor, cycloheximide (CHX) for 4 hours followed by measurement of SREBP1 and SREBP2 mRNA and **(B.)** protein level.

**C.** 4 $\beta$ -HC increases SREBP1 cytosolic and nuclear levels while not affecting SREBP2. Huh7 cells were treated with 4 $\beta$ -HC or 25-HC for 24hours followed cytosolic-nuclear fractionation to measure protein level of SREBP1 and SREBP2 cytoplasmic and nuclear levels.

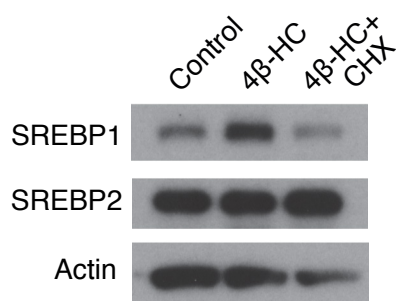
Cyto: Cytosolic. Nuc: Nuclear. Asterisk denotes unspecific band in SREBP2 nuclear blot. Bars are Mean+SD. Statistical significance calculated by one-way ANOVA. \*\*\*\*p<0.0001.

Figure 2

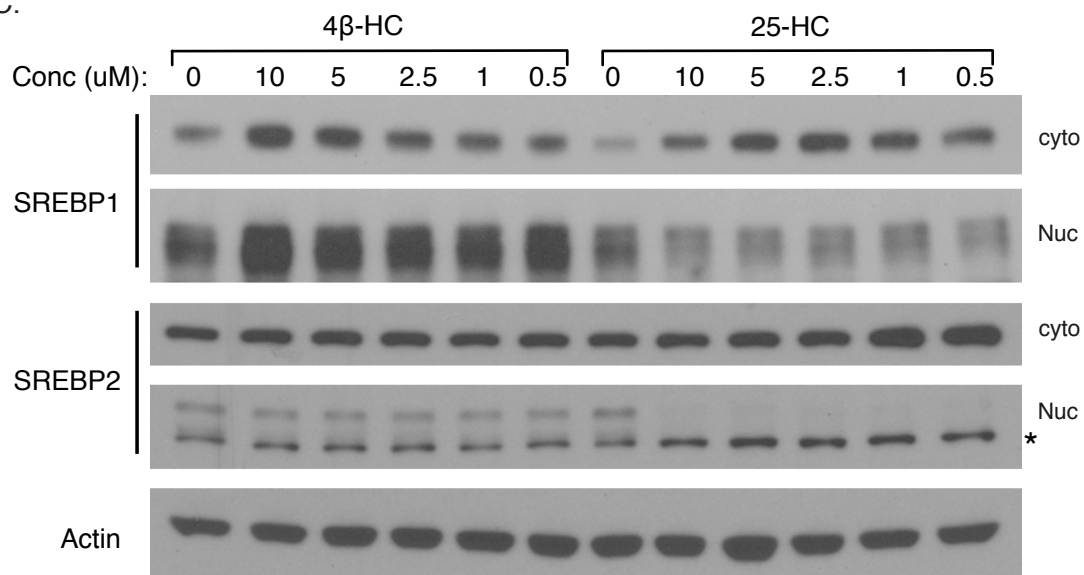
A.



B.



C.



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### Figure 3: 4 $\beta$ -HC induce lipogenic programs through the LXRs

**A.** 4 $\beta$ -HC interacts with LXR and RXR agonist and antagonists like an LXR ligand. Huh7 cells were treated with 20 $\mu$ M 4 $\beta$ -HC, RXR agonist, 9-cis-retinoic acid (9-RA), LXR antagonist (GSK-2033) and LXR agonist (GW3965). For convenience, Agonists are marked in green and antagonist are marked in red.

**B.** LXR $\alpha$  and LXR $\beta$  are required for SREBP1c induction by 4 $\beta$ -HC in Huh7 cells. Knockdown of LXR $\alpha$  or LXR $\beta$  by siRNA for 72 hours followed by treatment with 5 $\mu$ M 4 $\beta$ -HC for 24 hours followed by RT-PCR of SREBP1c. **C.** Knockdown efficiency was evaluated by measurement of LXR $\alpha$  and LXR $\beta$  protein levels

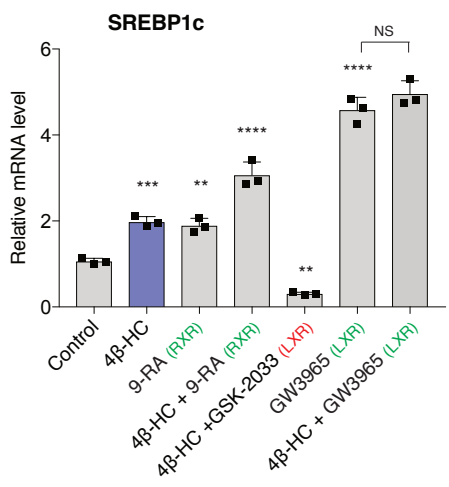
**D.** 4 $\beta$ -HC induction of lipogenic genes. Huh7 cell were treated for 24 hours with 4 $\beta$ -HC, 24-HC or LXR agonist (GW3965) followed by mRNA measurement of Fatty acid synthase (FASN), Stearoyl CoA Desaturase 1 (SCD1) and Lipin1 (LPIN1).

**E.** 4 $\beta$ -HC increases *de-novo* Lipogenesis. Huh7 treated for 24 hours with 5 $\mu$ M 4 $\beta$ -HC, 24-HC or LXR agonist (GW3965) with media containing C13 Glucose followed by Lipids extraction. C13 incorporation into TAGs was measured via Liquid chromatography Mass spec.

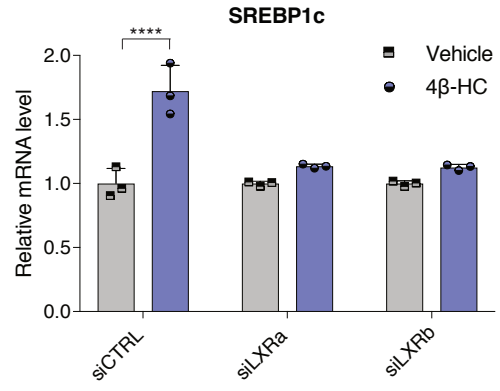
Asterisk denotes unspecific band in LXR $\alpha$  blot. Bars are Mean+SD. Statistical significance calculated by one-way ANOVA. \*p<0.05, \*\*p<0.01, \*\*\*\*p<0.0001.

Figure 3

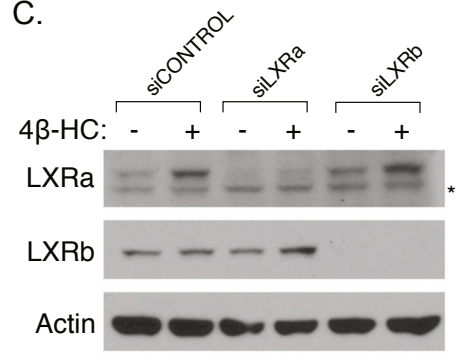
A.



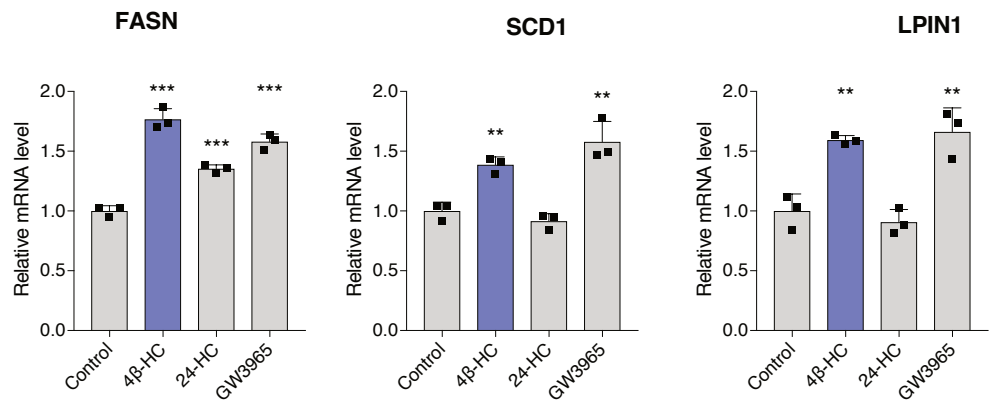
B.



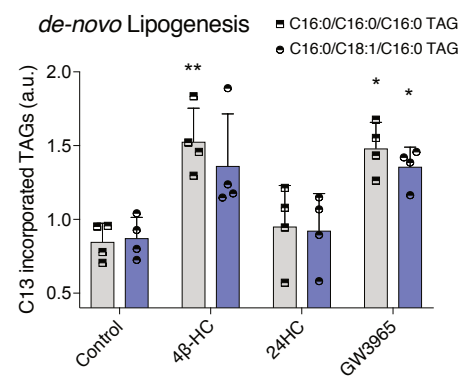
C.



D.



E.



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**Figure 4: 4 $\beta$ -HC induces lipid droplet formation and triglyceride accumulation**

**A.** 4 $\beta$ -HC increases Lipid droplet size and number. Huh7 cells were treated with 5 $\mu$ M 4 $\beta$ -HC with indicated drugs for 72 hours followed staining with lipid droplet dye, BODIPY 493/503 and visualization by confocal microscopy and **(B.)** quantified using ImageJ.

**C.** 4 $\beta$ -HC increases triglycerides (TAG) levels. Huh7 cells were treat as (C) followed by measurement of triglycerides, total cholesterol and protein levels using commercial kits.

**D.** 4 $\beta$ -HC increase lipid droplet in mice liver. Mice were fed normal chew with either Vehicle, 50mg/kg/day 4 $\beta$ -HC or 10mg/kg/day GW3965 for 5 days. Liver samples from were fixed and stained with BODIPY 493/503 and DAPI to observe lipid droplet and nuclei ultrastructure.

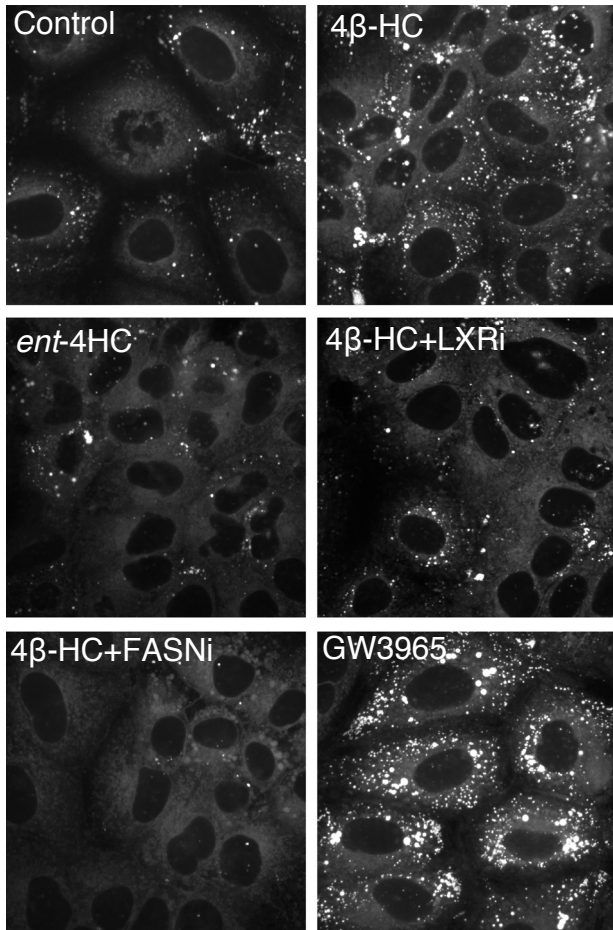
**E.** 4 $\beta$ -HC increases triglycerides (TAG) levels in mice liver treat above, followed by measurement of triglycerides, total cholesterol and protein levels using commercial kits.

For convenience, Agonist are marked in green and antagonist are marked in red. Bars are Mean+SD. Statistical significance calculated by one-way ANOVA. ent-4HC: stereo enantiomer-4HC, FASNi: TVB-3166 LXRi: GSK-2033, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.

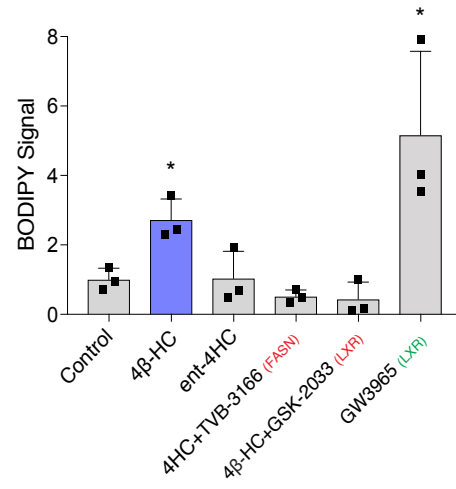


Figure 4

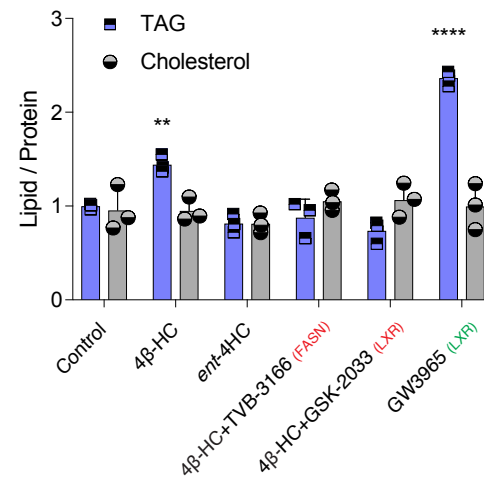
A.



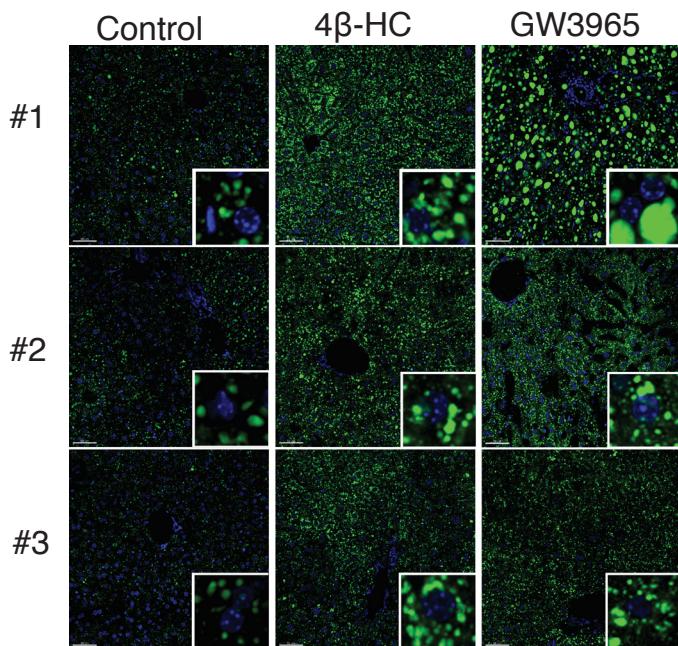
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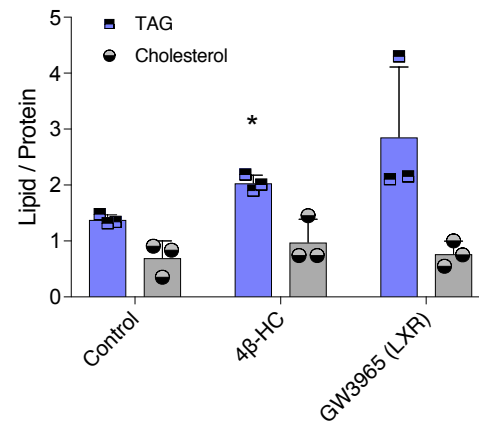
C.



D.



E.



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**Figure 5: 4 $\beta$ -HC acts in parallel to insulin-PI3K signaling to drive SREBP1c expression.**

**A.** SREBP1c transcription is additive by 4 $\beta$ -HC and Insulin. Primary hepatocytes were treated o/n with vehicle or 5 $\mu$ M 4 $\beta$ - followed with 6hrs stimulation with combinations of Insulin, PI3K inhibitor (LY294002) or Rapamycin. SREBP1c mRNA level was measured by RT-PCR.

**B.** 4 $\beta$ -HC and Insulin have an additive effect on SREBP1c expression and nuclear processing. Primary Hepatocytes were treated o/n with vehicle or 4 $\beta$ -HC followed by addition of Insulin for 40min. proteins were extracted and SREBP1 and AKT protein level were measured.

**C.** Primary hepatocytes were treated with 4 $\beta$ -HC and Insulin as described in (A) followed by RT-PCR measurement of Insig-2a mRNA level.

**D.** Insulin does not induce 4 $\beta$ -HC synthesis. Mice were fasted for 16hrs and then refed for 4 hours, followed by liver extraction and RT-PCR for SREBP1c mRNA level and (E.) 4 $\beta$ -HC levels by mass spectrometry.

**F.** Model: the 4 $\beta$ -HC-LXR pathway acts in parallel to the insulin-PI3K pathway to drive SREBP1c expression in an additive manner.

Bars are Mean+SD. Statistical significance calculated by one-way ANOVA. \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.

Figure 5

