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Research Article

Hepatology

Inflammation in response to excess low-density lipoproteins in the blood is an important driver of atherosclerosis development. Due to its ability to enhance ATP-binding cassette A1-dependent (ABCA1-dependent) reverse cholesterol transport (RCT), liver X receptor (LXR) is an attractive target for the treatment of atherosclerosis. However, LXR also upregulates the expression of sterol regulatory element-binding protein 1c (SREBP-1c), leading to increased hepatic triglyceride synthesis, an independent risk factor for atherosclerosis. Here, we developed a strategy to separate the favorable and unfavorable effects of LXR by exploiting the specificity of the coactivator thyroid hormone receptor-associated protein 80 (TRAP80). Using human hepatic cell lines, we determined that TRAP80 selectively promotes the transcription of *SREBP-1c* but not *ABCA1*. Adenovirus-mediated expression of shTRAP80 inhibited LXR-dependent SREBP-1c expression and RNA polymerase II recruitment to the LXR responsive element (LXRE) of *SREBP-1c*, but not to the LXRE of *ABCA1*. In murine models, liver-specific knockdown of TRAP80 ameliorated liver steatosis and hypertriglyceridemia induced by LXR activation and maintained RCT stimulation by the LXR ligand. Together, these data indicate that TRAP80 is a selective regulator of hepatic lipogenesis and is required for LXR-dependent *SREBP-1c* activation. Moreover, targeting the interaction between TRAP80 and LXR should facilitate the development of potential LXR agonists that effectively prevent atherosclerosis.

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Hepatic TRAP80 selectively regulates lipogenic activity of liver X receptor

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Inflammation in response to excess low-density lipoproteins in the blood is an important driver of atherosclerosis development. Due to its ability to enhance ATP-binding cassette A1-dependent (ABCA1-dependent) reverse cholesterol transport (RCT), liver X receptor (LXR) is an attractive target for the treatment of atherosclerosis. However, LXR also upregulates the expression of sterol regulatory element-binding protein 1c (SREBP-1c), leading to increased hepatic triglyceride synthesis, an independent risk factor for atherosclerosis. Here, we developed a strategy to separate the favorable and unfavorable effects of LXR by exploiting the specificity of the coactivator thyroid hormone receptor-associated protein 80 (TRAP80). Using human hepatic cell lines, we determined that TRAP80 selectively promotes the transcription of *SREBP-1c* but not *ABCA1*. Adenovirus-mediated expression of shTRAP80 inhibited LXR-dependent SREBP-1c expression and RNA polymerase II recruitment to the LXR responsive element (LXRE) of *SREBP-1c*, but not to the LXRE of *ABCA1*. In murine models, liver-specific knockdown of TRAP80 ameliorated liver steatosis and hypertriglyceridemia induced by LXR activation and maintained RCT stimulation by the LXR ligand. Together, these data indicate that TRAP80 is a selective regulator of hepatic lipogenesis and is required for LXR-dependent *SREBP-1c* activation. Moreover, targeting the interaction between TRAP80 and LXR should facilitate the development of potential LXR agonists that effectively prevent atherosclerosis.

Introduction

Liver X receptor (LXR) is a member of the nuclear receptor superfamily and acts as a cholesterol sensor to regulate the expression of several key proteins involved in lipid metabolism and inflammation (1, 2). Ligand-induced transactivation by nuclear receptors is a finely organized process that requires a variety of coactivators (3–5). Preferential functional association between particular nuclear receptors and coactivators may promote the specific regulation of individual genes under various conditions (6, 7). At present, a limited amount of information is available regarding coactivators of LXR and their mechanistic and physiological roles in LXR-mediated transactivation (8, 9).

Atherosclerosis is the leading cause of mortality in developed countries and is poised to become a worldwide health problem (10). LXR is a promising target for the treatment of atherosclerosis due to its ability to inhibit macrophage-driven inflammation and enhance ABCA1-dependent reverse cholesterol transport (RCT) (11–14). RCT is a pathway by which excess cellular cholesterol is transported from peripheral tissues to the liver for excretion in the bile and ultimately the feces, thereby helping to prevent atherosclerosis (15). Increased RCT mediated by ABCA1 upon treatment with the LXR ligands T0901317 or GW3965 contributes to an increase in serum HDL and a decrease in atherosclerosis (16, 17). Moreover, LXR exerts a further atheroprotective effect through inhibition of the macrophage inflammatory gene response (11,

18). These favorable effects of LXR agonists may be therapeutic in patients with atherosclerotic disease. However, LXR activation is also associated with increased mRNA levels of *SREBP-1c*, the master regulator of de novo fatty acid biosynthesis, leading to elevated serum and liver triglyceride levels (19–21). To optimize the therapeutic benefits of LXR agonists, it will be necessary to inhibit the unfavorable effects of LXR on fatty acid metabolism.

This study was initiated to clarify the mechanism of LXR-mediated transcription of metabolic genes, with particular focus on the details of the coactivator complex and a view toward modulating specific target gene expression. Here, we present 4 LXR α -interacting coactivators and demonstrate that 1 of these LXR α -interacting coactivators, TRAP80, is selectively recruited to the LXR responsive element (LXRE) of *SREBP-1c*, but not of *ABCA1*. TRAP80 knockdown by adenoviral shTRAP80 infection inhibited the transcription of lipogenic genes, including *SREBP-1c*, fatty acid synthase (*FAS*), and stearoyl CoA desaturase 1 (*SCD1*), but not the transcription of RCT-related genes such as *ABCA1*, *ABCG5*, and *ABCG8*. Furthermore, hepatic delivery of adenoviral shTRAP80 (Ad-shTRAP80) ameliorated hepatic steatosis induced by LXR-activated lipogenesis. Collectively, our data provide insight into the coactivator selectivity for transcriptional activation and suggest a strategy that could be exploited to amplify the beneficial effects of LXR agonists.

Results

Identification of TRAP80 and TRAP220 as direct LXR α -interacting proteins. To isolate nuclear proteins as possible coactivators that interact independently or cooperatively with LXR α , we incubated purified GST-fused LXR α ligand-binding domain (LBD) with

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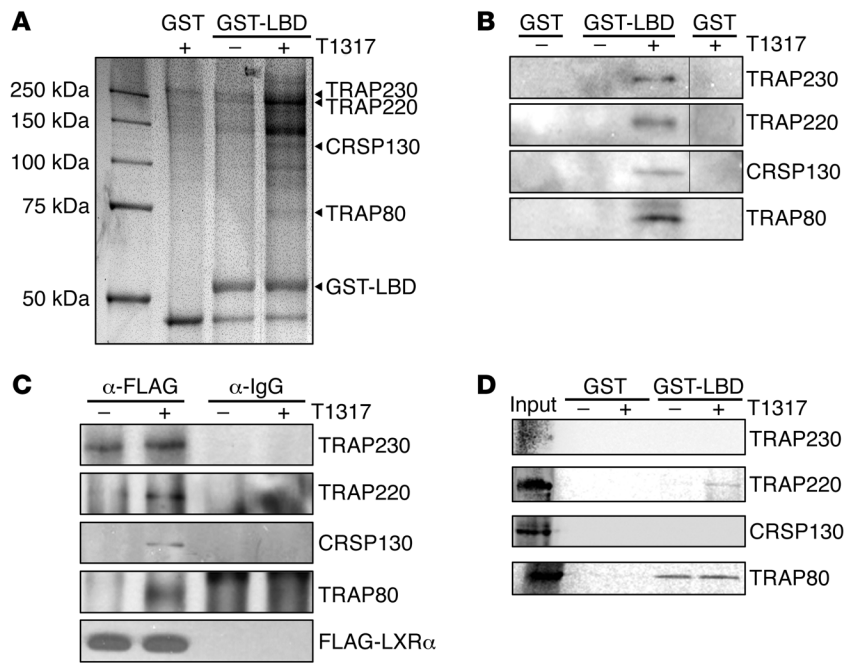


Figure 1. Purification and identification of proteins interacting with LXR α LBD. (A) Ligand-dependent interactions between LXR α LBD and several nuclear proteins from HepG2 cells. Immobilized GST-LXR α LBD was incubated with HepG2 nuclear extracts in the presence or absence of T0901317. Immobilized GST was used as a control protein in the presence of ligand. Arrowheads indicate LXR α -interacting proteins analyzed by MALDI-TOF. (B) Identification of LXR α LBD interactors by immunoblot analysis. The thin black lines indicate that the lanes were run on the same gel but were noncontiguous. The TRAP80 blot was derived from samples run on a parallel gel. (C) Immunoblot analyses of proteins immunoprecipitated from FLAG-LXR α -transfected HEK293T cells using anti-FLAG antibodies. (D) GST pull-down assays were used to evaluate the binding between GST-LXR α LBD and [35 S]-methionine-labeled interactors. GST-LBD, GST-LXR α LBD; T1317, T0901317.

HepG2 nuclear extracts in the presence or absence of the LXR agonist T0901317 (Figure 1A and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI173615DS1). Compared with GST alone, 4 proteins bound specifically to GST-LXR α LBD in a ligand-dependent manner (Figure 1A). Using matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) analysis, we identified these LXR α LBD-interacting proteins as TRAP230 (ARC240 and MED12), TRAP220 (DRIP205, CRSP200, PBP, and MED1), CRSP130 (DRIP130, ARC130, SUR2, and MED23), and TRAP80 (DRIP80, ARC77, CRSP77, and MED17) (Supplemental Table 1 and refs. 22–26). We confirmed the identities of the GST-LXR α LBD-bound proteins by immunoblot analyses with specific antibodies (Figure 1B). To verify that these interactions took place inside the cells, total lysates from FLAG-LXR α -transfected cells were immunoprecipitated with anti-FLAG antibodies and subsequently probed with antibodies against each interacting protein (Figure 1C). All 4 proteins interacted with LXR α in a ligand-dependent manner in the cells. These results demonstrate that LXR α interacts with TRAP230, TRAP220, CRSP130, and TRAP80 via its LBD in a ligand-dependent manner.

Next, we examined whether any of these proteins bind directly to the LXR α LBD using *in vitro*-translated and [35 S]-labeled interactors. Only 2 of the proteins, TRAP220 and TRAP80, interacted directly with the LXR α LBD (Figure 1D). Interestingly, [35 S]-TRAP220 bound to GST-LXR α LBD in a ligand-dependent manner, whereas the [35 S]-TRAP80 interaction with GST-LXR α LBD was ligand independent in a 2-component protein-protein interaction assay.

TRAP80 stimulates the transcriptional activity of the LXRE of SREBP-1c but not of the LXRE of ABCA1. To determine the functional relevance of the interaction of TRAP220 and TRAP80 with LXR α , we performed cotransfection and luciferase reporter assays. Both TRAP220 and TRAP80 stimulated the transcrip-

tional activity of LXR α on a synthetic LXRE promoter in a ligand-dependent manner (Figure 2A). We also examined their coactivator functions on 2 physiologically important natural promoters containing LXRE, specifically those of *SREBP-1c* and *ABCA1*. We observed that TRAP220 enhanced ligand-dependent transactivation of both the *SREBP-1c* and *ABCA1* promoters (Figure 2, B and D). By contrast, TRAP80 increased the transcriptional activity of the *SREBP-1c* promoter, but not that of the *ABCA1* promoter (Figure 2, B and D). Importantly, neither TRAP220 nor TRAP80 stimulated the transcription of *SREBP-1c* and *ABCA1* promoters containing mutations in the LXRE, indicating that their coactivator function, at least in these cases, is specifically dependent on LXR recruitment to the LXRE (Figure 2, C and E). We also examined this target gene-specific activity of TRAP80 using GW3965, a specific LXR ligand structurally unrelated to T0901317. Again, TRAP80 showed LXR-mediated selective activation of the *SREBP-1c* promoter by GW3965 in a dose-dependent manner (Figure 2F). Dose-dependent activation of the *ABCA1* promoter by GW3965 was not affected by TRAP80 (Figure 2G). Meanwhile, we found that TRAP230 and CRSP130, the indirect interactors of LXR α , stimulated both *SREBP-1c* and *ABCA1* LXRE promoters (Supplemental Figure 2).

In vivo transcription occurs on a nucleosomal template, i.e., chromatin. To investigate the recruitment of TRAP220 and TRAP80 in endogenous chromatin, we performed ChIP analyses of the *Srebp-1c* and *Abca1* promoter regions (Figure 3A). Both TRAP220 and TRAP80 were recruited to the LXRE of the endogenous *Srebp-1c* promoter in response to treatment with T0901317 or GW3965 (Figure 3, B and D). By contrast, TRAP220 was recruited to the LXRE of the *Abca1* promoter, but TRAP80 was not (Figure 3, C and E). We also investigated the recruitment of TRAP220 and TRAP80 under the condition of LXR α knockdown to confirm whether the recruitment of those coactivators is LXR α dependent. LXR α -specific knockdown in mouse primary hepatocytes

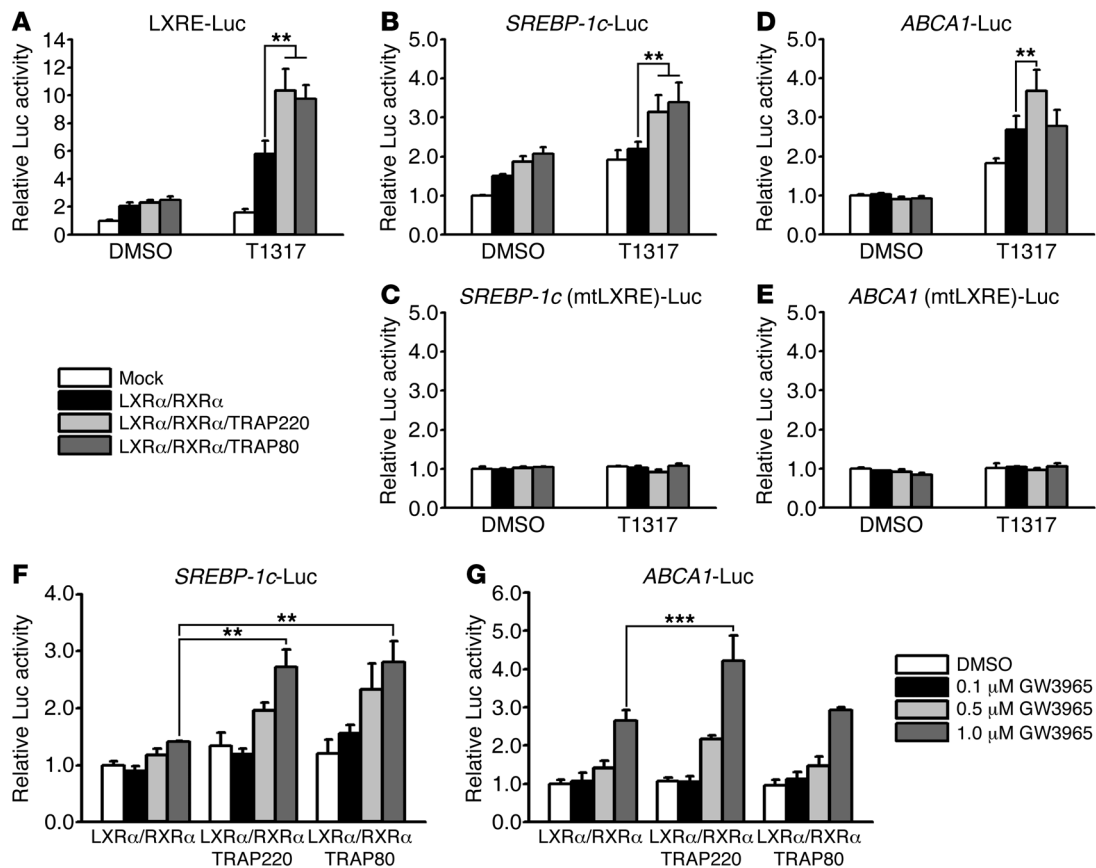


Figure 2. TRAP80 selectively stimulates *SREBP-1c* promoter activity in a ligand-dependent manner via LXRE. HepG2 cells were transfected with a luciferase reporter plasmid under the control of synthetic LXRE (A, $n = 7$), *SREBP-1c* WT promoter (B, $n = 9$; F, $n = 6$), *ABCA1* WT promoter (D, $n = 10$; G, $n = 6$), *SREBP-1c* mutant promoter (C, $n = 6$), or *ABCA1* mutant promoter (E, $n = 5$) with mutated DR-4 LXRE. T0901317 (A–E) or GW3965 (F and G) was used to activate the LXRs. Data are presented as the mean \pm SEM. T1317, T0901317; Luc, luciferase; mt, mutant. ** $P < 0.01$ and *** $P < 0.001$, by 1-way ANOVA.

by siRNA was validated by quantitative reverse transcriptase PCR (qRT-PCR) (Supplemental Figure 3). As a result, the recruitment of both TRAP220 and TRAP80 to *Srebp-1c* LXRE was not stimulated by LXR ligands under LXR α -knockdown conditions (Figure 3, B and D). We found that TRAP80 was not recruited to *Abca1* LXRE, regardless of the expression of LXR α (Figure 3E). Interestingly, we observed that the recruitment of TRAP220 to the *Abca1* LXRE was increased by LXR ligands, even under LXR α -knockdown conditions (Figure 3C). In conclusion, TRAP80 was selectively recruited in a ligand- and LXR α -dependent manner to the *Srebp-1c* promoter, but not to the *Abca1* promoter.

*TRAP80 regulates the recruitment of RNA polymerase II and histone modification of the LXRE region of *Srebp-1c*, but not that of *Abca1*.* To address the consequences of the differential effects of TRAP80 on the *Srebp-1c* and *Abca1* promoters, we introduced *TRAP80* cDNA or Ad-shTRAP80 into the cells and determined the levels of *Srebp-1c* and *Abca1* transcripts by qRT-PCR. TRAP80 overexpression significantly increased *Srebp-1c* mRNA expression, but did not affect *Abca1* mRNA levels (Figure 4A). Consistent with this, Ad-shTRAP80 significantly suppressed the T0901317-dependent increase of *Srebp-1c* transcription, but did not affect *Abca1* transcription (Figure 4B). Knockdown of TRAP80 using siRNA yielded results similar to those obtained with Ad-shTRAP80 (Supplemental Figure 4). To further inves-

tigate the role of TRAP80 in *Srebp-1c* and *Abca1* promoter activation, we performed ChIP assays using antibodies against RNA polymerase II (Pol II) or acetyl-histone H4 (H4Kac). While Ad-shTRAP80 abolished the T0901317-dependent recruitment of Pol II to the LXRE of the *Srebp-1c* promoter, we found that recruitment of Pol II to the LXRE of the *Abca1* promoter was not significantly altered (Figure 4C). The recruitment of transcriptional regulators to the promoter chromatin is coordinated by epigenetic information encoded in covalent modifications of histone proteins (27). Histone acetylation is a central switch that allows interconversion between transcriptionally permissive or repressive chromatin states (28). We found that infection of mouse primary hepatocytes with Ad-shTRAP80 consistently decreased T0901317-dependent acetylation of histone H4 lysine (H4K) in the vicinity of the LXRE of the *Srebp-1c* promoter (Figure 4D). However, ligand-dependent acetylation of H4K near the LXRE of the *Abca1* promoter was still significantly increased in the presence of shTRAP80, although this acetylation was slightly attenuated (Figure 4D). Meanwhile, we observed that LXR was recruited to the LXRE regions of the *Srebp-1c* and *Abca1* genes in a ligand-dependent manner, regardless of TRAP80 expression (Figure 4E). Taken together, these results suggest that TRAP80 selectively stimulates *SREBP-1c* expression by Pol II recruitment and chromatin remodeling.

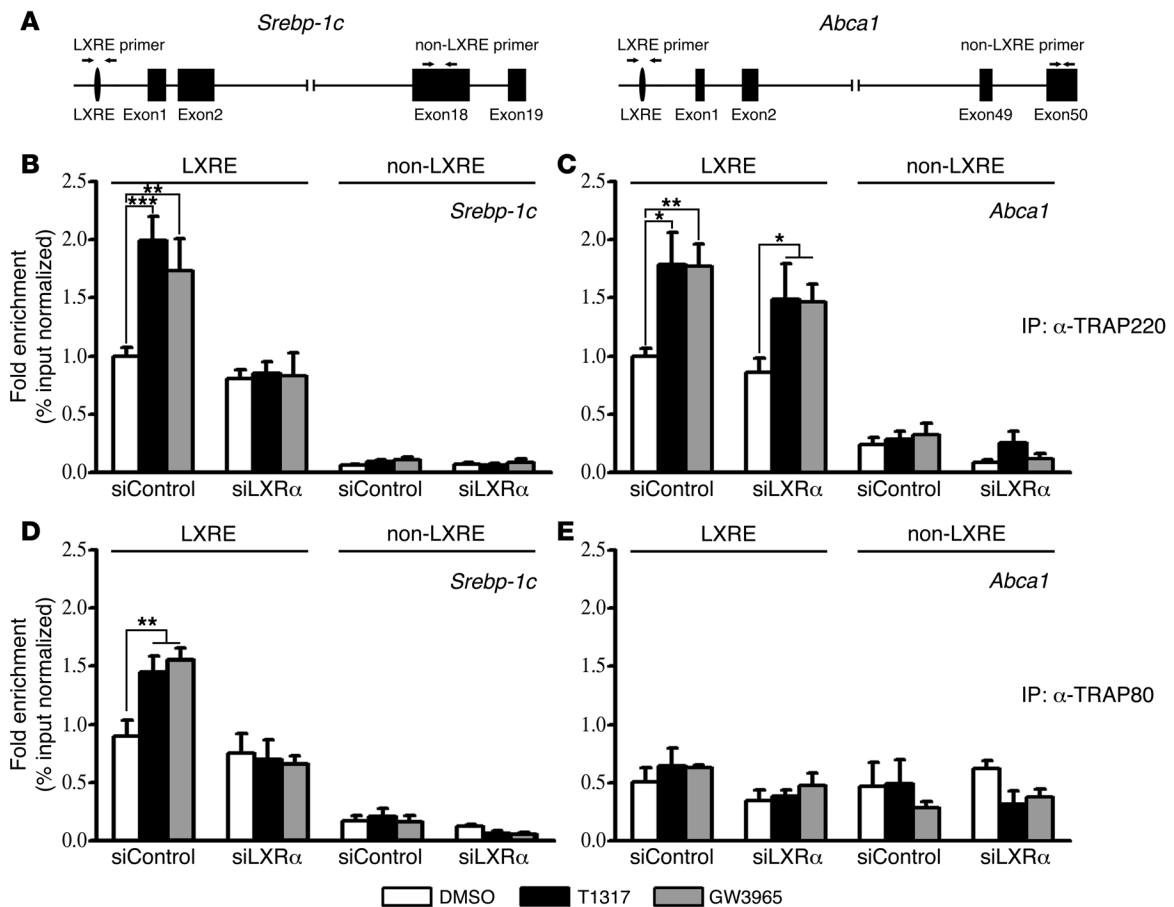


Figure 3. TRAP80 is selectively recruited to the LXRE of the *Srebp-1c* promoter. (A) Schematic diagram of primer locations used for the ChIP assay across the *Srebp-1c* or *Abca1* genomic structure. (B–E) ChIP assays were performed with anti-TRAP220 (B and C) or anti-TRAP80 (D and E) antibodies in mouse primary hepatocytes following LXRα knockdown by siRNA (sc-38829). Recruitment of TRAP220 or TRAP80 to the LXREs of the *Srebp-1c* and *Abca1* genes was determined by qPCR ($n = 3$). The level of recruitment is expressed as the fold enrichment relative to the recruitment of TRAP220 to the LXREs in the presence of DMSO alone. Data are presented as the mean \pm SEM. T1317, T0901317. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, by 1-way ANOVA.

Hepatic knockdown of TRAP80 represses LXR-dependent lipogenesis in the liver. To investigate the physiological effects of TRAP80 modulation, we administered Ad-shTRAP80 to the liver via tail vein injection (Supplemental Figure 5). Five days after Ad-shTRAP80 injection, GW3965 was administered for 3 days. Surprisingly, we found that Ad-shTRAP80 prevented GW3965-induced hepatic steatosis (Figure 5A) and significantly reduced hepatic triglyceride accumulation (Figure 5B). In addition, Ad-shTRAP80 reduced the GW3965-induced increase in serum triglyceride levels (Figure 5C). Separation of serum lipoprotein classes by fast protein liquid chromatography (FPLC) indicated that the decrease of serum triglycerides in Ad-shTRAP80-administered mice treated with GW3965 was attributed to a decrease of triglycerides in the VLDL fraction (Figure 5D). We then determined the effects of hepatic TRAP80 knockdown on the RCT process. The increases in both biliary cholesterol output and fecal neutral sterol loss by GW3965 were not affected by TRAP80 knockdown (Figure 5, E and F). In addition, we also observed an increase in HDL cholesterol by GW3965 in the mice injected with Ad-shTRAP80 (Figure 5G). The T0901317 treatment of Ad-shTRAP80-injected mice did not increase hepatic lipogenesis or hypertriglyceridemia, either (Supplemental Figure

6), whereas the level of anti-atherogenic HDL cholesterol was still increased by T0901317 under hepatic TRAP80-knockdown conditions (Supplemental Figure 6F). We also examined the effects of TRAP80 knockdown on the lipoprotein cholesterol and triglyceride profile in *ApoE*^{-/-} mice, an experimental animal model of atherosclerosis, and found that the increase in VLDL cholesterol and triglycerides by GW3965 was diminished in Ad-shTRAP80-injected *ApoE*^{-/-} mice (Supplemental Figure 7). These results suggest that the detrimental effects of LXR activation could be selectively controlled by TRAP80 modulation.

Ad-shTRAP80 selectively inhibits lipogenic gene expression in the liver, but does not affect the expression of RCT-related genes or antiinflammatory effects in macrophages. In line with the serum lipid profile, the GW3965- and T0901317-induced transcription of lipogenic genes (*Srebp-1c*, *Fas*, and *Scd1*) was repressed by Ad-shTRAP80 expression in the liver (Figure 6A and Supplemental Figure 8A). On the other hand, Ad-shTRAP80 did not decrease GW3965- or T0901317-induced transcription of RCT-related genes (*Abca1*, *Abcg5*, and *Abcg8*) (Figure 6B and Supplemental Figure 8B). Recently, inducible degrader of low-density lipoprotein receptor (*Idol*) was reported as a new LXR target gene that regulates hepatic cholesterol uptake by ubiquitination of the LDL

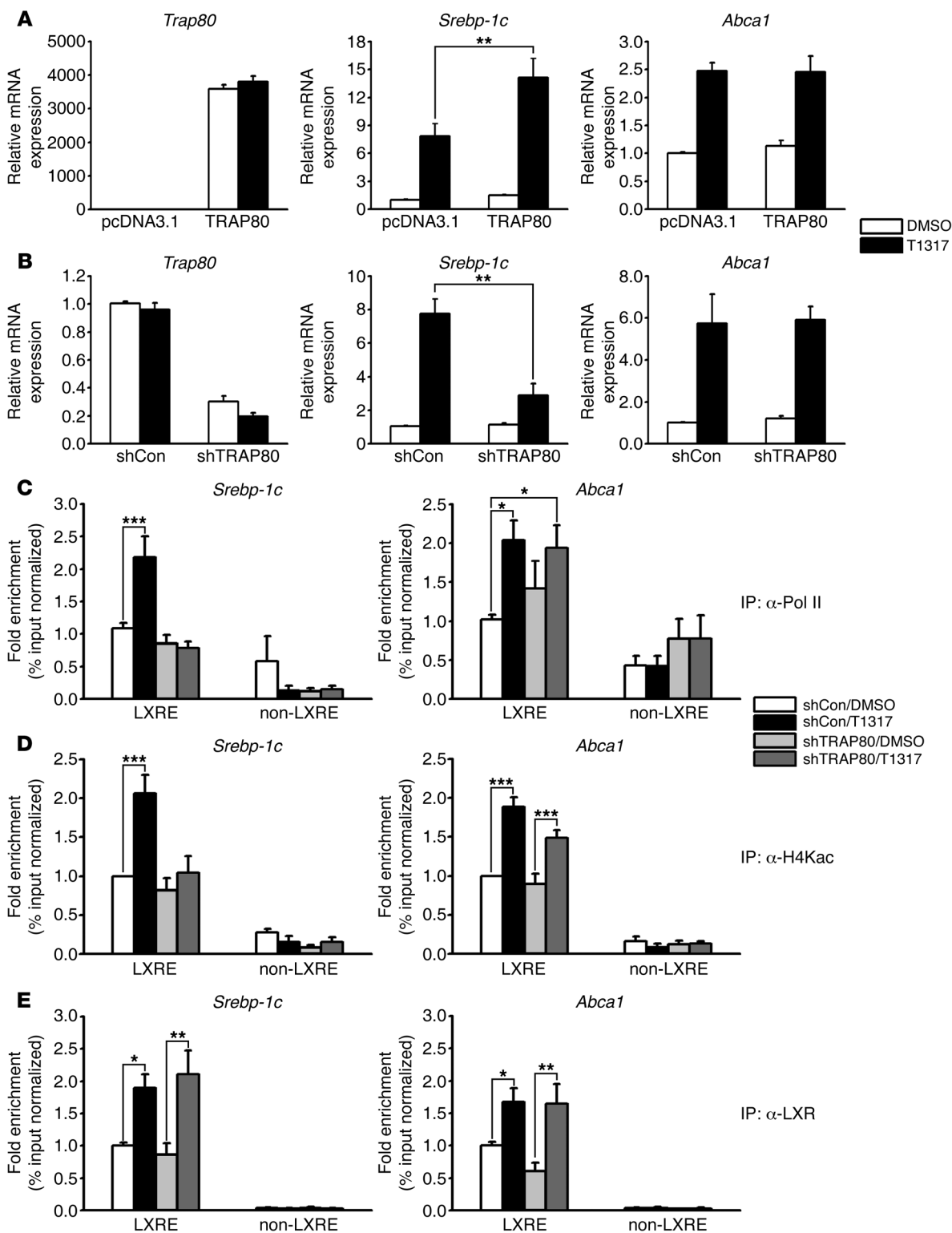


Figure 4. TRAP80 regulates *Srebp-1c* transcript levels by recruiting RNA Pol II. (A and B) Changes in *Srebp-1c* and *Abca1* mRNA levels following TRAP80 overexpression (A, $n = 5$) or infection with Ad-shTRAP80 (B, $n = 6$) in mouse primary hepatocytes. (C–E) ChIP assays were performed with anti-Pol II (C, $n = 5$), anti-H4Kac (D, $n = 5$), or anti-LXR α antibodies (E, $n = 4$) in mouse primary hepatocytes infected with Ad-shTRAP80. Abundance of each protein bound to *Srebp-1c* or *Abca1* promoters was determined by qPCR. Enrichment levels are expressed as the fold enrichment relative to the enrichment observed in controls (shCon/DMSO). Data are presented as the mean \pm SEM. T1317, T0901317. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ by 1-way ANOVA.

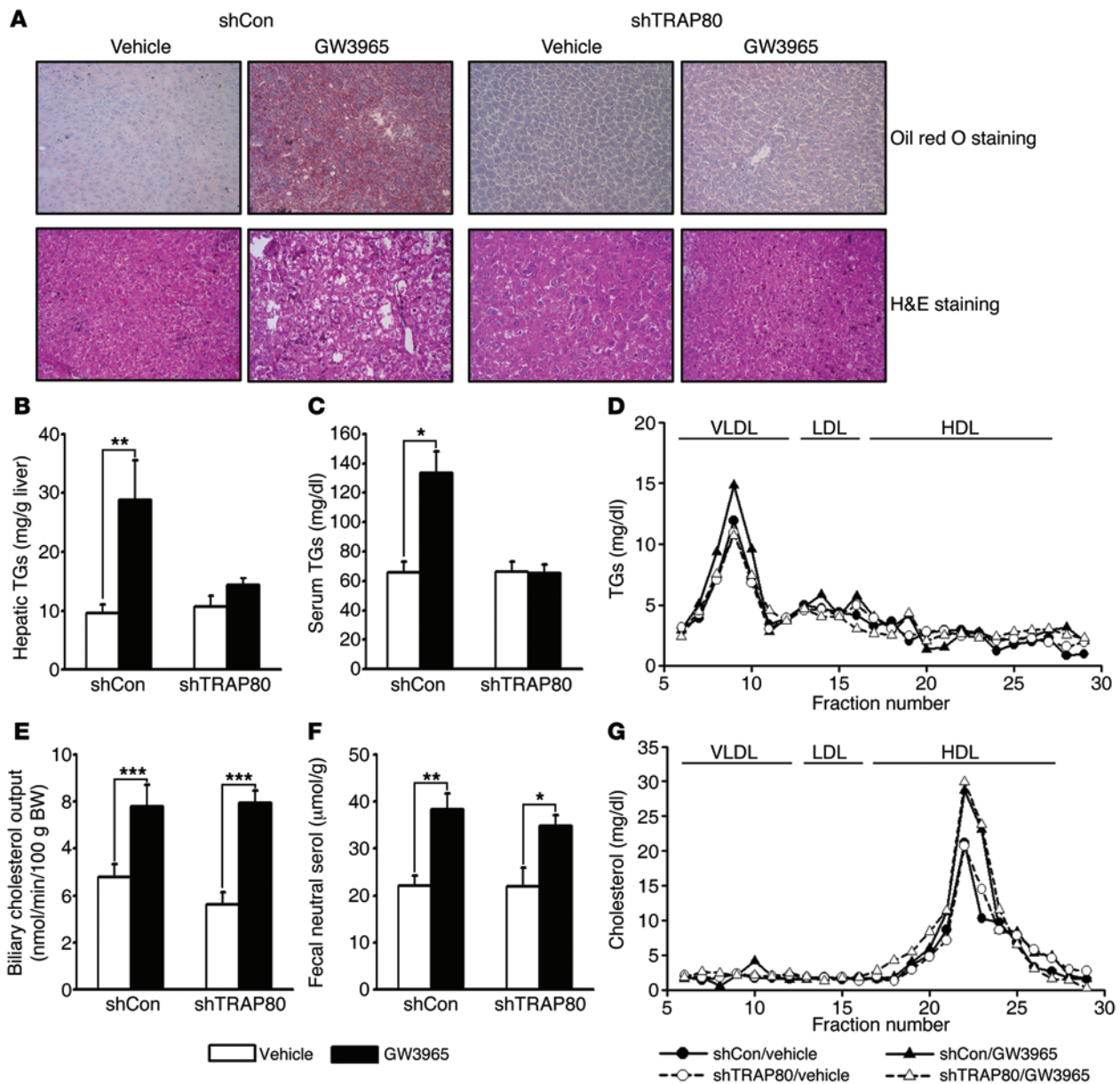


Figure 5. Knockdown of hepatic TRAP80 ameliorates GW3965-induced fatty liver and an aberrant serum lipid profile. (A) Representative images of H&E and oil red O-stained liver of Ad-shTRAP80-injected mice after administration of 50 mg/kg GW3965 for 3 days. Original magnification, $\times 100$. (B–D) Changes in hepatic triglyceride (B) and serum triglyceride (C and D) levels in mice injected with Ad-shTRAP80 via the tail vein ($n = 5$ for each group). (E–G) Effects of Ad-shTRAP80 on GW3965-mediated RCT. Biliary cholesterol output (E), fecal neutral sterol (F), and HDL cholesterol (G) were measured ($n = 5$ for each group). The lipoprotein profile of mouse serum was analyzed by FPLC (D and G). Data are presented as the mean \pm SEM. TG, triglyceride. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, by 2-tailed Student's t test.

receptor (LDLR) (29). The IDOL-mediated inhibition of cholesterol uptake is independent of and complementary to the SREBP pathway (30). Interestingly, we found that the increases in *Idol* expression by GW3965 or T0901317 were differently affected by TRAP80 knockdown (Figure 6B and Supplemental Figure 8B). Meanwhile, macrophages are the primary cell type that becomes overloaded with cholesterol in atherosclerotic lesions and thus mediates the activation of the inflammatory pathway (15, 31). We found that the T0901317-dependent transcription of RCT-related genes in mouse peritoneal macrophages was not affected by Ad-shTRAP80 (Figure 6C). In contrast, the LXR-dependent

increases of lipogenic genes were repressed in Ad-shTRAP80-infected macrophages (Figure 6D). Importantly, the inhibitory effects of T0901317 on the inflammatory gene response to LPS were reproduced in mouse peritoneal macrophages, even under TRAP80-knockdown conditions (Figure 6E). Taken together, these results suggest that TRAP80 selectively regulates the lipogenic activity of LXR, but not RCT or the antiinflammatory effects induced by LXR activation. On the basis of the results described herein, we propose a model for selective activation of the RCT process by simultaneous TRAP80 knockdown and treatment with LXR ligand (Figure 7).

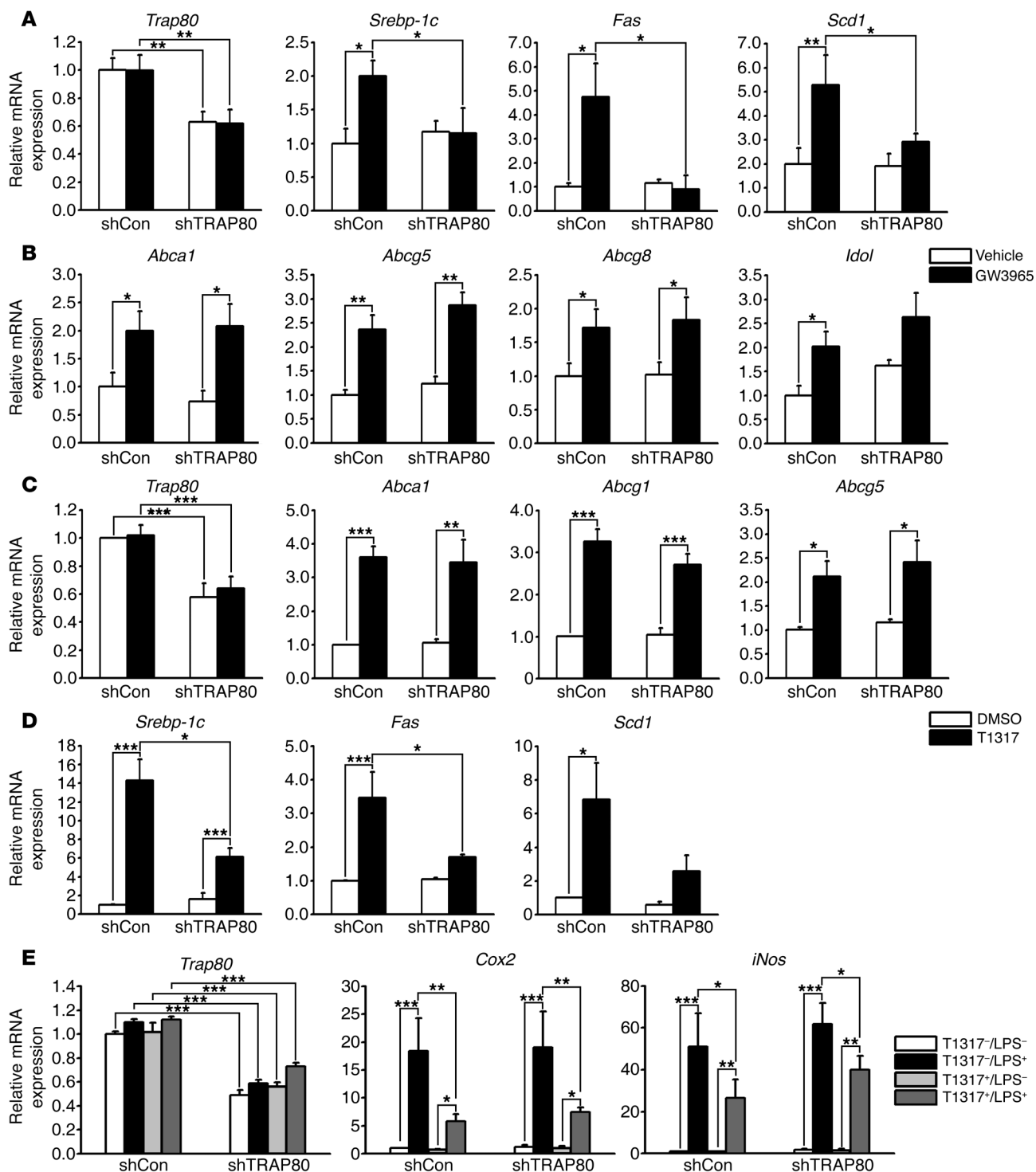


Figure 6. Effects of TRAP80 on the expression of metabolic and inflammatory genes in the liver and peritoneal macrophages. (A and B) Effects of TRAP80 knockdown on the transcript levels of lipogenesis- (A) or RCT-related genes (B) in the livers of mice treated with GW3965 ($n = 5$ for each group). mRNA levels were measured by qRT-PCR. (C and D) Effects of Ad-shTRAP80 on the transcript levels of RCT- (C) or lipogenesis-related genes (D) in mouse peritoneal macrophages treated with DMSO or $1 \mu\text{M}$ T0901317 ($n = 4$). (E) Effects of Ad-shTRAP80 on the transcript levels of inflammation-related genes in mouse peritoneal macrophages ($n = 4$). Macrophages were pretreated with $1 \mu\text{M}$ T0901317 for 18 hours and then challenged with 100 ng/ml LPS for another 24 hours. Data are presented as the mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, by 1-way ANOVA.

Discussion

In the present study, we identified LXR α -interacting nuclear proteins in HepG2 cells in an effort to elucidate the lipogenic processes mediated by LXR in the liver. There are 2 subtypes of LXR: LXR α

and LXR β . No obvious phenotype has yet been attributed to *Lxrb*-knockout mice. However, *Lxra*-knockout mice exhibited decreased serum triglyceride levels as well as decreased hepatic mRNA levels of multiple enzymes that participate in fatty acid synthesis,

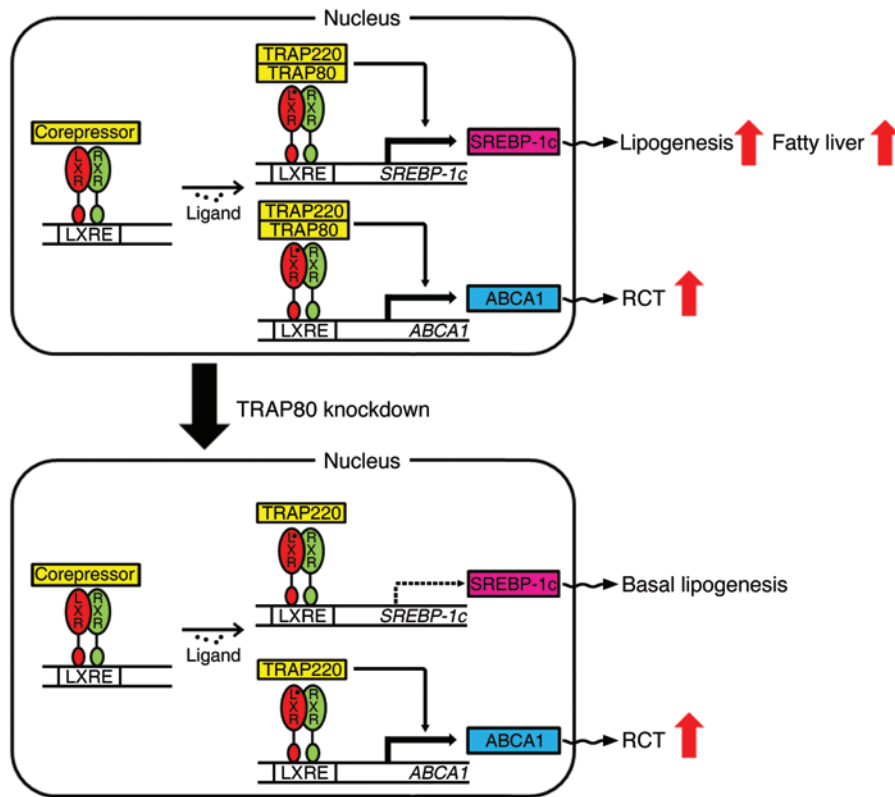


Figure 7. Working model for selective activation of RCT-related genes by simultaneous TRAP80 knockdown and treatment with LXR ligand. TRAP80 is essential for the activation of lipogenic genes including *SREBP-1c*, but not for RCT-related genes, such as *ABCA1*. Therefore, under conditions of TRAP80 knockdown, LXR ligand treatment selectively activates RCT-related genes.

including FAS (32). These findings suggest that LXR α is primarily responsible for hepatic triglyceride accumulation and hypertriglyceridemia, effects that have hampered the development and use of LXR agonists as therapeutic agents (13). The adverse lipogenic effects of LXR α are in part mediated by LXR-induced transcription of *SREBP-1c*, a transcription factor that also induces the transcription of numerous lipogenic genes (33–35). To circumvent the adverse effect of LXR agonists, it is necessary to understand the mechanism by which *SREBP-1c* is activated by LXR α and identify the proteins involved in this activation process.

In this regard, we report for the first time to our knowledge the role of TRAP80 in the transcriptional regulation of *SREBP-1c* by LXR α . TRAP80 was first isolated as a component of the thyroid hormone receptor-associated protein (TRAP), which was initially identified as a large multimeric complex that copurifies with the thyroid hormone receptor (TR) from HeLa cells and distinctly increases TR-mediated transcription in vitro (23). Later, TRAP80 was also shown to interact directly with p53 and VP16 activation domains (22). TRAP80, also called DRIP77, ARC77, or CRSP77, is also identified as a subunit of vitamin D receptor-interacting protein (DRIP), activator-recruited cofactor (ARC), or cofactor required for Sp1 activation (CRSP), respectively (24, 26, 36). ARC can be purified by affinity chromatography using the SREBP-1 α activation domain and has been shown to interact directly with VP16 and the NF- κ B p65 subunit (26). Although more recent studies implicate TRAP as a coactivator of a wide range of nuclear hor-

mone receptors as well as other classes of transcription factors, most studies focus on TRAP220/DRIP205, because it is thought to form an interface between the nuclear receptor and the TRAP complex and interact with nuclear receptors in a ligand-dependent manner (37). Consequently, very little is known about the other subunits of the TRAP complex, including TRAP80. TRAP80 is a 651-amino acid protein and is equivalent to the p78 component of the mouse Mediator (38). Our study shows that TRAP80 interacts with LXR α LBD in a ligand-dependent manner in nuclear extract-binding experiments and in a ligand-independent manner in 2-component GST pull-down assays. The same interaction pattern was seen between vitamin D receptor-LBD (VDR-LBD) and TRAP80/DRIP77 (36). This suggests that the ligand-dependent interactions between TRAP80 and LXR and VDR require nuclear component(s) other than TRAP80 and LXR or VDR alone. Interestingly, although full-length dTRAP80 of *Drosophila* interacts with retinoid X receptor (RXR) LBD only in the presence of the RXR ligand, 9-*cis* retinoic acid, a fragment of dTRAP80 possesses a sticky region that interacts with RXR LBD, regardless of the presence of ligand (39).

Here, we purified 4 coactivators with LXR α in the presence of ligand from hepatoma HepG2 cells and from HEK 293T cells transfected with FLAG-LXR α . Two of the coactivators, TRAP220 and TRAP80, interacted with LXR α directly and enhanced ligand-dependent transcription of the synthetic LXRE-driven reporter gene. However, while TRAP220 activated the transcription of both *SREBP-1c* and *ABCA1*, TRAP80 selectively activated *SREBP-1c*, but not *ABCA1*, although both are LXR target genes. This specificity of TRAP80 is attributed to selective recruitment to the *SREBP-1c* promoter, but not to the *ABCA1* promoter. The ligand-stimulated recruitment of TRAP80 to the *SREBP-1c* LXRE was completely dependent on LXR α . On the other hand, it is noteworthy that LXR α -specific knockdown did not block TRAP220 recruitment to the *ABCA1* promoter, while it completely blunted ligand-dependent recruitment of both TRAP220 and TRAP80 to the *SREBP-1c* promoter. Based on these observations, we hypothesized that LXR α plays a major role in T0901317- or GW3965-dependent activation of the *SREBP-1c* gene and recruitment of relevant coactivators to the LXRE region of *SREBP-1c*, while LXR β has a main role in the LXR ligand-dependent activation of the *ABCA1* gene via the recruitment of TRAP220.

The ChIP assay using anti-RNA Pol II and -H4KAc antibodies showed that TRAP80 could facilitate the recruitment of RNA Pol II via chromatin remodeling through acetylation of H4 near the LXRE region of the *Srebp-1c* gene. However, we observed that LXR was recruited to the LXRE regions of both *Srebp-1c* and *Abca1*

genes, irrespective of TRAP80 expression. This suggests that TRAP80 stimulates *SREBP-1c* transcription through the increase in RNA Pol II recruitment and histone acetylation, at least in part, but not through the enhancement of LXR binding. Consequently, Ad-shTRAP80 selectively decreased lipogenic gene expression, but did not affect the expression of RCT-related genes or the anti-inflammatory effects of the LXR ligand. Meanwhile, IDOL is the E3 ubiquitin ligase that stimulates LDLR degradation and thus inhibits cholesterol uptake. In our study, *Idol* expression was differently regulated by Ad-shTRAP80, depending on the LXR ligands. The meaning of this differential regulation of IDOL by Ad-shTRAP80 in the context of different LXR ligands remains to be determined.

These differential effects of TRAP80 on LXR target gene expression resulted in alleviation of fatty liver and potentiation of RCT activity in the combinatorial treatment with LXR agonist and shTRAP80. In terms of RCT, we measured the expression of ABC transporters (ABCA1, ABCG1, ABCG5, and ABCG8) and the levels of biliary cholesterol and fecal sterol. RCT is a multistep process that occurs across several tissues including macrophages, liver, and intestine. Moreover, the ABC transporters are involved in the overall RCT process, from macrophages to feces, not just in cholesterol efflux from macrophages. Thus, we investigated the very last steps in RCT — biliary cholesterol secretion and fecal sterol loss — to evaluate the effect of TRAP80 on LXR-stimulated RCT.

This study suggests that the manipulation of TRAP80 represents a mechanism by which SREBP-1c-mediated lipogenesis can be selectively repressed while preserving RCT stimulation by LXR α . Therefore, TRAP80 could be a promising target not only for the treatment of fatty liver, but also for the development of LXR agonists that effectively prevent atherosclerosis. In addition, the mechanism of coactivator selectivity described here might be used to modulate the specific expression of other key regulatory proteins responsible for the pathogenesis of a diverse array of diseases.

Methods

GST pull-down assays. GST pull-down assays were performed as described previously (25). Prior to incubation with the HepG2 nuclear extracts, glutathione-sepharose bead-immobilized GST and GST-LXR α LBD were normalized to equimolarity by quantitation using SDS-PAGE. After binding (20 mM HEPES [pH 7.6], 20% glycerol, 0.2 mM EDTA, 180 mM KCl, 1 mM DTT, 0.05% nonidet P-40, 0.5 mM PMSF, 10 μ M T0901317) and washing (20 mM HEPES [pH 7.6], 20% glycerol, 0.2 mM EDTA, 180 mM KCl, 1 mM DTT, 0.1% nonidet P-40, 0.5 mM PMSF), samples were eluted with 0.2% sarkosyl in wash buffer, resolved by 4% to 20% SDS-PAGE (Bio-Rad), and analyzed either by staining with Silver Stain Plus (Bio-Rad) or by immunoblot analysis.

Identification of LXR α LBD-interacting proteins by mass spectrometry. LXR α LBD-interacting proteins in silver-stained gel slices were subjected to in-gel trypsin digestion. Extracted peptides were desalted using a ZipTip (EMD Millipore), resuspended in matrix, and subjected to MALDI-TOF analysis using a Voyager Biospectrometry system (Applied Biosystems) to yield a peptide mass fingerprint. Data were processed with the Mascot program (www.matrixscience.com).

IP and immunoblot analyses. HEK293T cells were treated with 1 μ M T0901317 for 2 hours, washed in PBS, and lysed on ice for 10 minutes in lysis buffer consisting of 1% NP-40, 10% glycerol, and 50 mM Tris-Cl (pH 7.4). IP was performed by adding 6 μ g anti-FLAG

antibody (F7425; Sigma-Aldrich) and incubating overnight at 4°C in IP buffer (180 mM KCl, 1% NP-40, 10% glycerol, and 50 mM Tris-Cl [pH 7.4]). Immune complexes were precipitated with protein G-agarose (sc-2002; Santa Cruz Biotechnology Inc.), washed with IP buffer, and resuspended in reduced sample buffer. Proteins were then separated by SDS-PAGE, transferred onto PVDF membranes (Qbiogene), and detected with antibodies against TRAP230 (sc-5374), TRAP220 (sc-5334), CRSP130 (sc-12454), TRAP80 (ARP34192_P050; Aviva Systems Biology), β -actin (A5316; Sigma-Aldrich), and FLAG.

Reporter plasmid construction and luciferase reporter assay. Promoter-reporter constructs, including *SREBP-1c-Luc*, *SREBP-1c (mtLXRE)-Luc*, *ABCA1-Luc*, and *ABCA1 (mtLXRE)-Luc*, were generated using a PCR-cloning strategy. The PCR primer sequences are listed in Supplemental Table 2. The TRAP80 expression construct was provided by Jae U. Jung (University of Southern California, Los Angeles, California, USA). The TRAP230 and CRSP130 were provided by Robert Roeder (Rockefeller University, New York, New York, USA) and Mononari Uesugi (Kyoto University, Kyoto, Japan), respectively. The reporter gene and β -gal constructs were cotransfected into HepG2 cells with or without LXR α , RXR α , TRAP230, TRAP220, CRSP130, and TRAP80 expression constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Eighteen to 24 hours after transfection, cells were treated with T0901317 or GW3965 for 24 hours. Cells were then collected for the measurement of luciferase and β -gal activity. Luciferase activity was measured using a Luminometer Centro LB 960 (Berthold) and normalized against β -gal activity.

ChIP assay. ChIP assays were performed as described previously, with minor modifications (40). Briefly, cells were treated with 1% formaldehyde for 20 minutes at room temperature. Next, cells were washed with cold PBS and resuspended in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 1% SDS, and 10 mM EDTA, followed by incubation for 30 minutes at 4°C. Soluble chromatin from cells was prepared by sonication and immunoprecipitated with antibodies against TRAP220 (sc-5334; Santa Cruz Biotechnology Inc.), TRAP80 (sc-12453; Santa Cruz Biotechnology Inc.), RNA polymerase II (sc-900X; Santa Cruz Biotechnology Inc.), H4Kac (06-598; EMD Millipore), and LXR (sc-13068; Santa Cruz Biotechnology Inc.). The final DNA extracts were amplified by qPCR using primers specific for the LXRE and non-LXRE regions of *Srebp-1c* and *Abca1* (Supplemental Table 3). The ChIP-qPCR data were normalized to input DNA sample and expressed as a fold change.

Generation of TRAP80 Ad-shRNA. To construct Ad-shTRAP80, shRNA-coding oligonucleotides were synthesized. For insertion of the shRNA sequence into the adenoviral shuttle vector pRNAT-H1.1/Adeno (SD1219; GenScript Inc.), *MluI*/*HindIII* restriction sequences and a T5 terminal sequence were included in the shRNA-encoding oligonucleotide (Supplemental Table 2). The synthesized oligonucleotides were annealed and inserted into pRNAT-H1.1/Adeno. The shRNA-containing shuttle vector was then linearized using *PmeI*, and the linearized pRNAT1.1/Adeno was recombined with the pAdEasy-1 vector by in vivo homologous recombination in the *E. coli* strain BJ5183. The resulting DNA was digested with *PacI* and transfected into Ad-293 cells using Lipofectamine 2000. After 2 to 3 days, cGFP expression was observed in Ad-293 cells by a fluorescence microscopy. Cells were harvested and lysed by the freeze-thaw method. Adenovirus-containing supernatant was obtained by centrifugation and used to infect the Ad-293 cells. This process was repeated 3 times.

Supernatants obtained from each step were stored at -70°C . For the infection of mice and mouse primary hepatocytes, Ad-293 cells were infected with crude stock virus, lysed by freezing and thawing after 24 to 48 hours, and the viruses were purified using CsCl_2 gradients.

RNA isolation and qRT-PCR. Total RNA was purified from HepG2 cells, mouse primary hepatocytes, peritoneal macrophages, or adenovirus-infected livers using TRIzol (Invitrogen) and reverse transcribed using MMLV reverse transcriptase (Promega) according to the manufacturer's instructions. Quantitative gene expression analysis was performed with the LightCycler 480 System (Roche) using SYBR Green PCR Master Mix (Invitrogen). The PCR primers used in this study are listed in Supplemental Table 4. RPS29 (ribosomal protein S29) was used as an internal control.

Mice. Six- to 8-week-old male C57BL/6N mice were purchased from Orient Bio Inc. Six-week-old male *Apoe*-deficient spontaneously hyperlipidemic mice (C57BL/6.KOR/StmSlc-*Apoe*^{chl}; *apoE*^{-/-}) were purchased from Central Lab Animal Inc. Mice were maintained in a controlled environment with a 12-hour light/12-hour dark cycle and ad libitum access to water and standard rodent chow.

Generation of hepatic TRAP80-knockdown mice and induction of fatty liver by LXR activation. Recombinant adenovirus Ad-shTRAP80 or Ad-shCon was delivered to C57BL/6N or *Apoe*^{-/-} mice by tail vein injection at a dose of 1×10^{11} particles per 20 g of body weight. On the fifth day after virus injection, mice were treated with 50 mg/kg T0901317 or GW3965, administered daily by oral gavage for 3 days. T0901317 or GW3965 was dissolved in 0.5% methylcellulose (M0262; Sigma-Aldrich) to a final concentration of 3.33 mg/ml and administered at a dose of 0.3 ml per 20 g of body weight. On the eighth day after virus injection, blood was obtained by heart puncture. Livers were harvested, snap-frozen in liquid nitrogen, and stored at -70°C until analysis.

Metabolic analyses of mouse serum and liver samples. Serum triglycerides, total cholesterol, HDL-cholesterol, and LDL/VLDL cholesterol were measured in serum samples using analysis kits purchased from Asan Pharmaceutical (total and HDL cholesterol), Sigma-Aldrich (serum triglycerides), and BioVision (LDL/VLDL cholesterol). The biochemical separation of HDL and LDL/VLDL was achieved using LDL/VLDL precipitation buffer (K613-100-2; BioVision). The supernatant is an HDL fraction, and the precipitate is an LDL/VLDL fraction. To determine lipoprotein lipid distribution, pooled serum (50 μl) from mice was fractionated by FPLC gel filtration (ÄKTA System; GE Healthcare) on a Superose 6 3.2/300 column (29-0362-26; GE Healthcare) at a flow rate of 40 $\mu\text{l}/\text{minute}$. The cholesterol and triglyceride concentrations in the FPLC fractions were determined enzymatically using assay kits (K623-100; BioVision, for cholesterol; AM157S-K; Asan Pharmaceutical, for triglycerides). For the measurement of hepatic triglycerides, lipid components were extracted from

livers using chloroform containing 1% Triton X-100. After the chloroform was evaporated, hepatic triglycerides were dissolved in Triton X-100 and assayed using an analysis kit (TRO100; Sigma-Aldrich).

Measurement of biliary cholesterol output and fecal neutral sterol. For bile collection, mice were anesthetized, and the peritoneal cavity was opened. The common bile duct was tied off by silk ligature, and the gallbladder was nicked and cannulated with a PE-10 tube (427401; BD). Bile samples were collected for 30 minutes, while the body temperature was maintained by placing the animal under a heat lamp. Cholesterol in bile was determined by an enzymatic method using total cholesterol assay kits (AM202-K; Asan Pharmaceutical). For the measurement of fecal neutral sterol, feces were collected on the last 2 days before sacrificing the mice. Feces samples were dried, weighed, and ground, and 5 α -cholestane was added as an internal standard. The sample was then saponified and extracted using cyclohexane. After extraction, neutral sterols were converted to trimethylsilyl-ether (TMS-ether) derivatives for gas-liquid chromatographic analysis.

Statistics. Results are expressed as the mean \pm SEM. Statistical significance was assessed using an unpaired, 2-tailed Student's *t* test or 1-way ANOVA. Differences with *P* values of less than 0.05 were considered statistically significant.

Study approval. All animals were maintained and handled according to protocols approved by the IACUC of the Asan Institute for Life Sciences.

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