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GROWTH FACTORS, CYTOKINES, AND CELL CYCLE MOLECULES

Lymphocyte-Specific Protein-1 Suppresses Xenobiotic-Induced Constitutive Androstane Receptor and Subsequent Yes-Associated Protein–Activated Hepatocyte Proliferation

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Activation of constitutive androstane receptor (CAR) transcription factor by xenobiotics promotes hepatocellular proliferation, promotes hypertrophy without liver injury, and induces drug metabolism genes. Previous work demonstrated that lymphocyte-specific protein-1 (LSP1), an F-actin binding protein and gene involved in human hepatocellular carcinoma, suppresses hepatocellular proliferation after partial hepatectomy. The current study investigated the role of LSP1 in liver enlargement induced by chemical mitogens, a regenerative process independent of tissue loss. 1,4-Bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP), a direct CAR ligand and strong chemical mitogen, was administered to global *Lsp1* knockout and hepatocyte-specific *Lsp1* transgenic (TG) mice and measured cell proliferation, hypertrophy, and expression of CAR-dependent drug metabolism genes. TG livers displayed a significant decrease in Ki-67 labeling and liver/body weight ratios compared with wild type on day 2. Surprisingly, this was reversed by day 5, due to hepatocyte hypertrophy. There was no difference in CAR-regulated drug metabolism genes between wild type and TG. TG livers displayed increased Yes-associated protein (YAP) phosphorylation, decreased nuclear YAP, and direct interaction between LSP1 and YAP, suggesting LSP1 suppresses TCPOBOP-driven hepatocellular proliferation, but not hepatocyte volume, through YAP. Conversely, loss of LSP1 led to increased hepatocellular proliferation on days 2, 5, and 7. LSP1 selectively suppresses CAR-induced hepatocellular proliferation, but not drug metabolism, through the interaction of LSP1 with YAP, supporting the role of LSP1 as a selective growth suppressor. *(Am J Pathol 2022, 192: 887–903; https://doi.org/10.1016/j.ajpath.2022.03.010)*

Constitutive androstane receptor (CAR), a xenobiotic-recognizing nuclear receptor activated by several chemicals and drugs, including 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) and phenobarbital, is traditionally known for its essential role in inducing drug-metabolizing enzymes.[1,2] TCPOBOP, the strongest activator, binds directly to CAR, and subsequently leads to its nuclear translocation. In the nucleus, CAR binds with retinoid X receptor to induce selective gene transcription. This results in increased expression of phase I and II drug-metabolizing enzymes (eg, cytochrome p450s and sulfotransferases) as well as drug transporters to facilitate the detoxification and elimination of foreign substances.[1,3] In addition to its functions on drug metabolism, CAR is also known to regulate energy metabolism in the liver as well as induce hepatocellular proliferation and hypertrophy.[4,5] The ability of CAR to stimulate proliferation is different from regeneration induced by

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surgical or chemically induced liver injury because it is not driven by loss of tissue mass or injury.[6] Proliferation induced by CAR activation on a long-term basis is also known to promote carcinogenesis in the liver.[7] TCPOBOP, a classic CAR agonist, is recognized as the strongest chemical mitogen for the liver and stimulates hepatocellular proliferation to a level comparable to two-thirds partial hepatectomy in the mouse.[8] Within a week following TCPOBOP administration, the size of the liver doubles and then levels off up to 4 weeks after treatment.[4,7] This demonstrates that CAR activation and induction of gene transcription is able to increase the liver/body weight ratio and thus alter the hepatostat[6] without loss of tissue or injury. The underlying mechanisms, however, by which CAR affects hepatocellular proliferation are not completely understood.[9]

Leukocyte-specific protein-1 (LSP1) is an F-actin binding protein involved in human hepatocellular carcinoma[10,11] as well as bladder and breast cancer.[12–15] In lymphoma cell lines, LSP1 interacts with kinase suppressor of Ras to facilitate the localization of mitogen-activated protein kinase kinase (MEK) and extracellular signal-regulated kinase (ERK) to the cytoskeleton. This affects mitogen-activated protein kinase signaling, a pathway that normally functions to regulate proliferation, differentiation, and migration, and is dysregulated in hepatocellular carcinoma.[16,17] Additional functions of LSP1 include inhibition of neutrophil migration[18] and induction of apoptosis in B cells in response to B-cell receptor ligation.[19] Loss of LSP1 also accelerates skin wound healing.[20] Previous work has demonstrated that LSP1 suppresses hepatocellular proliferation following two-thirds hepatectomy and inhibits proliferation and migration of rat cancer cell lines *in vitro* [21,22] Additional studies have found genomic alterations of *LSP1* in breast cancer, colorectal cancer, and lung cancer, as well as its down-regulation in bladder cancer, resulting in drug resistance.[12,14,23–25] The *LSP1* gene is hypermethylated in hepatocellular carcinoma samples versus cirrhotic controls, providing additional indication that LSP1 plays a role in hepatocarcinogenesis.[26]

Given the previous findings of LSP1 acting as a suppressor of hepatocellular proliferation, hepatocyte-specific *Lsp1* transgenic mice as well as global *Lsp1* knockout mice were treated with TCPOBOP. Whether LSP1 could regulate CAR-driven TCPOBOP-stimulated proliferation and hypertrophy, and/or alter CAR-regulated drug metabolism, was investigated. The current findings indicate that LSP1 specifically regulates hepatocellular proliferation in this model, likely by regulating Yes-associated protein (YAP) localization. It does not, however, affect TCPOBOP- and CAR-associated hepatocyte hypertrophy or induction of drug-metabolizing enzymes.

Materials and Methods

Animals and TCPOBOP Treatment

Male, 129svJ [genetic control strain for *Lsp1* knockout (KO) mice], global *Lsp1* KO,[11] hepatocyte-specific *Lsp1* transgenic (TG) mice,[22] and C57 (genetic control strain for *Lsp1 TG* mice) ($n = 3$ to 6) between 15 and 20 weeks old were administered 3 mg/kg of TCPOBOP in corn oil by oral gavage. The *Lsp1* transgene was generated in C57 mice and is expressed specifically in hepatocytes under the albumin promoter and α-fetoprotein enhancer.[22] Livers were harvested at various time points [days 0 (untreated), 1, 2, 3, 5, and 7] following TCPOBOP administration. Liver tissue was processed for Western blot analysis, immunohistochemistry, real-time quantitative PCR, and RNA sequencing. Wild-type (WT) C57 and *Lsp1 TG* mice ($n = 3$) were given TCPOBOP; and on day 2 following TCPOBOP, livers were perfused using collagenase, as previously described.[27] The hepatocytes and nonparenchymal cells were separated by centrifugation and used for Western blot analysis, real-time quantitative PCR, and RNA sequencing. Studies were performed according to the guidelines published by the NIH in the *Guide for the Care and Use of Laboratory Animals*[28] and were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

Immunohistochemistry

Liver tissue was fixed in formalin and embedded in paraffin. Sections (4 μm thick) were stained with Ki-67 antibody (Cell Signaling Technologies, Danvers, MA; number 12202), as previously described.[29] Biotinylated secondary antibody, goat anti-rabbit, was purchased from Millipore (St. Louis, MO; number AP187B).

Immunofluorescence

Frozen liver tissue embedded in OCT was sectioned at 5 μm and fixed with 2% paraformaldehyde for 1 hour. Fixed tissue was incubated with total YAP antibody (number 4912; Cell Signaling Technologies) in 0.5% bovine serum albumin for 1 hour. Following primary antibody incubation, sections were incubated with donkey anti-rabbit secondary antibody conjugated to Cy3 (Jackson ImmunoResearch Labs, West Grove, PA) for 1 hour. DAPI stain was used to visualize the nuclei. Images were taken at ×200 magnification on the Olympus (Tokyo, Japan) Fluoview 1000-2 confocal microscope in the Center for Biologic Imaging at the University of Pittsburgh.

Table 1 List of Forward and Reverse Primers Used for Quantitative RT-PCR

Gene symbol	Forward primer sequence	Reverse primer sequence
Cyb2b10	5′-GGCACTGGAGAAATCAATCAAC-3′	5′-CTTGGGCTATTGGGAGGAAA-3′
Cyp2c55	5′-AATGATCTGGGGGTGATTTTCAG-3′	5′-GCGATCCTCGATGCTCCTC-3′

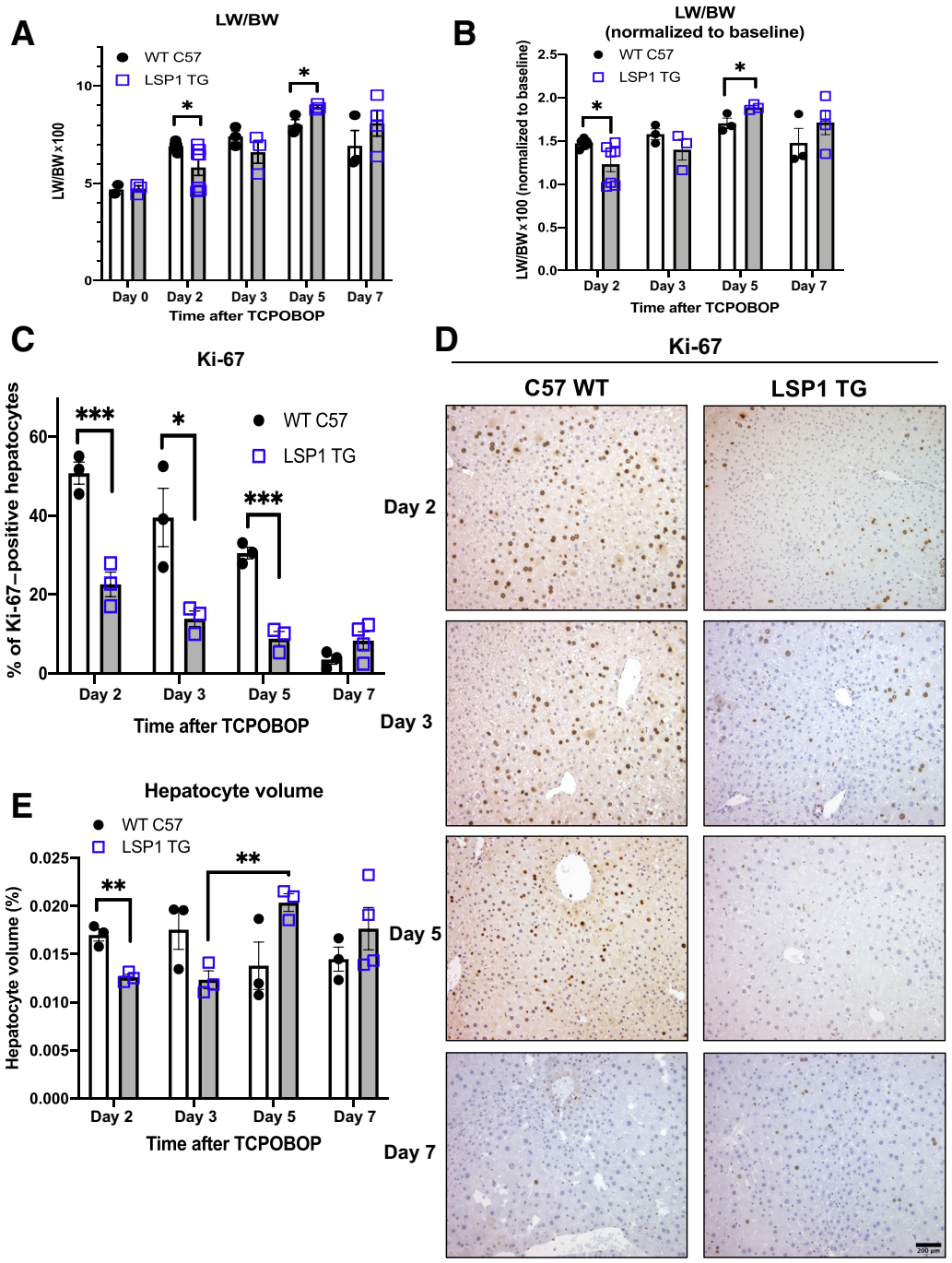
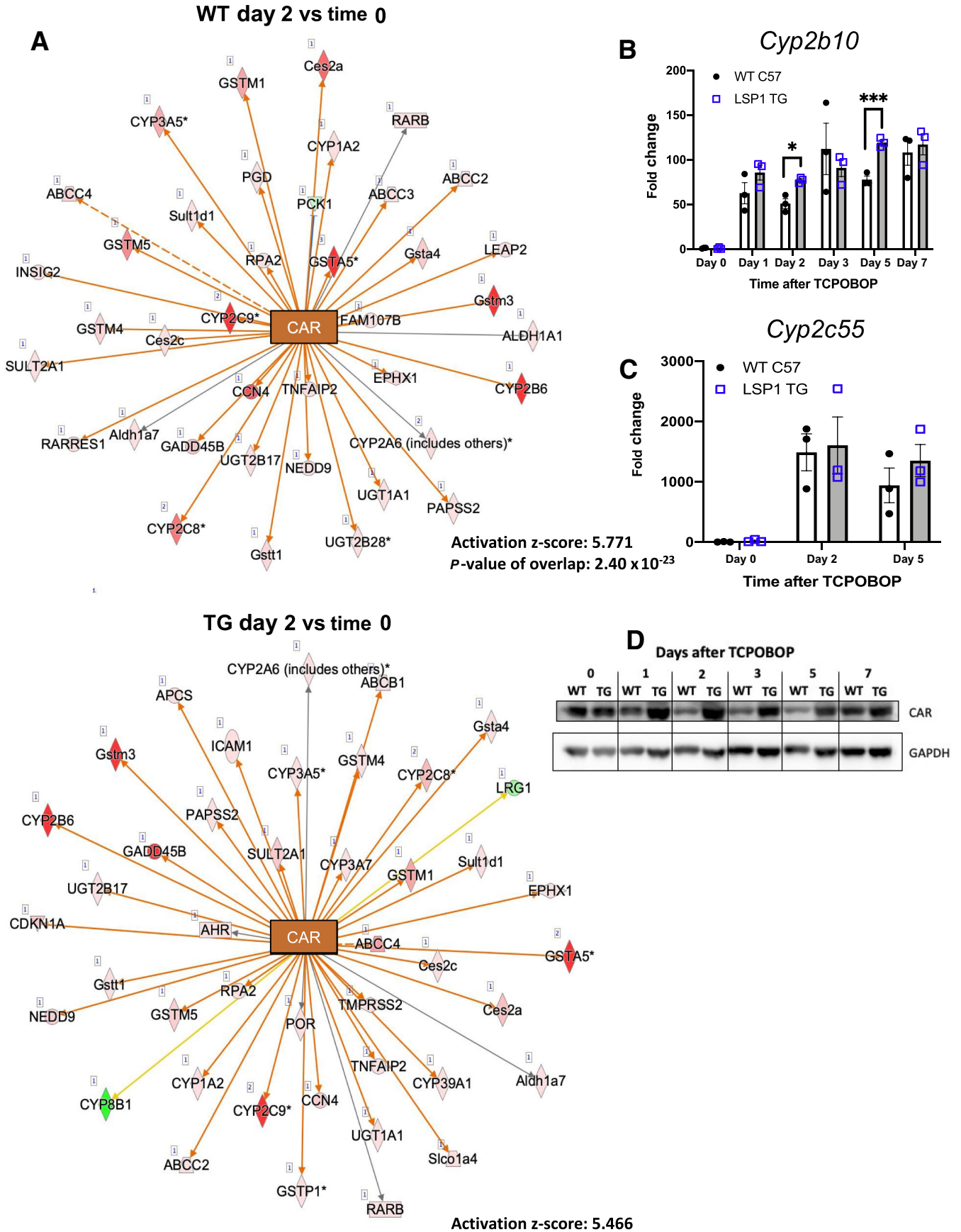


Figure 1 Effect of lymphocyte-specific protein-1 (LSP1) expression on 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP)–driven hepatocellular proliferation and hypertrophy. **A:** Liver weight (LW)/body weight (BW) ratios of wild-type (WT) and *Lsp1* transgenic (TG) animals. **B:** Liver/body weight ratios of WT and *Lsp1* TG animals normalized to time 0. **C:** Percentage of Ki-67–positive hepatocytes in WT and *Lsp1* TG livers after TCPOBOP. **D:** Representative images of Ki-67 immunohistochemistry. **E:** Average hepatocyte volume (number of hepatocytes per unit area). n = at least 3 animals per time point (**B**). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Scale bar = 200 μm (**D**). Original magnification, ×200 (**D**).

Protein Isolation and Western Blot Analysis

Protein isolation was performed using frozen liver tissue in radioimmunoprecipitation assay buffer with proteinase and phosphatase inhibitors. Pooled protein lysates were utilized for Western blot analysis. Antibodies utilized for Western blot analysis include the following: from Cell Signaling Technologies: cyclin D1 (number 2978), phosphorylated ERK (p44/42; number 9101), total ERK (number 4696), phosphorylated YAP (S127; number 4911), phosphorylated YAP (S397; number 13619), total YAP (number 14074), p16 (number 80772), p27 (number 3686), glyceraldehyde-3-phosphate dehydrogenase (number 5174), phosphorylated epidermal growth factor receptor (EGFR) 846 (number


A
WT day 2 vs time 0
Ces2a
GSTM1
CYP3A5*
RARB
CYP1A2
PGD
ABCC2
ABCC4
PCK1
ABCC3
Sult1d1
GSTM5
RPA2
GSTA5*
Gsta4
LEAP2
INSIG2
Gstm3
CYP2C9*
CAR
FAM107B
GSTM4
Ces2c
ALDH1A1
SULT2A1
EPHX1
CCN4
TNFAIP2
CYP2B6
RARRES1
Aldh1a7
GADD45B
CYP2A6 (includes others)*
UGT2B17
NEDD9
UGT1A1
PAPSS2
CYP2C8*
UGT2B28*
Gstt1
Activation z-score: 5.771
P-value of overlap: 2.40 x 10-23
B
Cyp2b10
WT C57
LSP1 TG
Fold change
200
150
100
50
0
*
***
Day 0
Day 1
Day 2
Day 3
Day 5
Day 7
Time after TCPOBOP
C
Cyp2c55
WT C57
LSP1 TG
Fold change
3000
2000
1000
0
Day 0
Day 2
Day 5
Time after TCPOBOP
TG day 2 vs time 0
CYP2A6 (includes others)*
ABCB1
APCS
Gsta4
ICAM1
Gstm3
GSTM4
CYP3A5*
CYP2C8*
LRG1
CYP2B6
PAPSS2
GADD45B
SULT2A1
CYP3A7
Sult1d1
GSTM1
UGT2B17
EPHX1
CDKN1A
AHR
CAR
ABCC4
GSTA5*
Ces2c
Gstt1
RPA2
TMPRSS2
Ces2a
NEDD9
GSTM5
POR
TNFAIP2
CYP1A2
CYP39A1
Aldh1a7
CYP8B1
CYP2C9*
CCN4
UGT1A1
Slco1a4
ABCC2
GSTP1*
RARB
Activation z-score: 5.466
P-value of overlap: 9.39 x 10-32
D
Days after TCPOBOP
0
1
2
3
5
7
WT TG WT TG WT TG WT TG WT TG WT TG
CAR
GAPDH

2231), phosphorylated EGFR 1068 (number 3777), total EGFR (number 4267), phosphorylated Met 1349 (number 3121), and total Met (number 8191). The antibody used for p21 was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX; sc6246). Total CAR (number PA5-95066) was purchased from Thermo Fisher Scientific (Waltham, MA).

Immunoprecipitation

Immunoprecipitation was performed using 500 μg of whole liver lysate in 500 μL of radioimmunoprecipitation assay buffer incubated with 10 μL of primary antibody overnight with end-over-end mixing at 4°C. Protein A/G agarose beads (Santa Cruz Biotechnology, Inc.) were added to the lysate the following day and incubated overnight with end-over-end mixing at 4°C. Pellet was collected by centrifugation at 1000 × *g* for 5 minutes and washed 3 times in radioimmunoprecipitation assay buffer with Halt protease and phosphatase inhibitors (Thermo Fisher Scientific) added. Following the final wash, 30 μL of 4× sample buffer was added to the pellet, and the samples were subsequently incubated at 95°C for 10 minutes and then run on a NuPAGE 4% to 12% Bis-Tris gel (Thermo Fisher Scientific).

Real-Time Quantitative PCR

Total RNA was isolated from frozen liver tissue using Trizol (Sigma, St. Louis, MO) following the manufacturer's protocol. RNA was utilized to produce cDNA using the First Strand Superscript Kit (Thermo Fisher Scientific). Real-time quantitative PCR analysis was performed using SYBR Green (Thermo Fisher Scientific) to determine mRNA levels of various targets following TCPOBOP administration. PCR primers used in this study are included in Table 1.

RNA Sequencing

RNA isolated from frozen liver tissue (time 0, day 2, and day 5) and WT and TG hepatocytes was sent to Novogene Corp., LTD (Beijing, China) for RNA sequencing. Ingenuity pathway analysis (IPA; Ingenuity Systems, Redwood City, CA) was utilized to analyze genes that were significantly altered by TCPOBOP treatment. For analysis of TCPOBOP-related genes, lists of significantly up-regulated and down-regulated genes from day 2 compared with time 0 as well as the corresponding fold changes were uploaded and analyzed by IPA. Gene sets that were significantly up-regulated and down-regulated on day 2 in the WT versus the *Lsp1 TG* isolated hepatocytes and day 5 in the WT129 versus the *Lsp1* KO liver tissue (with a 1.5-fold cutoff) were uploaded to the IPA software version 22.0 (Qiagen, Redwood City, CA). IPA analysis was utilized to predict canonical signaling pathways and upstream regulators that were altered because of changes in downstream gene expression. DAVID software version 6.8 (Frederick National Laboratory, Frederick, MD) was utilized to determine biological processes (Gene Ontology terms) that were enriched because of TCPOBOP compared with the *Mus musculus* reference gene list.

Statistical Analysis

Data are presented as the means ± SEM. All statistical analyses were performed using the unpaired *t*-test and GraphPad Prism software version 9.3.0 (San Diego, CA). Statistically significant differences were defined at $P < 0.05$. All experiments were performed at least three times, and each time point contains data from at least three independent animals.

Results

LSP1 Expression Suppresses TCPOBOP-Driven Hepatocellular Proliferation but Not Hypertrophy

In *Lsp1* transgenic mice, which express the transgene in hepatocytes under the albumin promoter and α-fetoprotein enhancer, there was decreased liver/body weight ratios on day 2 after administration of TCPOBOP in comparison to WT mice (5.8% to 6.9%, respectively). However, by day 5, the liver/body weight ratios of the TG animals were significantly higher than those of the WT mice (8.9% to 8%, respectively) (Figure 1, A and B). Hepatocellular proliferation driven by CAR activation was measured by Ki-67 immunohistochemistry. Following TCPOBOP administration, TG livers exhibited significantly less Ki-67−positive hepatocytes compared with WT hepatocytes on day 2 (20% versus 50%; $P = 0.003$), day 3 ($P = 0.03$), and day 5 ($P = 0.0007$) (Figure 1, C and D). Because hepatocyte proliferation did not account for the increased liver/body weight ratios in the TG at days 5 and 7, and TCPOBOP is known to not only promote hepatocellular proliferation but hypertrophy as well, the average volume of hepatocytes in both the WT and TG animals was measured following TCPOBOP treatment. TG hepatocytes were approximately twice the size of WT hepatocytes on day 5 and 35% larger on day 7 (Figure 1, D and E). We conclude that enhanced LSP1 expression in TG mice

Figure 2 Constitutive androstane receptor CAR) expression and activity is unaffected by lymphocyte-specific protein-1 (LSP1) expression. **A:** Ingenuity pathway analysis of RNA-sequencing data, demonstrating activation of CAR target genes in both wild-type (WT; **top panel**) and *Lsp1* transgenic (TG; **bottom panel**) groups on day 2 after 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) treatment in comparison to time 0. Quantitative RT-PCR data of CAR target genes. Number next to gene indicates number of isoforms. **B:** *Cyp2b10* in liver. **C:** *Cyp2c55* in liver. **D:** Western blot analysis of total CAR expression in WT and TG liver whole cell lysates. Samples are pooled from three independent livers. Genes marked with an **asterisk** indicate that multiple identifiers exist in the data set file map to a single gene in the Global Molecular Network. $n = 3$ (**D**). $^{*}P < 0.05$, $^{***}P \leq 0.001$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

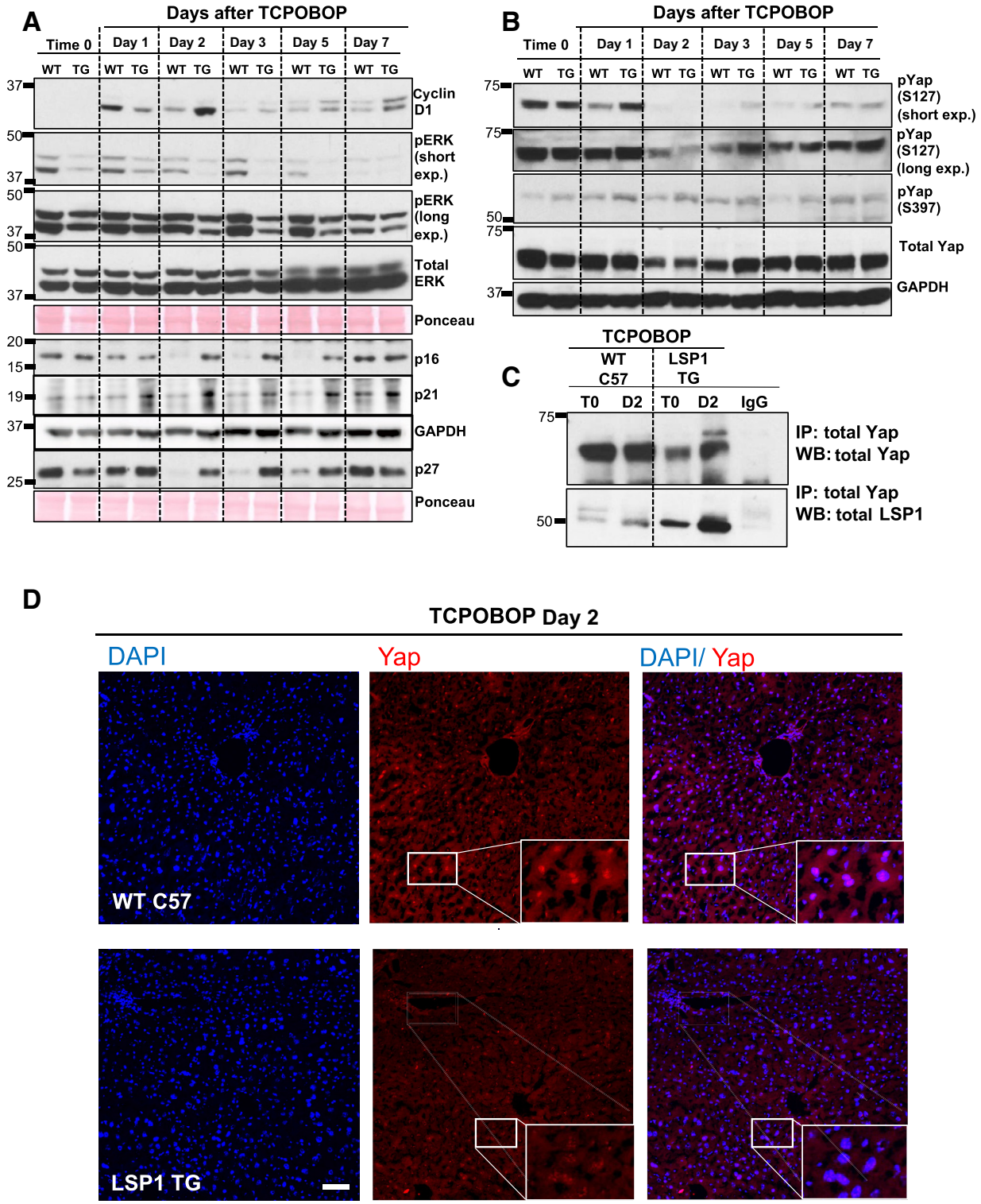


Figure 3 Lymphocyte-specific protein-1 (LSP1) expression results in increased expression of cell cycle inhibitors and decreased YAP in the nucleus. **A:** Western blot (WB) analysis of wild-type (WT) and *Lsp1* transgenic (TG) whole liver lysates following 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) for cyclin D1, phosphorylated extracellular signal-regulated kinase (pERK), total ERK, p16, p21, and p27. Ponceau S staining was used as a loading control. Each lane is a pooled sample. **B:** Western blot analysis for phosphorylated YAP (pYAP; S127) and pYAP (S397), with total YAP, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. **C:** Immunoprecipitation (IP) of total YAP in WT and *Lsp1* TG livers at time (T) 0 and day (D) 2 after TCPOBOP treatment. **Top panels:** Western blot analysis: total YAP. **Bottom panels:** Western blot analysis: total LSP1. IgG: control. Compared with the C57 control mice, there is enhanced coprecipitation of LSP1 protein with YAP at day 2 after TCPOBOP. **D:** Immunofluorescence of WT and *Lsp1* TG livers for total YAP (red). DAPI (nuclei) and merge of DAPI and total YAP. **Insets**: Magnified images from **boxed areas** to demonstrate YAP staining in the hepatocyte nuclei. n = at least 3 (**A**). Scale bar = 100 μm (**D**). Original magnifications, ×200 (**D**, **main images**); ×400 (**D**, **insets**). exp., exposure.

suppresses hepatocellular proliferation but not hypertrophy in response to TCPOBOP administration.

Increased LSP1 Expression Does Not Suppress CAR Expression or Its Drug Metabolism Functions in Response to TCPOBOP

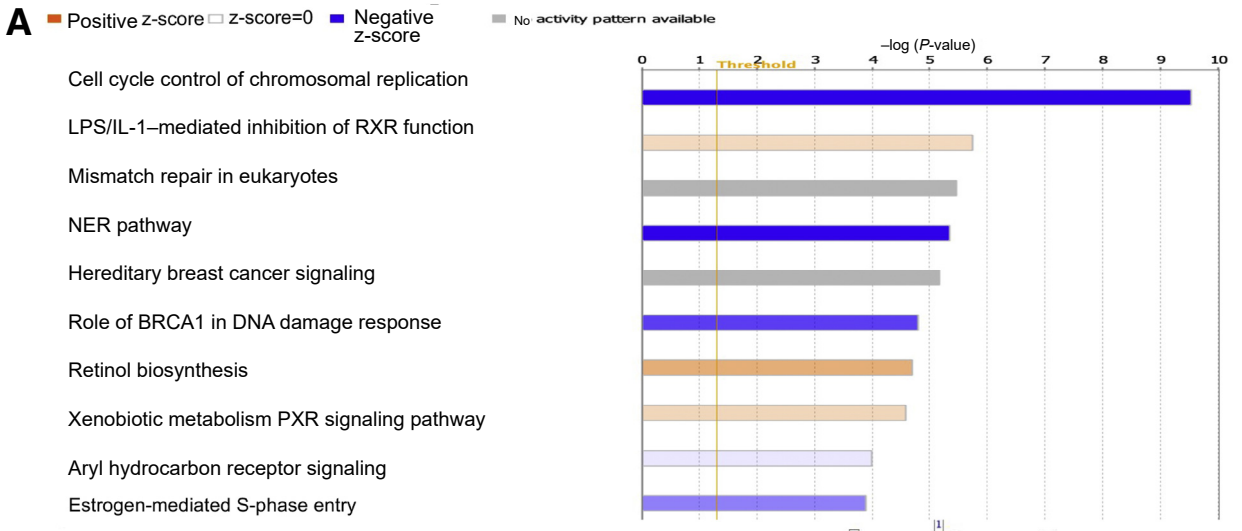
To determine whether the suppression of proliferation in *Lsp1 TG* livers after TCPOBOP treatment is due to decreased CAR expression and activation, CAR expression was measured by Western blot analysis as well as the ability of CAR to activate downstream transcriptional targets using IPA analysis of RNA-sequencing data. Comparing the differentially expressed genes of both the WT and TG on day 2 versus time 0, a significant activation of downstream CAR target genes, including the ones associated with drug metabolism in both the WT and TG livers (WT activation z-score, 5.771; TG activation z-score, 5.466) was observed (Figure 2A). Activation of typical CAR target genes, *Cyp2b10* and *Cyp2c55*,[30] by quantitative RT-PCR was confirmed in both *Lsp1* TG and WT livers (Figure 2, B and C). There was no suppression in CAR expression between the WT and TG in the same dates, as evidenced by Western blot analysis of all date time points; which was elevated relative to control (Figure 2D). Because *Lsp1* TG mice did not display suppressed levels of CAR expression nor downstream activation of target genes in response to TCPOBOP, the study showed that the observed decrease in proliferation in TG livers is not due to decreased CAR activation.

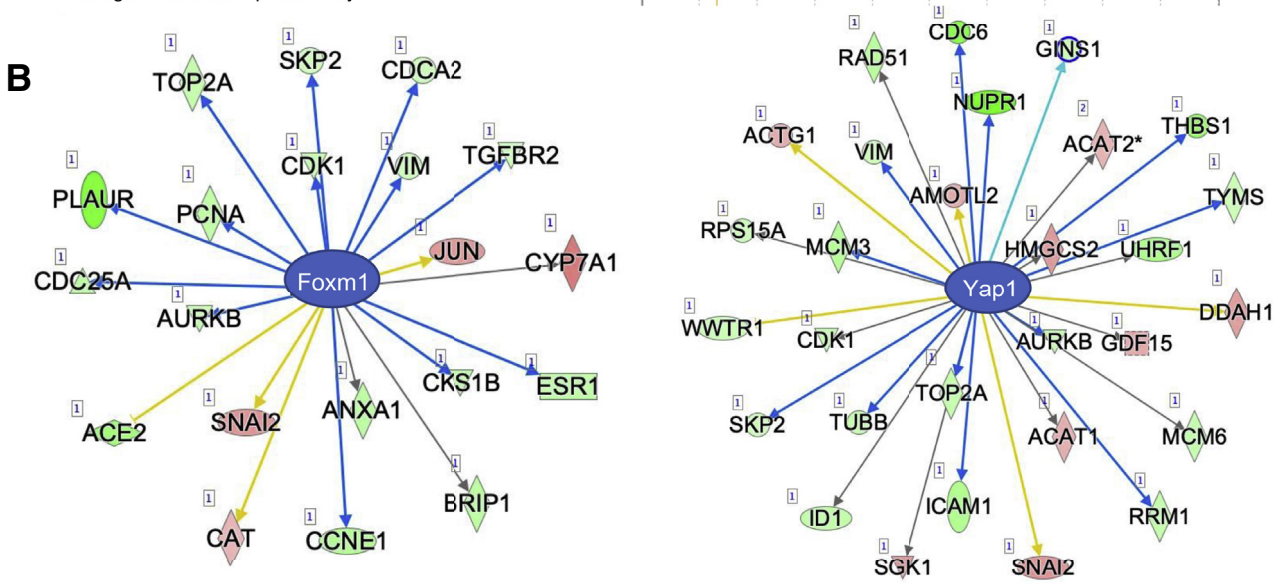
Increased Cell Cycle Inhibitor Expression, Decreased Nuclear YAP, and Increased YAP Interaction with LSP1 in *Lsp1* TG Livers following TCPOBOP

Because CAR activation and its ability to regulate drug metabolism genes is not suppressed by LSP1 expression, the role of LSP1 in regulating cell cycle progression after TCPOBOP administration was analyzed next. Western blot analyses demonstrated increased p16 (days 2, 3, and 5), p21 (all days), and p27 (days 2, 3, and 5) in the *Lsp1* TG livers in comparison to those in the WT controls (Figure 3A), suggesting inhibition of the cell cycle. Because LSP1 inhibits ERK activation in liver after hepatectomy,[21] LSP1 overexpression in hepatocytes was confirmed to lead to decreased ERK phosphorylation following TCPOBOP treatment at days 2, 3, 5, and 7 (Figure 3A). Examination of YAP and phosphorylated YAP by Western blot analysis showed no changes in total YAP but an increased phosphorylation of YAP at S397 at days 1, 2, 3, and 5, which is associated with cytoplasmic retention of YAP (Figure 3B). Immunofluorescence staining corroborated the loss of nuclear YAP in the TG hepatocytes on day 2 after TCPOBOP administration (Figure 3D). Immunoprecipitation of total YAP in WT and *Lsp1* TG livers following TCPOBOP treatment demonstrated an interaction between YAP and LSP1 that increased in the TG animals at day 2 after TCPOBOP (Figure 3C). LSP1 overexpression is cytoplasmic.[21] The findings in Figure 3 demonstrate that LSP1 may sequester YAP in the cytoplasm, preventing its translocation to the nucleus and subsequent activation of proliferation. *Lsp1* TG livers displayed suppressed EGFR activation in response to TCPOBOP, as evidenced by decreased phosphorylation of EGFR at the 845 and 1068 tyrosine sites compared with that in the WT (Supplemental Figure S1). There was no effect on the activation of MET, the hepatocyte growth factor receptor. Thus, the study shows that LSP1 expression leads to decreased hepatocellular proliferation induced by TCPOBOP, in part by causing increased expression of cell cycle inhibitor pathways, as well as decreased YAP translocation to the nucleus.

Lsp1 Transgenic Hepatocytes, but Not the Nonparenchymal Cells, Display Decreased Proliferation and Increased Cell Cycle Inhibitor Expression

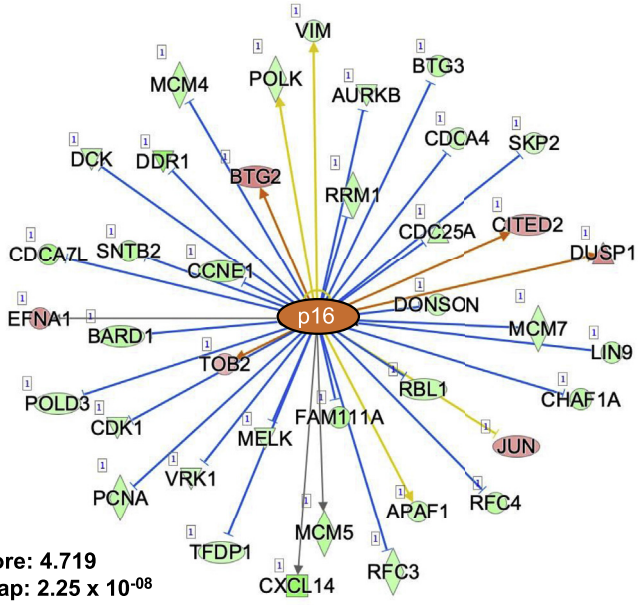
Although *Lsp1* TG preparations from whole livers displayed decreased cell cycle progression in response to treatment with TCPOBOP, cyclin D1 expression levels were increased in whole TG livers on days 2 to 7 compared with those in the WT (Figure 3A). In addition, IPA analysis of RNA-sequencing data from the *Lsp1* TG and WT livers did not show any alterations in cell cycle—related canonical pathways between the two groups. These results were unexpected because *Lsp1* TG livers displayed decreased proliferation of hepatocytes by Ki-67 immunohistochemistry at those time points (Figure 1, C and D). Because TG animals only express the *Lsp1* transgene in hepatocytes, both WT and *Lsp1* TG animals were treated with TCPOBOP and hepatocytes and nonparenchymal cells (NPCs) were isolated from these livers on day 2 after TCPOBOP (the day of the most enhanced cyclin D1 expression in TG animals) (Figure 3A), to determine whether NPCs affected the results. As shown in Figure 4, enrichment analysis of RNA-sequencing data using both the DAVID database and IPA demonstrated that, following TCPOBOP administration, *Lsp1* TG hepatocytes displayed decreased activation of DNA replication and cell cycle—related pathways, such as cell cycle control of chromosomal replication, DNA repair, DNA replication, and the nuclear excision repair pathway, compared with that in WT hepatocytes (Table 2 and Figure 4A). Further analyses demonstrated that in response to TCPOBOP, *Lsp1* TG hepatocytes displayed decreased activation of forkhead box M1 (Foxm1), E2F transcription factor 1 (E2f1), and Yap1 protein downstream pathways (Figure 4B and Supplemental Figure S2), as well as increased activation of the p16 (Cdkn2a) activated downstream pathways (Figure 4B), compared with those in WT hepatocytes. RNA-sequencing predicted certain upstream regulators to be activated in *Lsp1* TG versus those in WT hepatocytes such as cell cycle inhibitors p16 (*Cdkn2a*), p21 (*Cdkn1a*), p53 (*Tp53*), as well as *Hnf4α* and *Rb*. On the other hand, it predicted transcription factors involved in activating cell cycle progression, such as





Activation z-score: -2.412
***P*-value of overlap: 1.02 x 10^{-05}**

Activation z-score: -2.053
***P*-value of overlap: 7.00 x 10^{-05}**



Activation z-score: 4.719
***P*-value of overlap: 2.25 x 10^{-08}**

Table 2 Enrichment Analysis Utilizing the DAVID Database Displaying Biological Processes (GO Terms) that Differ between *Lsp1* TG and WT Hepatocytes Isolated on Day 2 after TCPOBOP

GO term (biological processes)	Genes, *N*	*P* value
DNA replication	32	8.40×10^{-14}
Cell cycle	75	6.10×10^{-12}
Metabolic process	54	3.90×10^{-8}
Cellular amino acid biosynthetic process	11	2.00×10^{-7}
Oxidation-reduction process	66	8.10×10^{-7}
Cellular response to DNA damage stimulus	42	6.20×10^{-5}
DNA unwinding involved in DNA replication	6	7.20×10^{-5}
DNA repair	34	1.00×10^{-4}
Cell division	38	1.10×10^{-4}
DNA replication initiation	8	1.50×10^{-4}
Lipid metabolic process	43	2.10×10^{-4}
Mitotic nuclear division	30	2.20×10^{-4}

GO, Gene Ontology; TCPOBOP, 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene; TG, transgenic; WT, wild type.

Table 3 IPA of RNA-Sequencing Data Demonstrating Upstream Regulators Predicted to be Changed in *Lsp1* TG versus WT Hepatocytes at Day 2 after TCPOBOP

Upstream regulator	Predicted activation state	Activation z-score	*P* value of overlap
p16 (*Cdkn2a*)	Activated	4.719	2.25×10^{-8}
Rb	Activated	3.940	3.72×10^{-8}
p53	Activated	3.006	2.14×10^{-10}
Hnf4a	Activated	2.763	4.46×10^{-10}
p21 (*Cdkn1a*)	Activated	2.650	2.45×10^{-18}
Erbb2	Inhibited	−5.188	8.03×10^{-13}
E2f1	Inhibited	−3.642	8.33×10^{-8}
Nrf2	Inhibited	−3.175	4.56×10^{-4}
Myc	Inhibited	−3.125	1.22×10^{-3}
Foxm1	Inhibited	−2.412	1.02×10^{-5}
E2f3	Inhibited	−2.382	3.43×10^{-5}
HGF	Inhibited	−2.276	5.02×10^{-9}
Yap	Inhibited	−2.053	7.00×10^{-5}

IPA, ingenuity pathway analysis; TCPOBOP, 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene; TG, transgenic; WT, wild type.

Foxm1, to be significantly attenuated in the *Lsp1* TG hepatocytes versus those in WT (activation z-score, −2.412; *P* value of overlap, 1.02×10^{-5}) (Table 3). RNA-sequencing also predicted inhibition of the upstream regulator Yap1 in *Lsp1* TG hepatocytes (activation z-score, −2.053; *P* value of overlap, 7.00×10^{-9}) (Figure 4B and Table 3). Yap1 is necessary to promote hepatocellular proliferation in response to TCPOBOP expression.[31] As indicated in Figure 5A, RNA levels of the cell cycle−related genes, aurora kinase B (*Aurkb*; $P = 0.026$) and cyclin E1 (*Ccne1*; $P = 0.027$), were significantly decreased in the TG hepatocytes. To demonstrate that these differences occur also at the protein level, Western blot analyses on both *Lsp1* TG and WT hepatocytes and NPCs were performed, which demonstrated that cyclin D1 expression was decreased in TG compared with that in the WT hepatocytes. However, cyclin D1 levels were high in both TG and control (C57) NPC populations (Figure 5B). Expression of cell cycle inhibitors, p21 and p27,[32] was elevated in the TG hepatocyte but not the NPCs. There was also a hepatocyte-specific increase in the phosphorylation of YAP at S127 and S397 (Figure 5, B and C). The cell cycle inhibitor p16 was increased only in NPCs but not in hepatocytes (Figure 5B). These data show that LSP1 expression inhibits proliferation of hepatocytes but not NPCs in response to TCPOBOP through increased expression of cell cycle inhibitors and decreased activation of pathways involved in cell division through a YAP-dependent mechanism.

Lsp1 KO Mice Exhibit Increased Hepatocellular Proliferation following TCPOBOP Administration

Because enhanced LSP1 expression in TG mice led to decreased hepatocellular proliferation in response to treatment with TCPOBOP, global *Lsp1* KO animals were treated with TCPOBOP to determine whether loss of LSP1 expression would conversely result in increased cell cycle progression. After normalizing liver/body weight ratios to the baseline ratios, *Lsp1* KO mice displayed significantly increased liver/body weight ratios compared with control mice at all time points following TCPOBOP treatment to both groups, except for day 3 (Figure 6, A and B). In addition, the percentage of Ki-67−positive hepatocytes was significantly increased in the *Lsp1* KO on days 2 and 5 compared with WT [day 2: WT versus KO, 22% versus 48% ($P = 0.0097$); day 5: WT versus KO, 9.6% versus 21% ($P = 0.033$)] (Figure 6, C and D). Hepatocellular volume was also significantly increased by 20% on day 5 in comparison to WT ($P = 0.005$) (Figure 6E). Therefore, loss of LSP1 protein expression in KO mice led to increased hepatocellular proliferation in response to TCPOBOP administration. Altered patterns of EGFR phosphorylation were also observed in the KO mice, with enhanced phosphorylation at days 1 and 2 at site 845, and days 5 and 7 for site 1068, after TCPOBOP (Supplemental Figure S3).

Figure 4 *Lsp1* transgenic (TG) hepatocytes exhibit decreased cell cycle activation and YAP activation after 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) treatment. **A:** Ingenuity pathway analysis (IPA)−based canonical signaling pathways predicted to be changed in *Lsp1* TG hepatocytes versus wild-type (WT) hepatocytes on day 2 after TCPOBOP. **B:** IPA of RNA-sequencing data displaying inhibition of Foxm1 and YAP1 and activation of p16 in *Lsp1* TG hepatocytes versus WT hepatocytes on day 2 after TCPOBOP. The number next to the genes indicates the number of isoforms. $n = 3$ per time point and strain (**B**). LPS, lipopolysaccharide; NER, nuclear excision repair; RXR, retinoid X receptor.

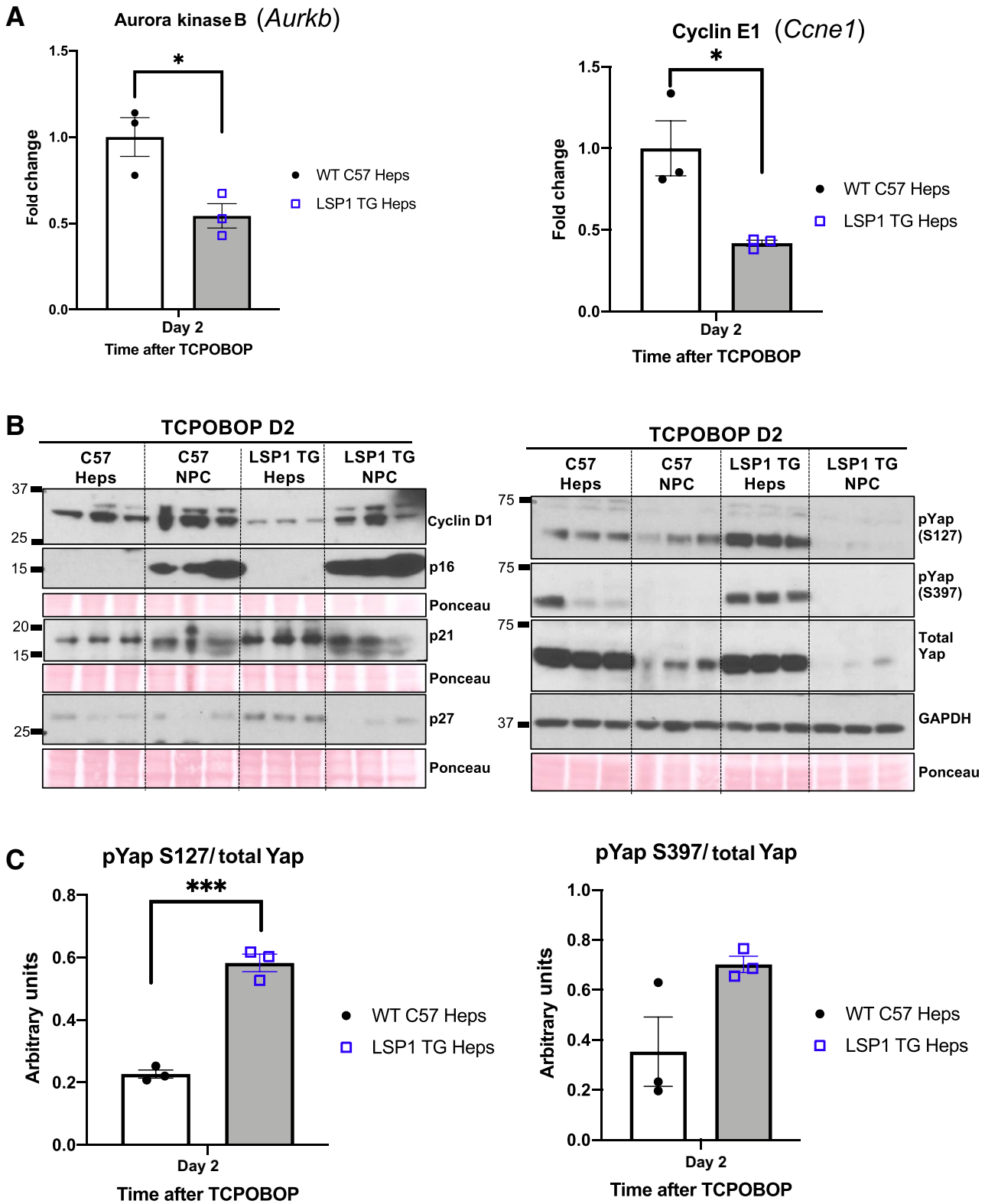


Figure 5 Lymphocyte-specific protein-1 (LSP1) expression in hepatocytes leads to increased activation of cell cycle inhibitors and increased YAP phosphorylation. **A:** RNA-sequencing data from wild-type (WT) and transgenic (TG) hepatocytes (Heps) on day 2 after 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) for aurora kinase B ($P = 0.026$) and cyclin E1 ($P = 0.027$). **B:** Western blot analyses of cyclin D1, p16, p21, p27, phosphorylated YAP (pYAP) S127, pYAP S397, total YAP, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in WT hepatocytes and nonparenchymal cells (NPCs) and *Lsp1* TG hepatocytes and NPCs isolated from livers on day 2 following TCPOBOP. Ponceau S staining was used as a secondary loading control. Each lane represents hepatocytes and NPCs isolated from one mouse. **C:** Quantification of Western blot analysis of pYAP S127/total YAP ($P = 0.0003$) and pYAP S397/total YAP ($P = 0.074$). $n = 3$ (**B**). $^{*}P < 0.05$, $^{***}P < 0.001$.

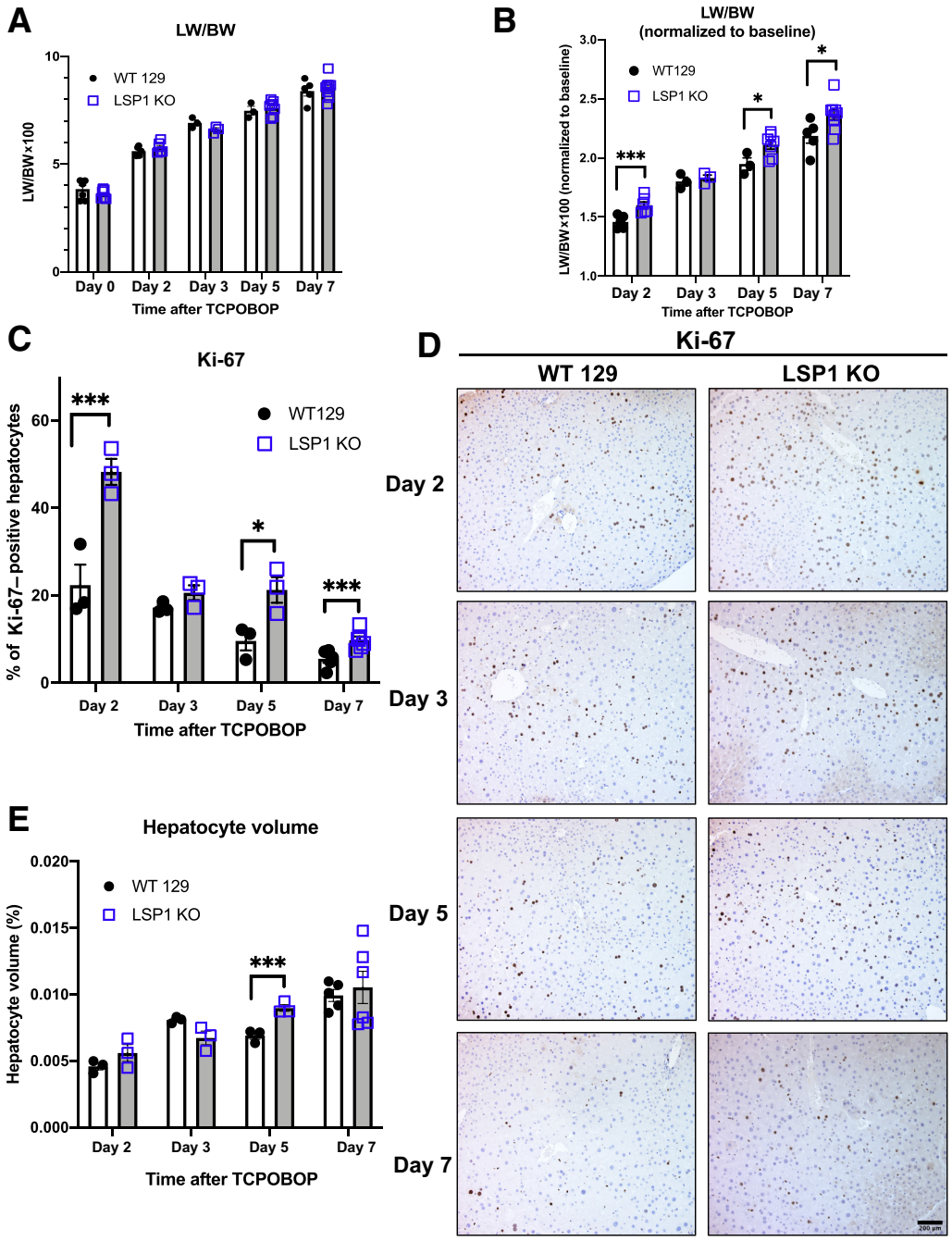


Figure 6 Loss of lymphocyte-specific protein-1 (LSP1) expression leads to increased 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP)–driven hepatocellular proliferation. **A:** Liver weight (LW)/body weight (BW) ratios of wild-type (WT) and *Lsp1* knockout (KO) animals. **B:** Liver/body weight ratios of WT and *Lsp1* KO animals normalized to time 0. **C:** Percentage of Ki-67–positive hepatocytes in WT and *Lsp1* KO livers after TCPOBOP. **D:** Representative images of Ki-67 immunohistochemistry. **E:** Average hepatocyte volume (number of hepatocytes per unit area). n = at least 3 animals per time point (**B**). $^{*}P < 0.05$, $^{***}P < 0.001$. Scale bar = 200 μm (**D**). Original magnification, ×200 (**D**).

CAR Expression and Role in Drug Metabolism Remains Functional in the Absence of LSP1 in Response to TCPOBOP

Both the KO and WT livers displayed robust activation of CAR pathways related to drug metabolism to a similar degree and increased activation of CAR target genes on day 2 following TCPOBOP treatment (WT: activation z-score, 5.697; P value of overlap, 1.52×10^{-36}; KO: activation z-score, 6.044; P value of overlap, 8.46×10^{-28}) (Figure 7A). Western blot analysis of total CAR expression was performed. Although there were changes in CAR expression in both KO and WT mice through the days after TCPOBOP treatment, there was no difference between the KO and WT livers at any time points following TCPOBOP (Figure 7B). Therefore, the data indicate that the increased

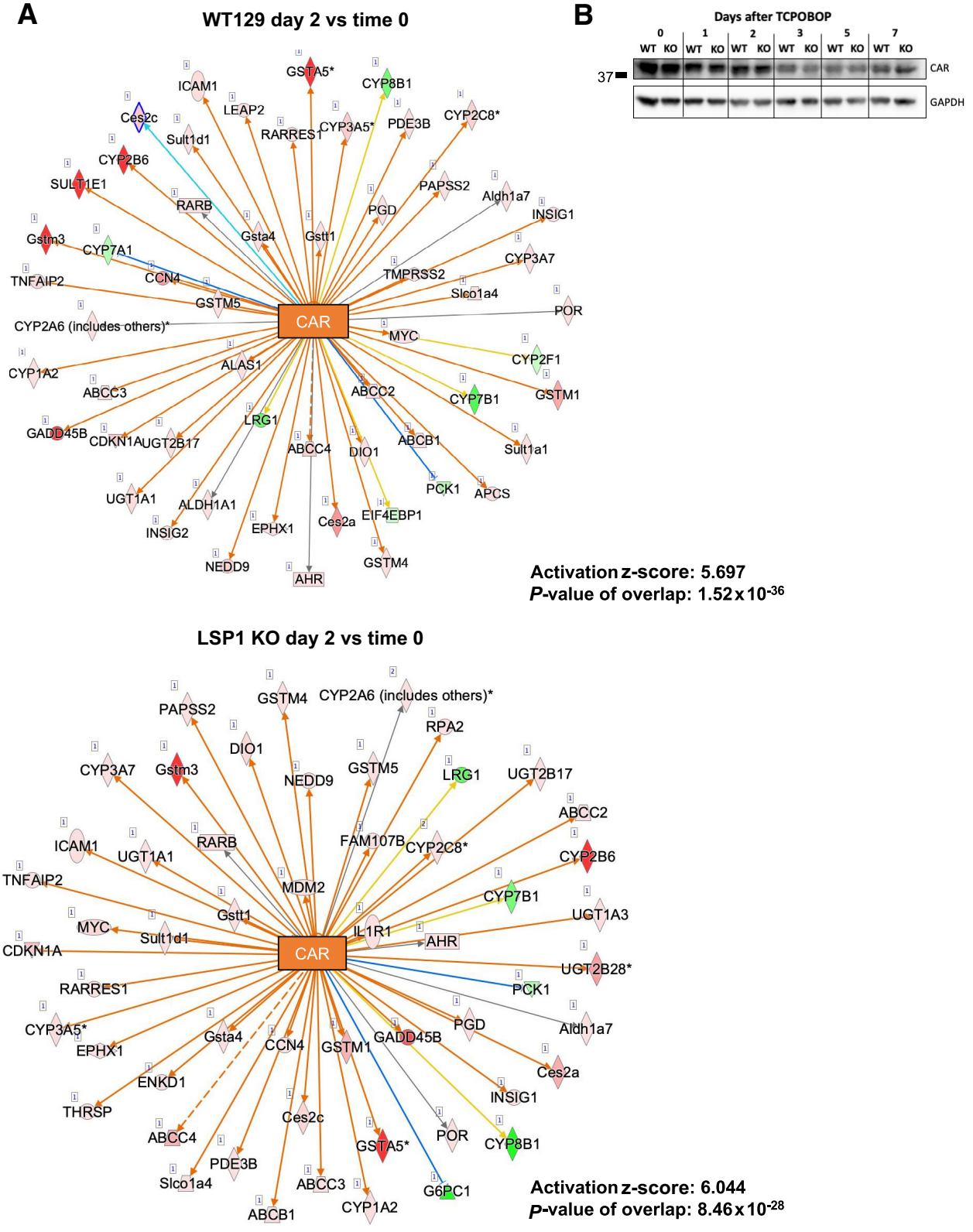


Figure 7 *Lsp1* knockout (KO) mice display no change in constitutive androstane receptor (CAR) activation but have increased cell cycle activation in response to 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP). **A:** Ingenuity pathway analysis of RNA-sequencing data of CAR target genes in both wild-type (WT; **top panel**) and *Lsp1* KO (**bottom panel**) livers on day 2 after TCPOBOP compared with time 0. The number next to the genes indicates the number of isoforms. **B:** Western blot analysis of total CAR expression in WT and KO liver whole cell lysates with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. Samples were pooled from three independent livers. Genes marked with an **asterisk** indicate that multiple identifiers exist in the data set file map to a single gene in the Global Molecular Network. $n = 3$ (**B**).

Table 4 Enrichment Analysis Utilizing the DAVID Database Displaying Biological Processes (GO Terms) that Differ between *Lsp1* KO and WT Livers Isolated on Day 5

GO term (biological processes)	Genes, *n*	*P* value
Cell cycle	113	4.70×10^{-22}
Mitotic nuclear division	59	1.50×10^{-14}
Cell division	70	3.80×10^{-14}
DNA replication	28	5.90×10^{-8}
Cell adhesion	65	3.80×10^{-7}
Chromosome segregation	21	2.00×10^{-6}
Cellular response to DNA damage stimulus	56	3.20×10^{-6}
Response to mechanical stimulus	17	3.90×10^{-6}
Mitotic chromosome condensation	8	8.60×10^{-6}
DNA repair	44	1.70×10^{-5}
DNA replication initiation	10	1.70×10^{-5}
Protein localization to kinetochore	7	1.70×10^{-5}

GO, Gene Ontology; KO, knockout; WT, wild type.

hepatocyte proliferation measured in the KO is not due to increased CAR activation per se or its ability to activate downstream target genes involved in drug metabolism.

Loss of LSP1 Leads to Increased Expression of Cell Cycle Related Genes

To understand how loss of LSP1 expression leads to increased hepatocyte proliferation in response to TCPOBOP, the study utilized both enrichment analysis using DAVID as well as IPA on RNA-sequencing data from day 5 after TCPOBOP to determine the biological processes and canonical pathways altered in the KO versus WT. The biological processes most altered in the KO were related to the cell cycle as well as mitotic nuclear division, cell division, and DNA replication (Table 4). The canonical pathways predicted to be most activated in the KO livers on day 5 following TCPOBOP administration were kinetochore metaphase signaling pathway (activation z-score, 2.887; *P* value of overlap, 4.86×10^{-14}) and cell cycle control of chromosomal replication (activation z-score, 4.123; *P* value of overlap, 3.02×10^{-8}) (Figure 8A). Upstream regulators inhibited in the KO livers in comparison to WT on day 5 were the cell cycle inhibitors, p16 (*Cdkn2a*; activation z-score, −3.502; *P* value of overlap, 1.85×10^{-6}) and p21 (*Cdkn1a*; activation z-score, −2.331; *P* value of overlap, 6.20×10^{-15}) (Table 5 and Supplemental Figure S4). Conversely, factors that promote cell cycle progression were activated in the KO versus WT mice on day 5, which included Foxm1 (activation z-score, 4.063; *P* value of overlap, 1.41×10^{-15}), cyclin D1 (*Ccnd1*; activation z-score, 4.382; *P* value of overlap, 1.02×10^{-23}), and E2f (activation z-score, 4.555; *P* value of overlap, 2.01×10^{-12}) (Table 5 and Supplemental Figures S5–S7). Interestingly, the transcription factor that displayed the greatest level of activation in the KO on day 5 after TCPOBOP was *Tbx2* (activation z-score, 5.253; *P* value of overlap, 5.81×10^{-29}), which has been shown to promote proliferation through the ERK signaling pathway.[33] Western blot analyses did not show changes in most cell cycle suppressor genes; however, total YAP expression and nuclear localization as measured by immunofluorescence was increased in the KO livers in comparison to control livers on day 5 after TCPOBOP (Figure 8, B and C). These data show that LSP1 loss leads to increased hepatocellular proliferation following TCPOBOP due to increased activation of pathways involved in cell cycle progression, including YAP.

Discussion

Despite multiple studies demonstrating the association of LSP1 with hepatocyte growth suppression, its precise mechanism of action remains elusive. Original studies related to the discovery of LSP1 and its functions showed that in lymphocytes LSP1 is an F-actin binding protein connecting the ERK pathway to the cytoskeleton.[16] Recent studies have also shown that modulation of the cytoskeleton activates pathways related to mechanotransduction at the level of individual cells, resulting in a variety of effects on pathways related to cell proliferation, including migration of YAP1 to the nucleus or inactivation of YAP1 by phosphorylation, leading to its degradation.[34,35] The findings from the current study provide another example of the important role played by cytoskeleton proteins in different aspects of cell proliferation and differentiation. LSP1 binds to F-actin in hepatocytes,[21] regulates phosphorylation of ERK,[16,22] and binds to MEK1 and ERK2 via kinase suppressor of Ras to target this complex to the cytoskeleton. The current study demonstrates the complexity of the regulation exerted on hepatocyte functions by LSP1. LSP1 is associated with F-actin and cytoskeletal regulation in a variety of cells besides hepatocytes.[36–39] It acts as a growth suppressor of hepatocyte proliferation not only in relation to liver regeneration[21,22] but also, as demonstrated in this work, in xenobiotic-induced hepatocyte proliferation. The effects of xenobiotics (as exemplified by TCPOBOP) on hepatocyte proliferation and liver size are an important issue related to regulation of therapeutic levels of drugs in clinical pharmacology.

The direct association of LSP1 with YAP seen herein is surprising; however, several recent studies have shown that regulation of YAP by the Hippo pathway is directly connected to structural and functional aspects of the cytoskeleton. Mechanotransduction has a direct impact on YAP activation or inactivation,[34,35,40,41] and YAP is involved in anchoring the actin cytoskeleton to the plasma membrane during the formation of focal adhesions to enable cell spreading.[41] Fascin1, an F-actin bundling protein, has recently been demonstrated to also mediate YAP activation due to changes in the extracellular matrix in the context of

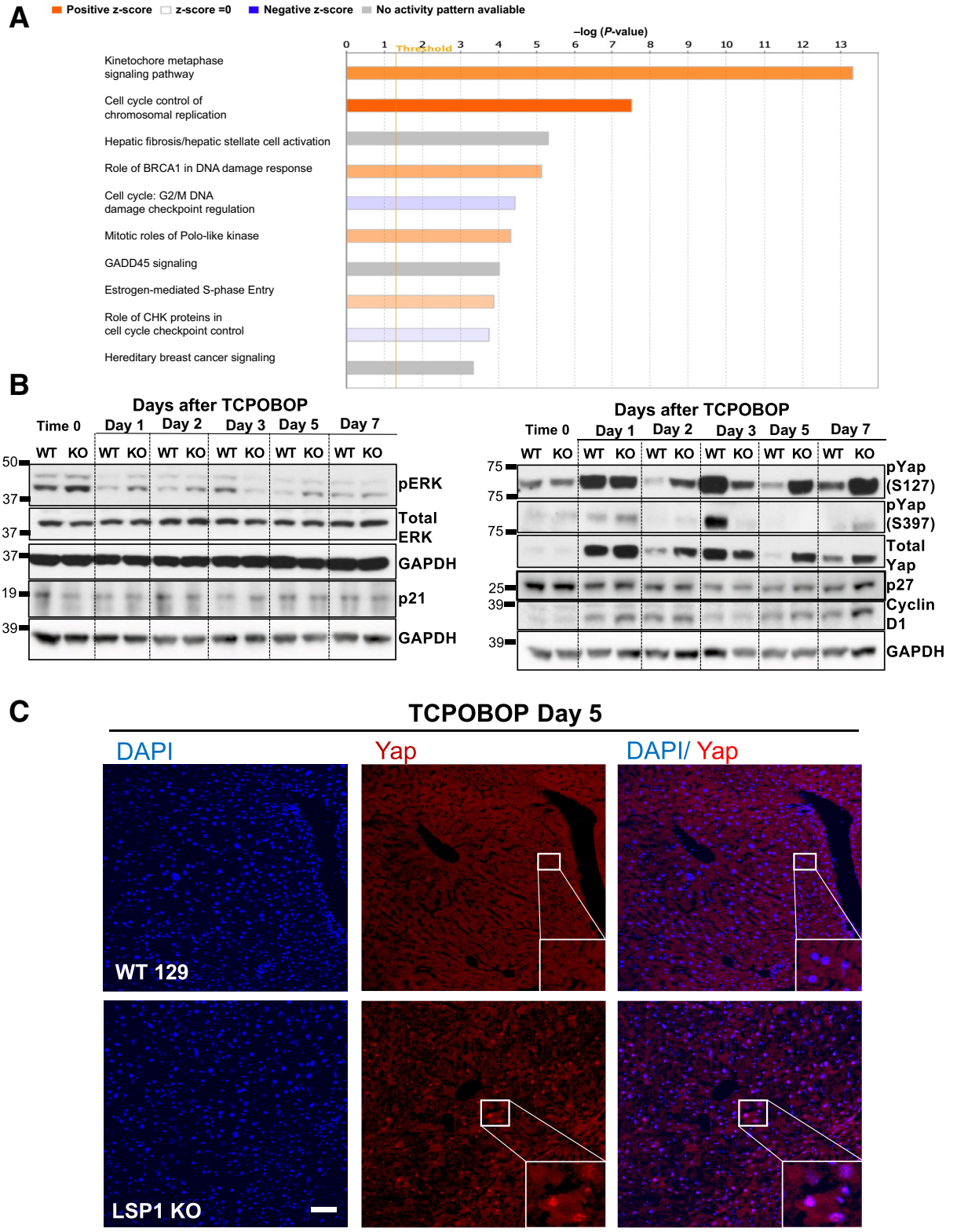


Figure 8 Increased cell cycle activation on day 5 following 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) in *Lsp1* knockout (KO) livers. **A:** Ingenuity pathway analysis–based canonical signaling pathways predicted to be changed in *Lsp1* KO versus wild-type (WT) livers on day 5 after TCPOBOP. **B:** Western blot analyses of phosphorylated extracellular signal-regulated kinase (pERK), total ERK, p21, phosphorylated YAP (pYAP) S127, pYAP S397, total YAP, p27, and cyclin D1 in WT and *Lsp1* KO whole liver lysates following TCPOBOP. Each lane contains a pooled sample. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), loading control. **C:** Immunofluorescence of WT and *Lsp1* KO livers for total YAP (red) on day 5. DAPI (nuclei) and merge of DAPI and total YAP. **Insets:** Magnified images from **boxed areas** to demonstrate YAP staining in the hepatocytes. $n = 3$ (**B**). Scale bar = 100 μm (**C**). Original magnifications, ×200 (**C**, **main images**); ×400 (**C**, **insets**).

Table 5 IPA of RNA-Sequencing Data Demonstrating Upstream Regulators Predicted to be Changed in *Lsp1* KO versus WT Livers on Day 5 after TCPOBOP Treatment

Upstream regulator	Predicted activation state	Activation z-score	*P* value of overlap
Tbx2	Activated	5.253	5.81×10^{-29}
RabI6	Activated	5.112	8.70×10^{-19}
E2f	Activated	4.555	2.01×10^{-12}
Cyclin D1 (ccnd1)	Activated	4.382	1.02×10^{-23}
Foxm1	Activated	4.063	1.41×10^{-15}
Hgf	Activated	3.720	1.80×10^{-18}
Erbb2	Activated	2.925	3.74×10^{-28}
p53	Inhibited	−4.488	3.95×10^{-21}
Rbl2	Inhibited	−3.780	5.01×10^{-9}
p16 (Cdkn2a)	Inhibited	−3.664	3.04×10^{-17}
Smarcb1	Inhibited	−3.604	9.66×10^{-15}
Rb	Inhibited	−3.447	6.44×10^{-13}
p21 (Cdkn1a)	Inhibited	−3.287	1.27×10^{-33}

IPA, ingenuity pathway analysis; KO, knockout; TCPOBOP, 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene; WT, wild type.

cholangiocarcinoma development.[42] Therefore, actin binding proteins and the cytoskeleton have an important function in modulating YAP activity, with LSP1 a newly identified and important modulator in this system.

Multiple studies have shown that CAR activation is associated with the following: i) proliferation of hepatocytes in the absence of liver injury, ii) induction of enzymes involved in drug metabolism, and iii) increases in overall liver mass. Although induction of enzymes associated with drug metabolism is directly controlled by CAR, the concomitant effects on hepatocyte proliferation and expansion of liver mass are elicited by CAR through recruitment of other regulators and signaling pathways directly associated with hepatocyte proliferation. Inhibition of receptor tyrosine kinases (MET and EGFR) dramatically decreases hepatocyte proliferation following activation of CAR by TCPOBOP.[4,5] The current study demonstrates that LSP1 also regulates activation of EGFR, linking EGFR activation to regulation by mechanotransduction (Supplemental Figures S1 and S3). These studies need to be explored further. A similar effect was seen in YAP KO mice.[4,31] The findings in the current work add to the overall role of LSP1 by demonstrating that direct binding of LSP1 to YAP is a mechanism by which LSP1, in TG mice, prevents YAP from moving to the nucleus, subsequently limiting its ability to interact with CAR and thus affecting CAR-induced hepatocyte proliferation. Combined, these results provide a background for further investigation that may explain the documented role of LSP1 in breast and urinary bladder cancers.[12–15]

Interestingly, LSP1 overexpression did not inhibit TCPOBOP-driven hepatocyte hypertrophy,[43–45] which led to increased liver/body weight ratios in the TG mice in the absence of increased proliferation. Increased hepatocyte hypertrophy is associated with enhanced expression of drug metabolism enzymes along with increased organelle size; however, the current results indicate that LSP1 does not affect CAR-driven drug metabolism.[46] Other studies have also demonstrated that CAR-driven proliferation and hypertrophy can be driven by different mechanisms. *Gadd45b*-KO mice display increased hepatocyte proliferation in response to TCPOBOP without increased hypertrophy, leading to smaller liver weights in the KO versus WT mice.[46] In addition, YAP-KO mice show decreased proliferation but no discernible difference in hepatocellular volume following TCPOBOP administration.[31] Therefore, signaling mechanisms controlling TCPOBOP-driven hepatocellular proliferation differ from those governing hypertrophy.

Lsp1 TG hepatocytes displayed inhibition of the YAP signaling pathway, which is corroborated by previous findings that loss of YAP expression in the hepatocytes leads to decreased proliferation after TCPOBOP.[31] LSP1 expression is down-regulated when YAP is overexpressed in the MCF10A human breast epithelial cell line.[47] This finding suggests a reciprocal regulation of functionality between YAP and CAR in specific tissues and circumstances. *Lsp1* TG hepatocytes also displayed decreased activation of Foxm1 signaling, which has been shown to facilitate TCPOBOP-driven proliferation by regulating cell cycle related genes, such as cyclins, cdc25 phosphatases, and regulators of mitosis, such as aurora kinase B.[48] In addition to its role in promoting transcription of cell cycle progressing genes, Foxm1 also inhibits expression of cell cycle inhibitors, such as p21,[48] which is up-regulated in the *Lsp1* TG hepatocytes after TCPOBOP. The current data demonstrate that Foxm1 is involved in the signaling mechanisms by which LSP1 affects hepatocyte proliferation.

These results demonstrate that the effect of global loss of LSP1 in transgenic animals was opposite to that observed in the WT animals. *Lsp1* KO livers displayed decreased induction of cell cycle inhibitor pathways, including p16 and p21. In addition, YAP translocation to the nucleus was enhanced in the absence of LSP1 expression in response to TCPOBOP. YAP activation and deactivation are affected by changes in the cytoskeleton.[49] Therefore, LSP1 may affect YAP signaling through modulation of the actin cytoskeleton. Even though the liver responses of KO mice were symmetrically different from those of TG mice, the possibility that the global elimination of LSP1 may have caused changes in extrahepatic sites that may have affected the liver responses shown in this study cannot be entirely ruled out. Expression of LSP1 has been primarily examined in lymphoid cells.[16] LSP1 expression is also seen in bladder and breast in the context of broader studies, where LSP1 is present along with many other unrelated genes.[12–15] In none of these studies, however, was there any analysis of global signaling changes that may affect liver-specific gene expression. This issue may be addressed with detailed evaluation of LSP1 function in other tissues.

Overall, the current study demonstrated that LSP1 is a protein with many functionalities. Its effects on ERK phosphorylation and activation have been demonstrated in many other studies. In normal liver, LSP1 is critical in regulating mitogenic effects through EGFR and MET.[21] Its effects on the Hippo/YAP pathway have not been previously described, and the findings presented in this study demonstrate that LSP1 should be considered as a complex regulator causing decreased mitogenic responses through both receptor tyrosine kinases (MET and EGFR) and the YAP/Hippo pathway. In view of the F-actin binding of LSP1[36] and its effects on the cytoskeleton, LSP1 is an important contributor to the mechanosensory effects of regulation of cell proliferation and function. Because LSP1 is involved in breast,[13–15] bladder,[12] and liver[10,21,22,24,26] cancers, understanding how LSP1 functions to suppress hepatocellular proliferation can provide new avenues of investigation to develop novel therapeutics.

Author Contributions

All authors had access to all of the data and have approved the final article.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.ajpath.2022.03.010*.

References

1. Kazantseva YA, Pustylnyak YA, Pustylnyak VO: Role of nuclear constitutive androstane receptor in regulation of hepatocyte proliferation and hepatocarcinogenesis. Biochemistry (Mosc) 2016, 81: 338–347
2. Tzameli I, Pissios P, Schuetz EG, Moore DD: The xenobiotic compound 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene is an agonist ligand for the nuclear receptor CAR. Mol Cell Biol 2000, 20:2951–2958
3. Locker J, Tian J, Carver R, Concas D, Cossu C, Ledda-Columbano GM, Columbano A: A common set of immediate-early response genes in liver regeneration and hyperplasia. Hepatology 2003, 38:314–325
4. Bhushan B, Stoops JW, Mars WM, Orr A, Bowen WC, Paranjpe S, Michalopoulos GK: TCPOBOP-induced hepatomegaly and hepatocyte proliferation are attenuated by combined disruption of MET and EGFR signaling. Hepatology 2019, 69:1702–1718
5. Donthamsetty S, Bhave VS, Kliment CS, Bowen WC, Mars WM, Bell AW, Stewart RE, Orr A, Wu C, Michalopoulos GK: Excessive hepatomegaly of mice with hepatocyte-targeted elimination of integrin linked kinase following treatment with 1,4-bis [2-(3,5-dichaloropyridyloxy)] benzene. Hepatology 2011, 53:587–595
6. Michalopoulos GK, Bhushan B: Liver regeneration: biological and pathological mechanisms and implications. Nat Rev Gastroenterol Hepatol 2021, 18:40–55
7. Dong B, Lee JS, Park YY, Yang F, Xu G, Huang W, Finegold MJ, Moore DD: Activating CAR and beta-catenin induces uncontrolled liver growth and tumorigenesis. Nat Commun 2015, 6:5944
8. Ledda-Columbano GM, Pibiri M, Loi R, Perra A, Shinozuka H, Columbano A: Early increase in cyclin-D1 expression and accelerated entry of mouse hepatocytes into S phase after administration of the mitogen 1, 4-bis[2-(3,5-dichloropyridyloxy)] benzene. Am J Pathol 2000, 156:91–97
9. Michalopoulos GK: Hepatostat: liver regeneration and normal liver tissue maintenance. Hepatology 2017, 65:1384–1392
10. Nalesnik MA, Tseng G, Ding Y, Xiang GS, Zheng ZL, Yu Y, Marsh JW, Michalopoulos GK, Luo JH: Gene deletions and amplifications in human hepatocellular carcinomas: correlation with hepatocyte growth regulation. Am J Pathol 2012, 180:1495–1508
11. Jongstra-Bilen J, Misener VL, Wang C, Ginzberg H, Auerbach A, Joyner AL, Downey GP, Jongstra J: LSP1 modulates leukocyte populations in resting and inflamed peritoneum. Blood 2000, 96: 1827–1835
12. Zeng SX, Zhu Y, Ma AH, Yu W, Zhang H, Lin TY, Shi W, Tepper CG, Henderson PT, Airhart S, Guo JM, Xu CL, deVere White RW, Pan CX: The phosphatidylinositol 3-kinase pathway as a potential therapeutic target in bladder cancer. Clin Cancer Res 2017, 23:6580–6591
13. Mulligan AM, Couch FJ, Barrowdale D, Domchek SM, Eccles D, Nevanlinna H, et al: Common breast cancer susceptibility alleles are associated with tumour subtypes in BRCA1 and BRCA2 mutation carriers: results from the Consortium of Investigators of Modifiers of BRCA1/2. Breast Cancer Res 2011, 13:R110
14. Tang J, Li H, Luo J, Mei H, Peng L, Li X: The LSP1 rs3817198 T > C polymorphism contributes to increased breast cancer risk: a meta-analysis of twelve studies. Oncotarget 2016, 7:63960–63967
15. Nickels S, Truong T, Hein R, Stevens K, Buck K, Behrens S, et al: Evidence of gene-environment interactions between common breast cancer susceptibility loci and established environmental risk factors. PLoS Genet 2013, 9:e1003284
16. Harrison RE, Sikorski BA, Jongstra J: Leukocyte-specific protein 1 targets the ERK/MAP kinase scaffold protein KSR and MEK1 and ERK2 to the actin cytoskeleton. J Cell Sci 2004, 117:2151–2157
17. Guegan JP, Fremin C, Baffet G: The MAPK MEK1/2-ERK1/2 pathway and its implication in hepatocyte cell cycle control. Int J Hepatol 2012, 2012:328372
18. Wang C, Hayashi H, Harrison R, Chiu B, Chan JR, Ostergaard HL, Inman RD, Jongstra J, Cybulsky MI, Jongstra-Bilen J: Modulation of Mac-1 (CD11b/CD18)-mediated adhesion by the leukocyte-specific protein 1 is key to its role in neutrophil polarization and chemotaxis. J Immunol 2002, 169:415–423
19. Wu JL, Wu HY, Tsai DY, Chiang MF, Chen YJ, Gao S, Lin CC, Lin CH, Khoo KH, Chen YJ, Lin KI: Temporal regulation of Lsp1 O-GlcNAcylation and phosphorylation during apoptosis of activated B cells. Nat Commun 2016, 7:12526
20. Wang J, Jiao H, Stewart TL, Lyons MV, Shankowsky HA, Scott PG, Tredget EE: Accelerated wound healing in leukocyte-specific, protein 1-deficient mouse is associated with increased infiltration of leukocytes and fibrocytes. J Leukoc Biol 2007, 82:1554–1563
21. Koral K, Paranjpe S, Bowen WC, Mars W, Luo J, Michalopoulos GK: Leukocyte-specific protein 1: a novel regulator of hepatocellular proliferation and migration deleted in human hepatocellular carcinoma. Hepatology 2015, 61:537–547
22. Koral K, Haynes M, Bowen WC, Orr A, Mars W, Michalopoulos GK: Lymphocyte-specific protein-1 controls sorafenib sensitivity and hepatocellular proliferation through extracellular signal-regulated kinase 1/2 activation. Am J Pathol 2018, 188: 2074–2086
23. Wang Q, Ye J, Fang D, Lv L, Wu W, Shi D, Li Y, Yang L, Bian X, Wu J, Jiang X, Wang K, Wang Q, Hodson MP, Thibaut LM, Ho JWK, Giannoulatou E, Li L: Multi-omic profiling reveals associations between the gut mucosal microbiome, the metabolome, and host DNA methylation associated gene expression in patients with colorectal cancer. BMC Microbiol 2020, 20:83
24. Zhang H, Wang Y, Liu Z, Yao B, Dou C, Xu M, Li Q, Jia Y, Wu S, Tu K, Liu Q: Lymphocyte-specific protein 1 inhibits the growth of hepatocellular carcinoma by suppressing ERK1/2 phosphorylation. FEBS Open Bio 2016, 6:1227–1237

25. Park SL, Fesinmeyer MD, Timofeeva M, Caberto CP, Kocarnik JM, Han Y, et al: Pleiotropic associations of risk variants identified for other cancers with lung cancer risk: the PAGE and TRICL Consortia. J Natl Cancer Inst 2014, 106:dju061
26. Lubecka K, Flower K, Beetch M, Qiu J, Kurzava L, Buvala H, Ruhayel A, Gawrieh S, Liangpunsakul S, Gonzalez T, McCabe G, Chalasani N, Flanagan JM, Stefanska B: Loci-specific differences in blood DNA methylation in HBV-negative populations at risk for hepatocellular carcinoma development. Epigenetics 2018, 13: 605−626
27. Novicki DL, Rosenberg MR, Michalopoulos G: Inhibition of DNA synthesis by chemical carcinogens in cultures of initiated and normal proliferating rat hepatocytes. Cancer Res 1985, 45:337−344
28. Committee for the Update of the Guide for the Care and Use of Laboratory Animals; National Research Council: Guide for the Care and Use of Laboratory Animals. Eighth Edition. Washington, DC, National Academies Press, 2011
29. Limaye PB, Bowen WC, Orr AV, Luo J, Tseng GC, Michalopoulos GK: Mechanisms of hepatocyte growth factor-mediated and epidermal growth factor-mediated signaling in transdifferentiation of rat hepatocytes to biliary epithelium. Hepatology 2008, 47:1702−1713
30. Tojima H, Kakizaki S, Yamazaki Y, Takizawa D, Horiguchi N, Sato K, Mori M: Ligand dependent hepatic gene expression profiles of nuclear receptors CAR and PXR. Toxicol Lett 2012, 212:288−297
31. Bhushan B, Molina L, Koral K, Stoops JW, Mars WM, Banerjee S, Orr A, Paranjpe S, Monga SP, Locker J, Michalopoulos GK: YAP is crucial for CAR-driven hepatocyte proliferation, but not for induction of drug metabolism genes in mice. Hepatology 2021, 73:2005−2022
32. Abbastabar M, Kheyrollah M, Azizian K, Bagherlou N, Tehrani SS, Maniati M, Karimian A: Multiple functions of p27 in cell cycle, apoptosis, epigenetic modification and transcriptional regulation for the control of cell growth: a double-edged sword protein. DNA Repair (Amst) 2018, 69:63−72
33. Liu X, Miao Z, Wang Z, Zhao T, Xu Y, Song Y, Huang J, Zhang J, Xu H, Wu J, Xu H: TBX2 overexpression promotes proliferation and invasion through epithelial-mesenchymal transition and ERK signaling pathway. Exp Ther Med 2019, 17:723−729
34. Wagh K, Ishikawa M, Garcia DA, Stavreva DA, Upadhyaya A, Hager GL: Mechanical regulation of transcription: recent advances. Trends Cell Biol 2021, 31:457−472
35. Totaro A, Panciera T, Piccolo S: YAP/TAZ upstream signals and downstream responses. Nat Cell Biol 2018, 20:888−899
36. Jongstra-Bilen J, Janmey PA, Hartwig JH, Galea S, Jongstra J: The lymphocyte-specific protein LSP1 binds to F-actin and to the cytoskeleton through its COOH-terminal basic domain. J Cell Biol 1992, 118:1443−1453
37. Jongstra-Bilen J, Jongstra J: Leukocyte-specific protein 1 (LSP1): a regulator of leukocyte emigration in inflammation. Immunol Res 2006, 35:65−74
38. Cervero P, Wiesner C, Bouissou A, Poincloux R, Linder S: Lymphocyte-specific protein 1 regulates mechanosensory oscillation of podosomes and actin isoform-based actomyosin symmetry breaking. Nat Commun 2018, 9:515
39. Scharinger K, Maxeiner S, Schalla C, Rutten S, Zenke M, Sechi A: LSP1-myosin1e bimolecular complex regulates focal adhesion dynamics and cell migration. FASEB J 2021, 35:e21268
40. Croci O, De Fazio S, Biagioni F, Donato E, Caganova M, Curti L, Doni M, Sberna S, Aldeghi D, Biancotto C, Verrecchia A, Olivero D, Amati B, Campaner S: Transcriptional integration of mitogenic and mechanical signals by Myc and YAP. Genes Dev 2017, 31: 2017−2022
41. Nardone G, Oliver-De La Cruz J, Vrbsky J, Martini C, Pribyl J, Skladal P, Pesl M, Caluori G, Pagliari S, Martino F, Maceckova Z, Hajduch M, Sanz-Garcia A, Pugno NM, Stokin GB, Forte G: YAP regulates cell mechanics by controlling focal adhesion assembly. Nat Commun 2017, 8:15321
42. Pocaterra A, Scattolin G, Romani P, Ament C, Ribback S, Chen X, Evert M, Calvisi DF, Dupont S: Fascin1 empowers YAP mechanotransduction and promotes cholangiocarcinoma development. Commun Biol 2021, 4:763
43. Blanco-Bose WE, Murphy MJ, Ehninger A, Offner S, Dubey C, Huang W, Moore DD, Trumpp A: C-Myc and its target FoxM1 are critical downstream effectors of constitutive androstane receptor (CAR) mediated direct liver hyperplasia. Hepatology 2008, 48: 1302−1311
44. Qatanani M, Moore DD: CAR, the continuously advancing receptor, in drug metabolism and disease. Curr Drug Metab 2005, 6:329−339
45. Huang W, Zhang J, Washington M, Liu J, Parant JM, Lozano G, Moore DD: Xenobiotic stress induces hepatomegaly and liver tumors via the nuclear receptor constitutive androstane receptor. Mol Endocrinol 2005, 19:1646−1653
46. Tian J, Huang H, Hoffman B, Liebermann DA, Ledda-Columbano GM, Columbano A, Locker J: Gadd45beta is an inducible coactivator of transcription that facilitates rapid liver growth in mice. J Clin Invest 2011, 121:4491−4502
47. Hao Y, Chun A, Cheung K, Rashidi B, Yang X: Tumor suppressor LATS1 is a negative regulator of oncogene YAP. J Biol Chem 2008, 283:5496−5509
48. Costa RH, Kalinichenko VV, Tan Y, Wang IC: The CAR nuclear receptor and hepatocyte proliferation. Hepatology 2005, 42: 1004−1008
49. Sun S, Irvine KD: Cellular organization and cytoskeletal regulation of the hippo signaling network. Trends Cell Biol 2016, 26:694−704