Cancer Research

LSD1 Stimulates Cancer-Associated Fibroblasts to Drive Notch3-Dependent Self-Renewal of Liver Cancer Stem–like Cells

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Abstract

Cancer stem-like cells (CSC) in hepatocellular carcinoma (HCC) are thought to mediate therapeutic resistance and poor survival outcomes, but their intrinsic and extrinsic control is not well understood. In this study, we found that the chromatin modification factor LSD1 is highly expressed in HCC CSC where it decreases during differentiation. LSD1 was responsible for maintaining CSC self-renewal and tumorigenicity in HCC, and its overexpression was sufficient to drive self-renewal of non-CSC. Levels of acetylated LSD1 were low in CSC with high LSD1 activity, and these CSC were capable of self-renewal. Notch signaling activated LSD1 through induction of the sirtuin SIRT1, leading to deacetylation and activation of LSD1 and CSC self-renewal. Notably, we found that LSD1 expression was increased in cancer-associated fibroblasts (CAF) as an upstream driver of Notch3-mediated CSC self-renewal. In clinical specimens of HCC, the presence of CAF, LSD1, and Notch3 strongly associated with poor patient survival. Overall, our results reveal that CAF-induced expression of Notch3 is responsible for LSD1 activation in CSC, driving their self-renewal in HCC.

Significance: These seminal findings illuminate a complex pathway in the tissue microenvironment of liver cancer, which is responsible for orchestrating the self-renewal of stem-like cancer cells, with potential implications to improve therapy and limit relapses. *Cancer Res; 78(4); 938–49. ©2017 AACR.*

Introduction

Hepatocellular carcinoma (HCC), the most common and lethal primary tumor, remains a challenging disease with poor prognosis. Cancer stem cells (CSC) within the tumor bulk display the capacity to self-renewal, differentiate, and give rise to a new tumor (1). Liver CSCs are enriched in certain defined markers (2–8). Although the mechanisms related to the self-renewal of liver CSCs have been partially elucidated (2–8), how this process is intrinsically and extrinsically regulated remains largely elusive.

A novel component of epigenetic dysregulation in cancer was uncovered by studies on lysine demethylases, which play a central role in the self-renewal and tumorigenicity of CSCs. The first identified lysine demethylase 1 (LSD1) is a histone-modifying enzyme that is responsible for demethylating histone H3 lysine 4 (H3K4) and histone H3 lysine 9 (H3K9), which are involved in genome instability, stem cell biology, and cancer (9–11). LSD1 is an essential epigenetic regulator of pluripotency in embryonic stem cells (ESC) and hematopoietic stem cells (12–14). Many studies have highlighted the important role of LSD1 in pluripotent cancer cells and have provided evidence that LSD1 inhibition might represent a therapeutic strategy for cancer treatment (15–17). Interestingly, LSD1 is highly expressed in $Lgr5^+$ cancer cells and suppresses negative regulators of β-catenin to promote HCC stemness and development (18). Despite these advances, the role of LSD1 in self-renewal of liver CSCs remains largely unknown.

Notch signaling plays an important role in development by modulating cell-fate determination, cell survival, and proliferation (19). Notch receptors, of which there are four in mammals (Notch1-4), are activated after binding to different ligands [Delta-like 1 (DLL1), DLL3, DLL4, JAG1, and JAG2]. Notch proteins and their ligands interact with neighboring cells, indicating a requirement for direct cell–cell contact. Upon ligand binding, the intracellular Notch domain is cleaved, after which it translocates to the nucleus to regulate downstream target gene transcription (20). Notch proteins and ligands are upregulated in several cancers, and the roles of Notch signaling in tumor cells include both tumor-promoting and tumor-suppressing activities, depending on the tumor type (21, 22). In HCC, Notch receptors tend to be overexpressed, and the expression of their ligands correlates with aggressive phenotypes (23). Recently, some studies showed that Notch1 could promote HCC cell growth, metastasis, and stemness via activation of the Stat3 and Wnt/beta-catenin signaling pathways (24, 25) and Notch3 could regulate stemness of tumor cells via the inactivation of the Wnt/beta-catenin pathway (26). Our previous study showed that Sox9 acts as a downstream of regulator Notch signaling through inhibition of Numb, thereby directing symmetrical cell division and promoting liver CSCs self-renewal (27). All of these studies suggested that the Notch signaling pathway promotes self-renewal of liver CSCs. But molecular mechanisms in modulation of self-renewal of liver CSCs by the Notch signaling pathway are still poorly understood.

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Therefore, we hypothesized that LSD1 might link the intrinsic and extrinsic regulations of liver CSCs. Accordingly, we demonstrated that cancer-associated fibroblasts (CAF) activate Notch3 signaling, which subsequently maintains LSD1 stability by inducing its deacetylation, thereby promoting self-renewal and tumor growth of liver CSCs.

Materials and Methods

Animal

All NOD/SCID mice used in this research were obtained from the Third Military Medical University and were maintained in pathogen-free conditions. All procedures were performed according to protocols approved by the Institutional Review Board of the Southwest Hospital, Third Military Medical University and conformed with the NIH guidelines on the ethical use of animals. Mice, 4 to 5 weeks of age, were used for cancer cell implantation. Tumors were dissected at the end of the experiments and were weighed.

Cell culture, lentiviral constructs, and transductions

293T and human liver cancer cell lines (PLC/PRF/5 and Huh7) were purchased from the ATCC, where they were characterized by DNA fingerprinting and isozyme detection. 293T, HCC/ExV cell lines (PLC/PRF/5 and Huh7) and HCC primary cell samples (T1115 and T1224) were grown in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37°C in a humidified atmosphere containing 5% CO_2. No mycoplasma contamination was observed in these cell lines. Cells were authenticated by short tandem repeat profiling. Immediately after receipt, they were expanded and frozen to be revived every 3 to 4 months.

Lentiviral vectors containing short hairpin RNAs against human LSD1, SIRT1, Notch1, Notch2, Notch3, and Notch4 were packaged into recombinant lentivirus as described previously (26). Lentiviral target sequences are listed in Supplementary Table S1.

In vivo tumor formation

Generally, 1×10^6 cells, or 100 to 10,000 cells, were mixed with Matrigel, and were injected subcutaneously into the flanks of NOD/SCID mice. mCherry-GFP-labeled PLC/PRF/5 cells and HCC-associated fibroblasts (the ratio 1:3) were subcutaneously inoculated into the flanks of NOD/SCID mice. Tumors grew for approximately 4 weeks or longer until they reached an appropriate size of 1,500 mm^3. Tumor size was measured with digital calipers and calculated based on the following formula: length $\times$ $(\text{width})^2 \times \pi/6$.

Immunoprecipitation

Cells were collected and lysed in lysis buffer (Thermo Fisher Scientific) supplemented with protease inhibitors, incubated on ice for 30 minutes, and cleared by centrifugation at 13,500 rpm at 4°C for 15 minutes. Total protein lysate (600 μg) was subjected to immunoprecipitation with agarose-immobilized antibodies (anti-LSD1, SIRT1, Ub, Ac-Lysine antibody, or isotype control antibodies) overnight at 4°C. After incubation, protein immunocomplexes were washed four times with 800 μL of wash buffer (Thermo Fisher Scientific). Protein complexes were analyzed by western blotting using 8% to 15% SDS-PAGE.

LSD1 activity assay

LSD1 activity was measured using a Histone Demethylase KDM1/LSD1 Activity Quantification Assay Kit according to the manufacturer's recommendations (Abcam, ab113457). Briefly, nuclear extract was harvested and incubated with substrate and assay buffer. After washing wells, capture antibody and detection antibody were added. After developing solution was added, measurements were performed using the Thermo Fisher Scientific Varioskan Flash fluorescence spectrophotometer.

Treatment of cells with inhibitors *in vitro*

Different amounts of inhibitor(s) were dissolved in dimethyl sulfoxide or ethanol. Cells were plated in 6-well plates. When cells reached 60% confluence, they were treated with the appropriate dose of inhibitor at different times. After treatment, cells were collected for protein extraction and immunoblotting.

IHC and immunofluorescence staining

Tumor tissues were harvested, fixed in 10% formalin, and embedded in paraffin for IHC assays. IHC staining was performed using a DAKO Autostainer (DAKO) with DAKO $LSAB^+$ and diaminobenzadine as the chromogen. IF staining was performed on tumor cells cultured on chamber slides (Thermo Fisher Scientific). The primary antibody was rabbit anti-human LSD1, Ki-67, Notch3, and PDGFα (Cell Signaling Technology) or mouse anti-human α-SMA (clone 1A4, IgG2α, Dako). Secondary antibodies were goat anti-mouse IgG (H+L) conjugated with Alexa Fluor 568 or goat anti-rabbit IgG (H+L) conjugated with Alexa Fluor 568.

Statistical analysis

Data are presented as mean ± SEM. Statistical comparisons between experimental groups were analyzed by a Student test. A P value < 0.05 was considered statistically significant. Log-rank analysis was used to determine statistical significance of Kaplan–Meier survival curves.

Results

LSD1 is highly expressed in liver CSCs and correlates with poor prognosis for HCC patients

To determine whether LSD1 expression is elevated in liver CSCs, we isolated these cells and non-CSCs form HCC cell lines and patient-derived primary HCCs using our established P_{Nanog}-green fluorescent protein (GFP) CSC isolation system (5). Immunoblotting and IF revealed higher levels of LSD1 in liver CSCs *in vitro*, as compared with that in non-CSCs (Fig. 1A); similar results were found in oncosphere cells derived from HCC cell lines and primary HCC samples (Fig. 1B). In addition, we observed high levels of LSD1 in the first and second xenograft tumors derived from liver CSCs by immunoblotting and IHC (Fig. 1C and D). During liver CSC differentiation using conventional conditions (27), we observed a gradual decrease in LSD1 expression. Meanwhile, albumin expression, a marker of hepatocellular differentiation, increased gradually (Fig. 1E). We next analyzed the clinical significance of LSD1 expression in human HCC patients. This marker was highly expressed in HCC tumors, but was almost undetectable in noncancerous tissues, and this difference was statistically significant (Fig. 1F). These data indicate that LSD1 is highly expressed in liver CSCs and HCC tissues.

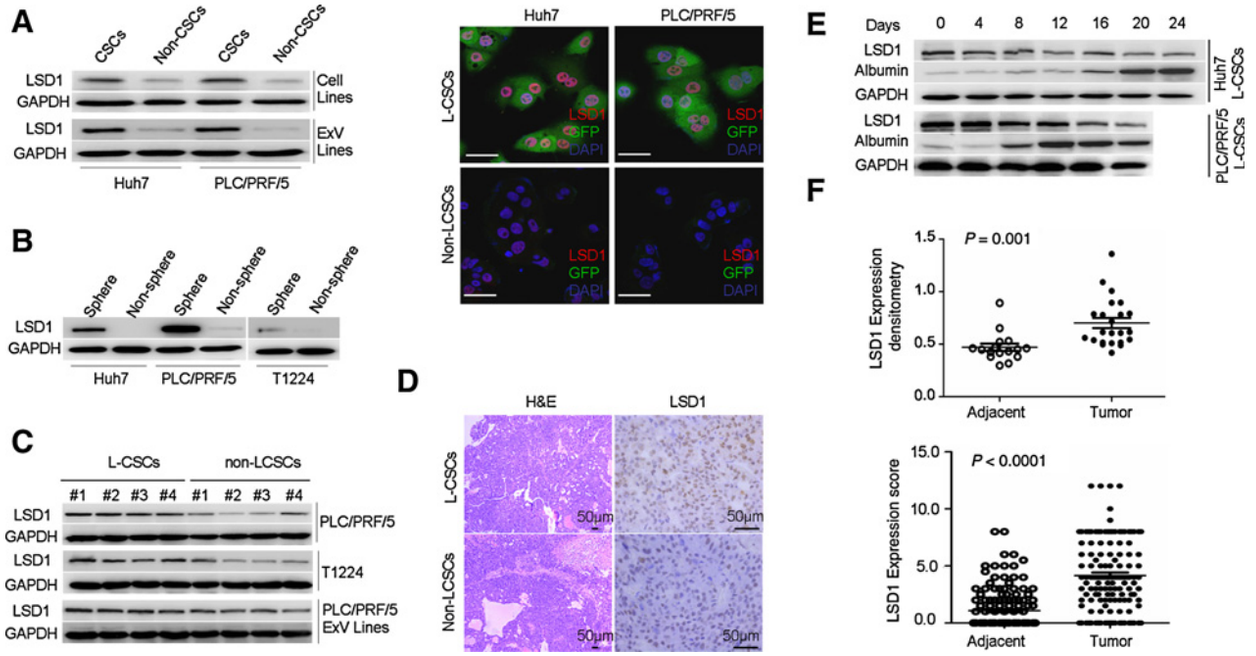


Figure 1.

LSD1 is highly expressed in liver CSCs and HCC tumors. **A,** Left, immunoblotting analysis of LSD1 in liver CSC and non-CSC populations purified from different HCC cell lines or ExV lines (digested tumors were cultured *in vitro* to generate *ex vivo* cell lines) by FACS. Right, Immunofluorescence staining for LSD1 expression in liver CSC and non-CSC populations purified from different HCC cell lines by FACS. Scale bars, 50 μm. **B,** Immunoblotting analysis of LSD1 in oncosphere and nononcosphere cells derived from different HCC cell lines or primary cell line. **C,** Immunoblotting analysis of LSD1 in liver CSC and non-CSC tumors derived from PLC/PRF/5 and T1224 cells or an ExV line. **D,** IHC staining for LSD1 expression in liver CSC and non-CSC tumors derived from PLC/PRF/5 cells generated *in vivo*. Representative images are shown. Scale bars, 50 μm. **E,** Immunoblotting analysis of LSD1 expression at different times in FACS-purified liver CSCs cultured *in vitro*. **F,** Top, immunoblotting analysis of LSD1 protein levels in adjacent tissues and liver cancer tissues. For quantification, densitometry signals were normalized to those of glyceraldehyde 3-phosphate dehydrogenase using the GeneTools image analysis program (SynGene). Bottom, Statistical analysis of LSD1 expression determined by IHC in adjacent tissues and liver cancer tissues from HCC patients (Student *t* test).

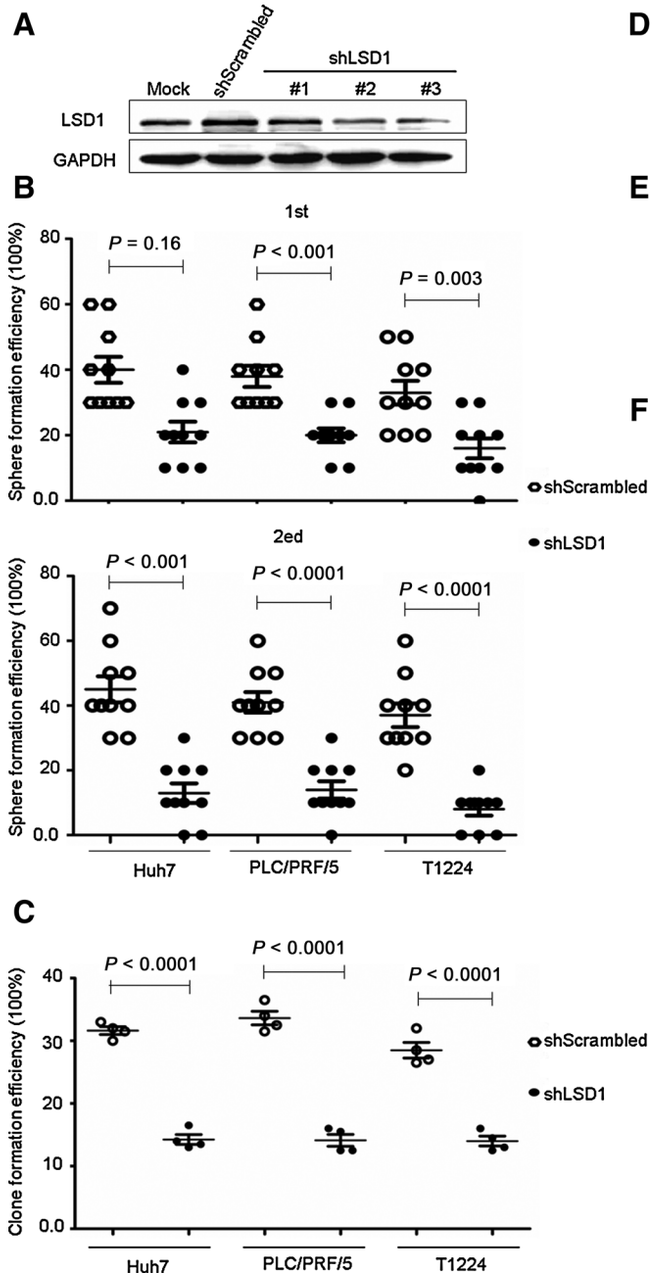
LSD1 is required for the self-renewal and tumorigenic potential of liver CSCs

To further test if LSD1 plays a critical role in liver CSC self-renewal and tumorigenicity, we treated different HCC subpopulations with shLSD1 or LSD1 overexpression vectors. Three shRNAs targeting LSD1 were designed (Fig. 2A), and shLSD1 (#3) resulted in the strongest reduction in LSD1 protein, and was used in subsequent experiments. Regarding the relative self-renewal potential of liver CSCs, knockdown (KD) of LSD1 decreased sphere and clone formation (Fig. 2B and C). We next established a xenograft tumor model to examine the role of LSD1 in tumor development in NOD/SCID mice. We observed that with LSD1-KD liver CSCs more cells and a longer incubation time was required *in vivo* to generate tumors, as compared with those with control liver CSCs (Supplementary Fig. S1A and S1B). IHC staining confirmed lower levels of LSD1 and Ki-67 in xenograft tumors derived from LSD1-KD liver CSCs, compared with those in tumors derived from control liver CSCs (Supplementary Fig. S1C), which is consistent with previous observations (18). In keeping with these findings, limiting dilution analysis showed that control cell populations were significantly enriched in CSCs, compared with that in their LSD1-KD counterparts (Fig. 2D and E). These findings indicate that LSD1 depletion abrogates liver CSC stemness.

To further determine whether LSD1 can restore self-renewal in liver non-CSCs, we infected these cells with lentivirus vectors for overexpression or silencing of LSD1. We observed that expression of exogenous LSD1 significantly restored tumor growth in liver non-CSCs (Fig. 2F and Supplementary Fig. S1D and S1E). However, with LSD1-KD, the tumorigenic potential of liver non-CSCs was inhibited (Fig. 2F and Supplementary Fig. S1D and S1E). Collectively, LSD1 promotes the self-renewal of liver CSCs and *in vivo* tumor propagation.

LSD1 controls the expression of stem cell transcription factors via demethylase activity

It has been reported that pluripotent transcription factors (SOX2, Nanog, c-Myc, and OCT4) function in liver CSCs (5, 28, 29). To demonstrate the molecular mechanisms involved in the LSD1-mediated self-renewal and tumorigenic potential of liver CSCs, we first examined the expression of these genes in liver CSCs and non-CSCs upon LSD1 KD or overexpression. We observed that knockdown LSD1 decreased the expression of these factors and increased the differentiation marker albumin in liver CSCs (Supplementary Fig. S2A and S2B). Overexpression of LSD1 in liver non-CSCs increased stem cell transcription factors and decreased albumin expression (Supplementary Fig. S2C and S2D). Furthermore, we found that the LSD1 inhibitor CBB1007 could suppress expression of these stem cell transcription factors in a dose- and time-dependent manner (Supplementary Fig. S2E). Previous results showed that SOX2 confers sensitivity to LSD1 inhibition in cancer cells (30). We



D

Cell Dose	shScrambled	shLSD1
1×10^2	2/10	0/10
1×10^3	4/10	1/10
1×10^4	7/9	6/10

E

Cell populations	CSC frequency			P
	Lower	Estimate	Upper	
shScrambled	7923	3859	1879	0.034
shLSD1	23289	10850	5055	

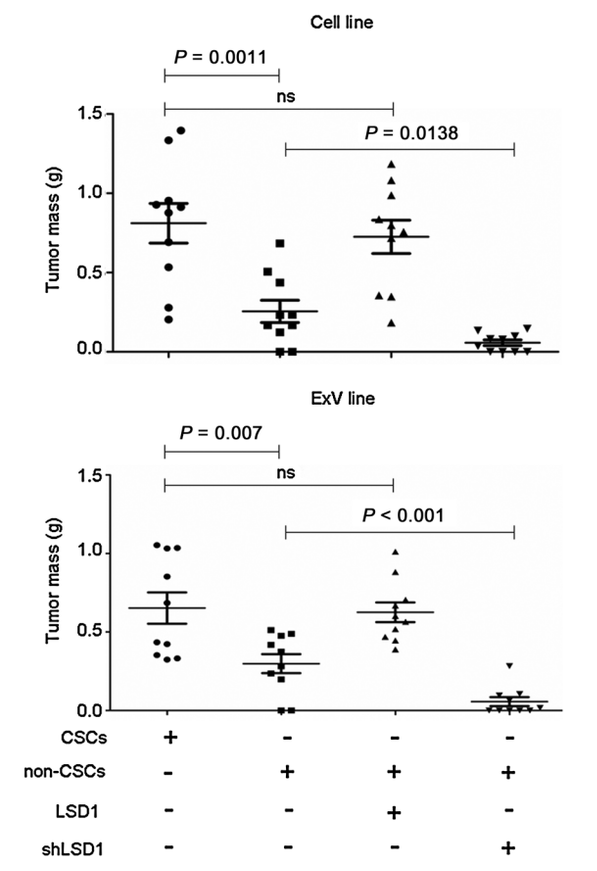


Figure 2.

LSD1 is essential for the maintenance of liver CSC self-renewal and tumorigenicity. **A,** Immunoblotting analysis of LSD1 protein levels in PLC/PRF/5 CSCs after infection with lentivirus expressing different shRNAs against LSD1. **B** and **C,** Sphere formation efficiency, in the first and second generations (**B**), and clone formation efficiency (**C**) assays with LSD1-knockdown (KD) and shScrambled controls in liver CSCs were performed after 2 weeks. **D** and **E,** Effects of shScrambled and LSD1-KD on the tumor-forming frequency of PLC/PRF/5 CSCs, as determined by limiting dilution assays. CSC frequency was calculated using the ELDA (http://bioinf.wehi.edu.au/software/elda). **F,** Purified non-CSCs from PLC/PRF/5 cells infected with LSD1 or shLSD1 vectors were analyzed for tumorigenic potential; liver CSC populations served as the positive control group. Final tumor masses are represented ($n = 10$/group). Data are shown as mean ± SEM.

found that LSD1 was enriched in the transcriptional regulatory region of the SOX2 gene and that the regulatory region was regulated directly by modifications to bivalent H3K4 and H3K9 methylation via the demethylase activity of LSD1 in liver CSCs (Supplementary Fig. S2F). This represents a region that was previously reported to act as a distal enhancer of SOX2 expression in breast cancer cells (31). These results suggest that LSD1 regulates the expression of stem cell–associated transcription factors via chromatin-based epigenetic changes, which functions to maintain liver CSC self-renewal.

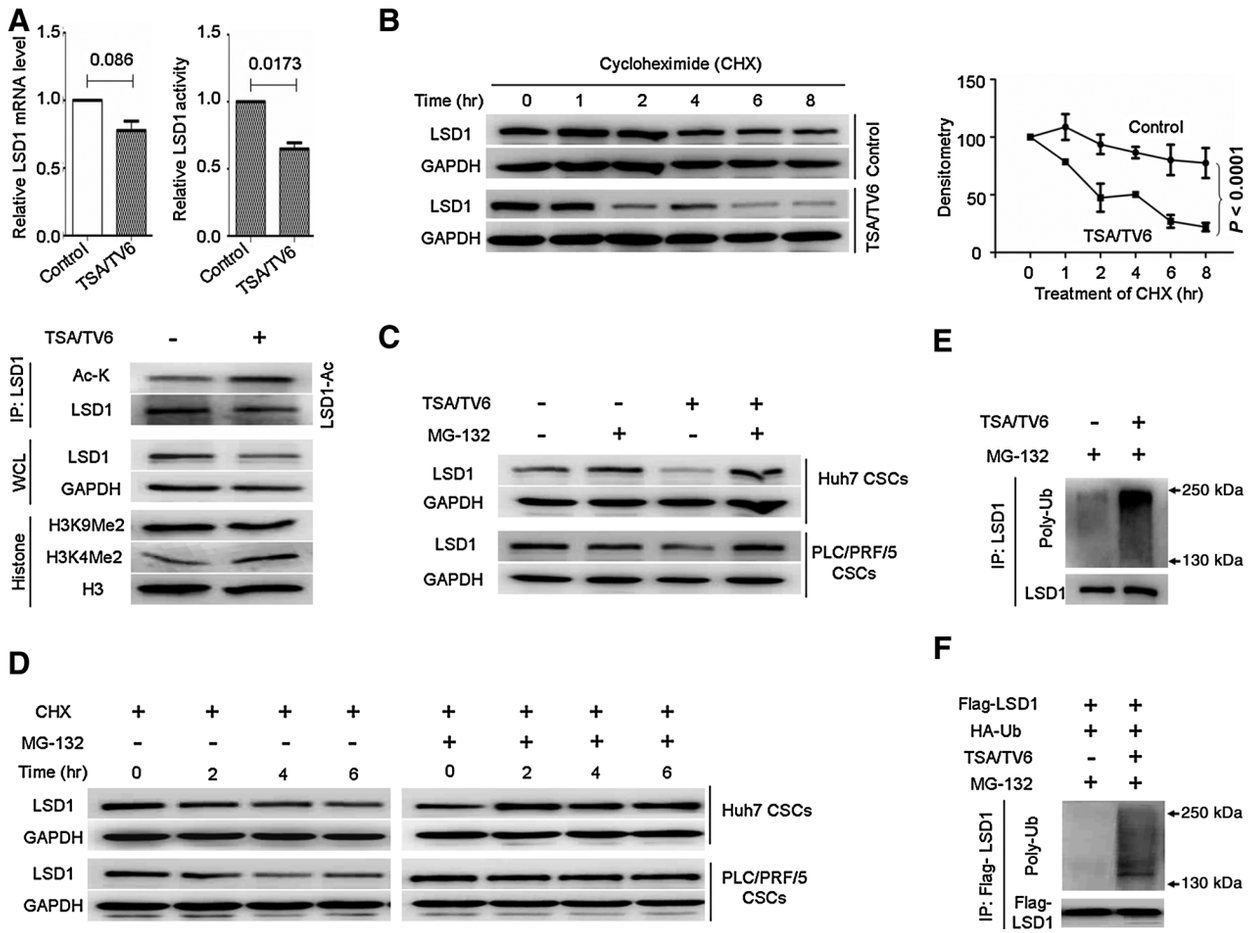


Figure 3.

Acetylation level of LSD1 affects its enzymatic activity and stability. **A,** Acetylation inhibits LSD1 enzyme activity. PLC/PRF/5 CSCs were treated with TSA/TV6 for 18 hours, qRT-PCR analysis LSD1 expression or LSD1 was purified by immunoprecipitation, which was followed by enzyme assays and Western blotting with the indicated antibodies. **B,** LSD1 is less stable in liver CSCs after treatment with TSA/TV6. Cells were maintained in serum-free medium for 18 hours, after which medium was replaced with 15% FBS/DMEM for 4 hours, and cells were then treated with 10 μg/mL cycloheximide (CHX) at the indicated time points before subjecting lysates to Western blotting. The experiments were repeated three times and band intensities were measured. Statistical analysis is provided on the right. **C,** Huh7 and PLC/PRF/5 CSCs were treated with TSA/TV6 for 18 hours and then treated with 10 μmol/L MG-132 for 6 hours. Cell lysates were immunoblotted with the indicated antibodies. **D,** Endogenous LSD1 was examined after treatment with 10 μg/mL cycloheximide or cycloheximide plus 10 μmol/L MG-132. Cells were harvested at the indicated time points. **E,** PLC/PRF/5 CSCs were treated with or without TSA/TV6 for 18 hours. Cells were treated with 10 μmol/L MG-132 for 6 hours before harvesting. Lysates were immunoprecipitated with LSD1 antibody conjugated beads and Western blotting was performed with the indicated antibodies. **F,** 293T cells were cotransfected with plasmids expressing HA-Ub (ubiquitin) and LSD1-Flag and treated with or without TSA/TV6 for 18 hours. Cells were treated with 10 μmol/L MG-132 for 6 hours before harvesting. LSD1 was immunoprecipitated and the polyubiquitylation of LSD1 was detected by Western blotting.

Acetylation modification regulates LSD1 enzyme activity and protein stability

Given the critical role of LSD1 in liver CSCs, we sought to investigate its regulation. Recent studies have revealed a broad role of lysine acetylation in metabolic enzyme regulation, which is involved in intermediate CSCs self-renewal and tumorigenicity (32–34). LSD1 acetylation has been reported in the phosphosite database (Supplementary Fig. S3A). We confirmed this acetylation event in HCC cell lines and patient tumor tissues (Supplementary Fig. S3B) by immunoblotting with an anti-acetyllysine antibody. To determine the effect of LSD1 acetylation on its activity and expression, we treated liver CSCs with trichostatin A (TSA), an inhibitor of histone deacetylase HDAC I and II, and tenovin 6 (TV-6), an inhibitor of the SIRT family of deacetylases. Upon treatment, LSD1 acetylation was increased and enzyme activity and protein expression were decreased (Fig. 3A). However, this treatment did not change the messenger RNA level of LSD1 (Fig. 3A). These results suggest that acetylation inhibits LSD1 expression and enzyme activity.

To further explore the acetylation-related mechanism of LSD1 regulation, we tested whether LSD1 is degraded in liver CSCs. Degradation is important for the activation of signaling and protein trafficking. Protein polyubiquitination is an important posttranslational modification that results in protein degradation. Previous studies showed that LSD1 is subjected to proteasomal degradation by the E3 ubiquitin ligase Jade-2 (35) and is stabilized by the deubiquitinase USP28 (11). Considering this model, we blocked protein synthesis using cycloheximide (CHX),

and the results showed that LSD1 is relatively stable in liver CSCs with a half-life longer than that with TSA/TV6 treatment (Fig. 3B). Furthermore, treatment of cells with the proteosomal inhibitor MG-132 increased LSD1 protein stability (Fig. 3C and D). Through ubiquitylation assays, we found that TSA/TV6 treatment strongly increased of polyubiquitylation of LSD1 (Fig. 3E). A similar result was observed when 293T cells were transfected with a Flag-LSD1 construct after HDAC inhibitor treatment, confirming that LSD1 is degraded by the ubiquitin-proteosome system (UPS; Fig. 3F). These results suggest that the acetylation-mediated decrease in LSD1 expression is dependent on UPS, which targets LSD1 for ubiquitylation and degradation.

SIRT1 deacetylates LSD1 to increase its stability

To identify which deacetylase is responsible for LSD1 regulation, we first determined how the inhibition of either SIRT or HDAC could affect LSD1 levels. Treatment of cells with the SIRT inhibitor TV6, but not the HDAC inhibitor TSA, decreased LSD1 expression and increased LSD1 acetylation (Supplementary Fig. S4A), indicating that a SIRT deacetylase is probably involved in LSD1 deacetylation. Our recent results showed that SIRT1 serves as a key regulator of self-renewal and tumorigenicity in liver CSCs (29). It has been demonstrated that LSD1 and SIRT1 can interact directly to regulate gene expression (36). We confirmed the interaction between endogenous LSD1 and SIRT1 proteins by coimmunoprecipitation experiments using liver CSCs (Supplementary Fig. S4B). Therefore, to test the effect of SIRT1 on LSD1 expression and protein stability, we used shRNA to suppress SIRT1 gene expression in liver CSCs. We found that protein levels of LSD1 decreased and acetylation increased with SIRT1 KD (Supplementary Fig. S4C). Furthermore, overexpression of WT SIRT1, but not the inactive H363Y mutant (catalytically inactive), increased LSD1 levels (Supplementary Fig. S4D). We also found LSD1 expression to be downregulated after treatment with the SIRT1-specific inhibitor EX-527 (Supplementary Fig. S4E). LSD1 protein stability was dramatically decreased upon SIRT1 KD by shRNA (Supplementary Fig. S4F). Treatment with MG-132 increased the stability of LSD1, compared with that with only SIRT1 KD (Supplementary Fig. S4G). These results suggest that SIRT1 can inhibit LSD1 deacetylation and might function to control LSD1 protein stability.

Notch activates LSD1 by inducing deacetylation to promote liver CSC self-renewal

We and others have recently reported that Notch signaling can regulate the self-renewal of liver CSCs (24, 27, 37). This prompted us to determine if LSD1 acetylation is modulated by Notch signaling. Inhibition of Notch signaling using DAPT or Compound E (γ-secretase inhibitors that block activation of the Notch intracellular domain) decreased the protein expression and reduced the enzymatic activity of LSD1, accompanied by decrease of Notch targeting gene HEY1 (Fig. 4A). DAPT or Compound E treatment also downregulated SIRT1 mRNA and protein expression (Fig. 4A and Supplementary Fig. S5A). When Notch signaling was activated by JAG-1 (an extracellular ligand of Notch), LSD1 expression was induced, whereas SIRT1 protein levels were increased (Fig. 4B). Furthermore, we also observed that SIRT1 deacetylase activity plays an import role in the regulation of LSD1 expression by Notch signaling (Supplementary Fig. S5B). To gain mechanistic insights, we performed ChIP assay on endogenous Notch signaling relative genes (RBP-J and Notch3) and investigated whether they could bind the SIRT1 promoter. Our results showed significantly decreased binding of RBP-J and Notch3 to the SIRT1 promoter (Supplementary Fig. S5C),

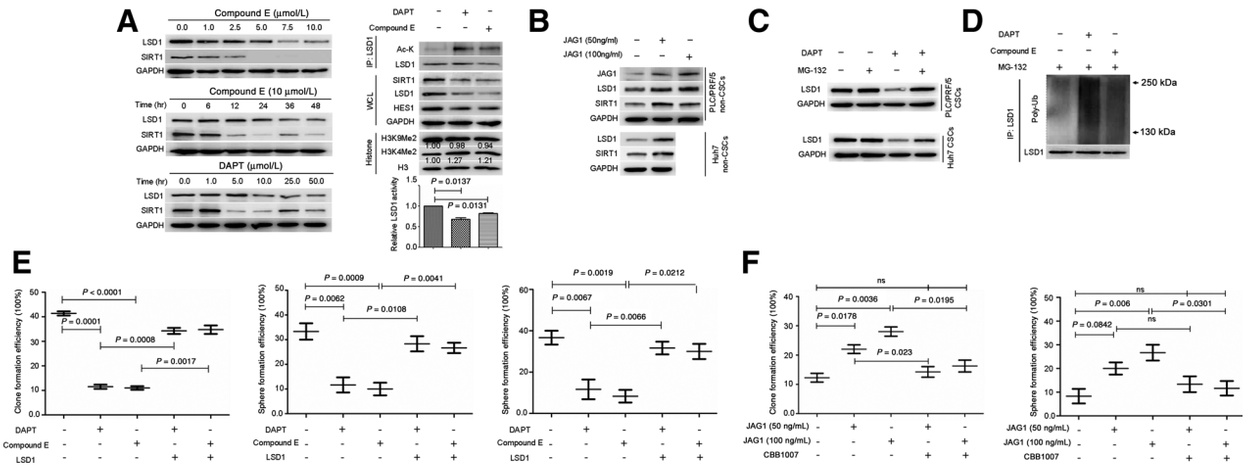


Figure 4.

Notch activates LSD1 by inducing deacetylation to promote liver CSCs self-renewal. **A,** Left immunoblotting analysis of LSD1 and SIRT1 in PLC/PRF/5 CSCs after treatment with DAPT for the indicated. Right, PLC/PRF/5 CSCs were treated with DAPT or Compound E for 48 hours. Lysates were immunoprecipitated with LSD1 antibody–conjugated beads and immunoblotting with the indicated antibodies and enzyme activity was performed by Histone Demethylase KDM1/LSD1 Activity Quantification Assay Kit. **B,** Immunoblotting analysis of LSD1 and SIRT1 in PLC/PRF/5 non-CSCs after treatment with JAG-1 for the indicated. **C,** Immunoblotting analysis of LSD1 in liver CSCs after treatment with or without DAPT for 48 hours. Cells were treated with 10 μmol/L MG-132 for 6 hours before harvesting. **D,** PLC/PRF/5 CSCs were treated with or without DAPT for 48 hours. Cells were treated with 10 μmol/L MG-132 for 6 hours before harvesting. Lysates were immunoprecipitated using LSD1 antibody–conjugated beads and Western blotting was performed with the indicated antibodies. **E,** PLC/PRF/5 and Huh7 CSCs were treated with DAPT or Compound E or plus with overexpression of LSD1; clone (left) and sphere (right) formation efficiency were analyzed after culture for 14 days. **F,** PLC/PRF/5 non-CSCs were treated with JAG-1 or plus with CBB1007, and cells were analyzed for clone (left) and sphere (right) formation efficiency after culture 14 days. Data are shown as mean ± SEM.

indicating that Notch signaling might transcriptionally regulate SIRT1 expression.

We then investigated the effect of Notch signaling on endogenous LSD1 protein stability. LSD1 protein stability was dramatically decreased when Notch signaling was inhibited by DAPT (Supplementary Fig. S6A). As expected, we found that treatment with MG-132 increased the stability of LSD1 upon DPAT treatment (Fig. 4C). Furthermore, LSD1 poly-ubiquitylation was found to be increased with DAPT and Compound E treatment (Fig. 4D). Together, the above data suggest a model in which Notch signaling activates LSD1 (via deacetylation) by inducing SIRT1 expression.

To address the function of the Notch pathway in liver CSC populations and self-renewal, we treated these cells with DAPT or Compound E and assessed clone and sphere formation. Results showed that inhibiting the Notch pathway reduced both clone and sphere formation (Fig. 4E), whereas activating the Notch pathway with JAG-1 promoted stem cell self-renewal (Fig. 4F). These data are consistent with our and other previous reports indicating a positive role for the Notch pathway in liver CSC self-renewal (24, 25, 27). Notably, compared with inhibition of the Notch pathway, overexpression of exogenous LSD1 dampened the response to DAPT or Compound E in terms of clone and sphere formation (Fig. 4E). Similarly, we found that non-CSCs were not increased in their self-renewal capacity in response to the Notch ligand JAG-1 if LSD1 expression was inhibited (Fig. 4F). This further supported the notion that Notch signaling promotes liver CSC self-renewal, at least in part by regulating the deacetylation of LSD1.

Notch3 plays a critical role in liver CSC self-renewal and tumorigenicity

GSIs inhibit the activation of all Notch receptors (Notch1-4), which are all highly expressed in sphere cells, as compared with that in nonsphere cells (Supplementary Fig. S6B). To determine whether specific Notch receptors play nonredundant roles in liver CSC self-renewal and LSD1 expression, these cells were transduced with individual shRNAs targeting each of the four Notch genes (Supplementary Fig. S6C). Suppressing Notch1, Notch2, or Notch4 did not result in decreased LSD1 expression (Supplementary Fig. S6D). In contrast, Notch3-KD led to a 2- to 3-fold decrease in LSD1 expression (Supplementary Fig. S6D). In addition, Notch3-KD significantly decreased sphere- and clone-forming ability (Fig. 5A), which is similar with previous observation

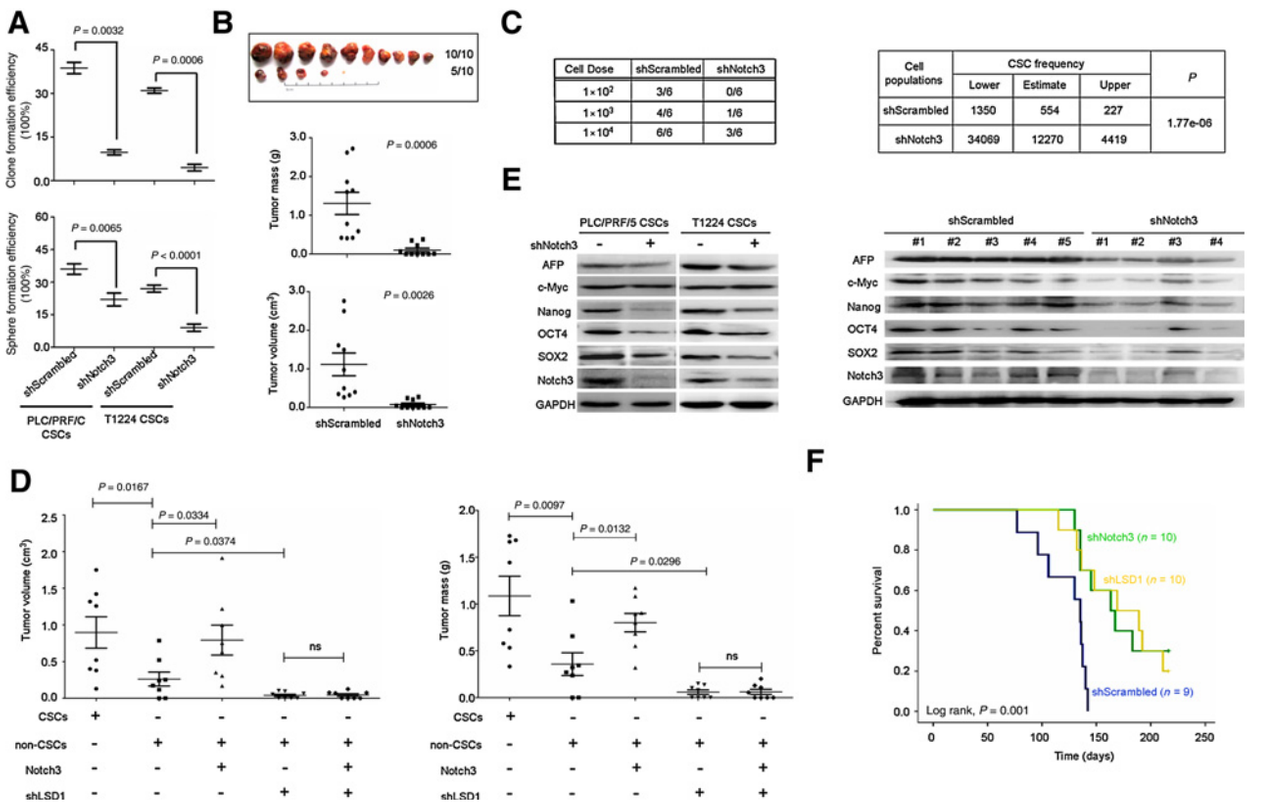


Figure 5.

Role of Notch3 in self-renewal and tumorigenicity of liver CSCs. **A,** Clone (top) and sphere (bottom) formation efficiency assays using Notch3-KD (knockdown) and shScrambled-control-infected liver CSCs were performed after 2 weeks. **B,** Knockdown of Notch3 expression inhibits tumor growth *in vivo*. Equal numbers of either shScrambled- or shNotch3-infected CSCs (1×10^6) were injected into NOD/SCID mice. Representative xenograft tumors (top); tumor weight (middle) and tumor volume (bottom) were measured at the end of experiments ($n = 10$/group). **C,** Effects of shScrambled and Notch3-KD expression on tumor-forming ability of PLC/PRF/5 CSCs, as determined by limiting dilution assays. **D,** Purified non-CSCs from PLC/PRF/5 cells infected with Notch3 or shLSD1 or both were analyzed for tumorigenic potential using liver CSC populations as the positive control group. ($n = 8$/group). **E,** Immunoblotting analysis of indicated proteins in PLC/PRF/5 and T1224 CSCs (left panel) or derived from Fig. 6B (right). **F,** Kaplan–Meier survival analysis of mice injected with the indicated PLC/PRF/5 CSCs ($n = 10$/group). Data, mean ± SEM.

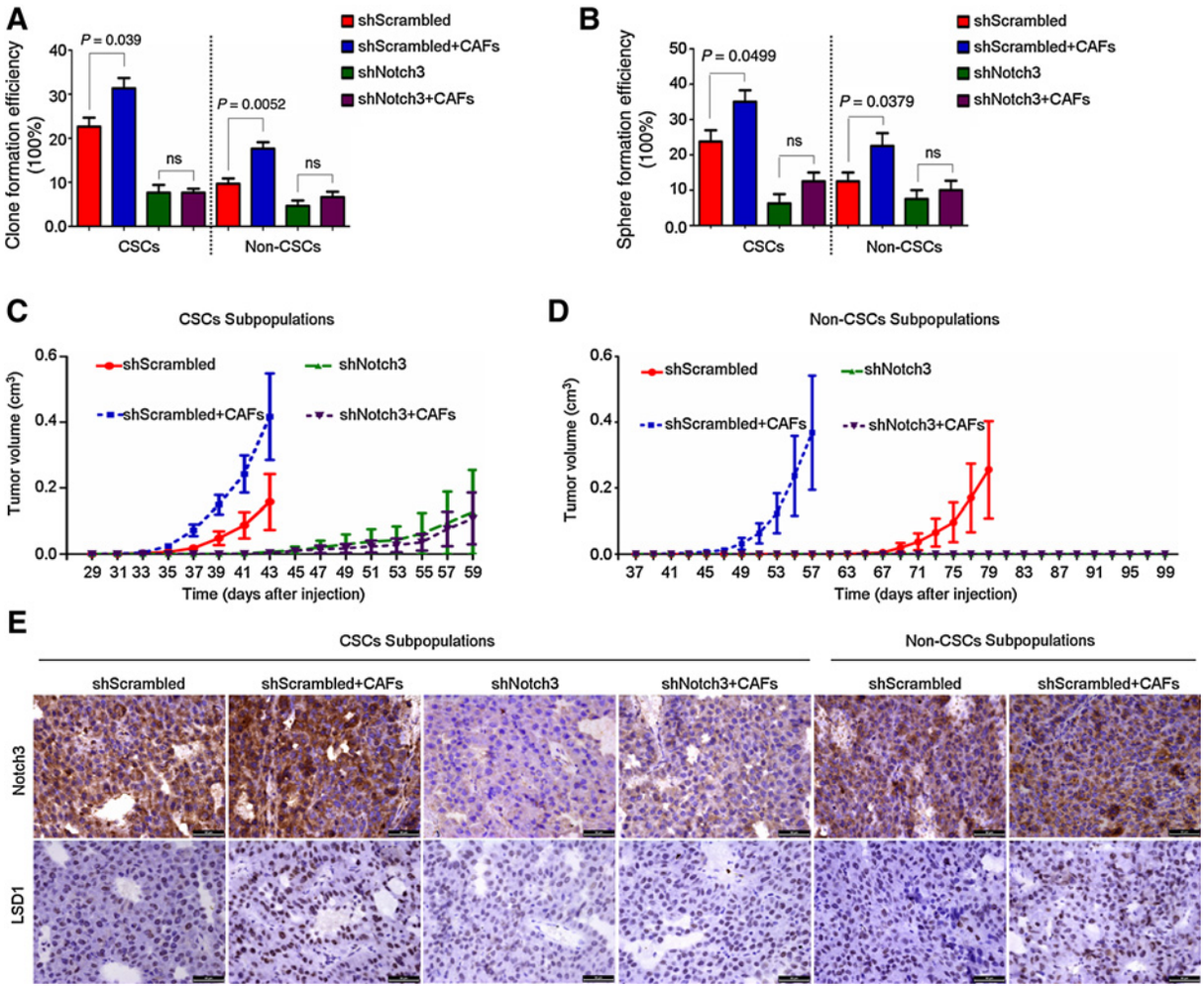


Figure 6.

CAFs maintain liver CSCs self-renewal and tumor growth via Notch3 signaling. **A** and **B,** Effect of Notch3 on CAF-induced liver CSC or non-CSC self-renewal *in vitro*. Clone (**A**) and sphere (**B**) formation efficiency assays of tumor cells, as assessed by FACS assessing mCherry$^+$ cells from the coculture of PLC/PRF/5-mCherry$^+$/GFP$^+$ (or -mCherry$^+$/GFP$^-$) cells and CAFs after 2 weeks with the indicated. **C** and **D,** Effect of Notch3 on CAF-induced liver CSC (**C**) or non-CSC (**D**) tumorigenicity *in vivo*. Tumor cells with or without CAFs were inoculated subcutaneously into mice, and tumor volume was monitored every 2 days with the indicated ($n = 10$/group). **E,** IHC staining of mouse tumor samples for LSD1 and Notch3. Representative images are shown. Scale bars, 50 μm. Data, mean ± SEM.

(26). The importance of Notch3 for liver CSC function was further investigated *in vivo*. Notch3-KD led to a 10-fold reduction in both weight and size of tumors in recipient mice (Fig. 5B). Limiting dilution analysis showed that control liver cell populations were significantly enriched for CSCs, as compared with their Notch3-KD counterparts (Fig. 5C). In addition, expression of exogenous Notch3 significantly restored tumor growth in liver non-CSCs (Fig. 5D). Furthermore, we observed that Notch3-KD decreased the expression of stem cell transcription factors *in vitro* and *in vivo* (Fig. 5E). Animals implanted with shLSD1 liver CSCs survived longer than that those receiving control liver CSCs (with scrambled shRNA; Fig. 5F). Kaplan–Meier analysis also revealed a statistically significant increase in the survival of mice injected with Notch3-KD cells, as compared with that in animals receiving control cells (shScramble; Fig. 5F). These data indicate that a Notch3-dependent pathway is essential for liver CSC self-renewal and *in vivo* tumor propagation.

We further confirmed whether the induction of tumorigenicity through the activation of Notch3 signaling occurs via LSD1. For this, we found that the ability of Notch3 to induce tumorigenicity in liver non-CSCs was abolished with LSD1-KD (Fig. 5D). In addition, LSD1 exhibited diminished stability after Notch3-KD in a CHX pulse-chase assay (Supplementary Fig. S6A). Treatment with MG-132 increased the stability of LSD1 protein, compared with that with only Notch3-KD (Supplementary Fig. S6E). Taken together, these data indicate that Notch3 is important for the maintenance of self-renewal and tumorigenicity, and that this is at least in part dependent upon the induction of LSD1.

CAFs maintain liver CSCs self-renewal and tumor growth by activating Notch3 signaling

CAFs are one of the major cellular components of the tumor microenvironment. These (as well as other cells within the niche) stimulate stemness through the activation of the Notch signaling

pathway, which is dependent on direct cell–cell contact (38). To test if HCC-associated CAFs play a role in the self-renewal of liver CSCs through Notch3 signaling, we isolated CAFs from fresh HCC tissues. IF staining showed that isolated CAFs expressed alpha-smooth muscle actin (α-SMA) and platelet-derived growth factor receptor-alpha (PDGFRα; Supplementary Fig. S7A).

When Cherry-GFP–labeled PLC/PRF/5 cells were directly cocultured with primary CAFs *in vitro*, functional assays showed that CAFs could inhibit liver CSC ($Cherry^+$-GFP^+) differentiation and enhance liver non-CSC ($Cherry^+$-GFP^-) self-renewal. However, CAF-mediated maintenance of the liver CSC state and enhanced liver non-CSC self-renewal were abolished when shNotch3 was applied (Fig. 6A and B). Next, we inoculated this mixture of $Cherry^+$-GFP^+ liver CSCs (or $Cherry^+$-GFP^- liver non-CSCs) and primary CAFs into NOD/SCID mice. Similar to *in vitro* results, CAFs enhanced liver CSC or non-CSC tumorigenicity by activating Notch3 signaling *in vivo* (Fig. 6C and D, respectively). IHC staining demonstrated the existence of SMA+ fibroblasts in established tumors (through the injection of both CAFs and cancer cells; Supplementary Fig. S7B). Thus, CAFs enhance liver CSC self-renewal and tumor growth by activating Notch3 signaling.

We further tested if CAF-mediated activation Notch3 signaling is involved in the regulation of LSD1 expression. Indeed, in xenograft tumors, LSD1 expression was increased in cancer cells that were coinoculated with primary CAFs; however, this effect was abolished upon Notch3 KD in liver CSCs and non-CSCs using shRNA (Fig. 6E). Taken together, these results suggest that CAF-mediated activation of Notch3 signaling and LSD1 functionally converge to promote HCC tumorigenicity.

Clinical impact of CAFs, Notch3, and LSD1 on HCC outcomes

Lastly, we investigated the clinical relevance of our findings using tissues obtained from HCC patients. We performed IHC staining for LSD1, Notch3, and α-SMA using 148 HCC specimens. We observed that α-SMA expression was strongly correlated with LSD1 and Notch3 expression (Fig. 7A and B). We also found positive correlations between LSD1 and Notch3 expression (Fig. 7B). Based on Kaplan–Meier analysis, we found that high levels of LSD1, Notch3, and α-SMA were significantly correlated with poor survival (Fig. 7C), which is consistent with a previous observation (26, 39). We further observed that high LSD1/Notch3, LSD1/α-SMA, and Notch3/α-SMA were associated with the poorest outcome, compared with that with the other possible combinations (Fig. 7D). Collectively, our results suggest that the density of stromal fibroblasts and Notch3/LSD1 expression affect HCC patient survival.

Discussion

High LSD1 activity is a property commonly associated with CSCs. Although the precise mechanism through which LSD1 regulates CSCs is not fully understood, it likely acts as an epigenetic mediator that is involved in stem cell maintenance and/or differentiation. High LSD1 activity in stem cells could be maintained by increased protein expression and/or posttranslational activation. However, posttranslational regulation of LSD1 in CSCs has not been investigated. In this report, we identified a novel mechanism of LSD1 regulation at the posttranslational level. Our data show that LSD1 is modified by lysine acetylation, and its enzyme activity and stabilization are inhibited by this modification. Moreover, we identified that Notch3 signaling plays an important role in LSD1 deacetylation and stabilization. Thus, this report establishes lysine acetylation as an *in vivo* biochemical mechanism regulating LSD1 activity.

LSD1 expression is elevated in many human cancers and promotes proliferation and drug resistance, in addition to facilitating transformation *in vitro* and *in vivo* (9, 40–42). In this study, we revealed that LSD1 is strongly expressed in liver CSCs and HCC tissues, which is consistent with a recent report (18). Furthermore, our results reveal that LSD1 has important functions in the regulation of liver CSC stemness; specifically, LSD1 downregulation inhibited both self-renewal and tumorigenicity, suggesting a critical role for this protein in these processes. Moreover, our findings indicated that acetylation plays a critical role in the posttranslational regulation of LSD1 expression by two mechanisms. First, acetylation inhibits LSD1 expression and enzymatic activity. Second, acetylation stimulates degradation of LSD1 via the proteosomal pathway, thus affecting its stability. Notably, acetylation of LSD1 was induced in liver non-CSCs, suggesting that decreased LSD1 protein acetylation in these cells might contribute to elevated expression and activity, as well as enhanced tumorigenicity. However, we currently have not uncovered the underlying molecular mechanism through which LSD1 acetylation site(s) are more prominent in stem-like cells. Further study is required to understand this.

Notch signaling is activated and plays an oncogenic role in HCC (23, 43). We and others have identified that activation of this signaling pathway is required for the maintenance of liver CSC self-renewal by either activation of SOX9 or Stat3 and Wnt/ β-catenin signaling pathways (24, 25, 27, 44). In this study, we further determined that LSD1 acts as an essential mediator of this process. Interestingly, our study revealed that Notch signaling can regulate LSD1 acetylation. Mechanistically, this appeared to be mediated by Notch signaling-induced SIRT1 expression. Inhibition of Notch signaling decreased SIRT1 levels and concomitantly increased LSD1 acetylation. Notch signaling was found to promote CSC self-renewal through the transcriptional regulation of downstream target genes that participate in cell-fate determination, cell survival, and proliferation. Our data suggest that Notch signaling promotes liver CSC self-renewal at least in part by inducing SIRT1 and consequently activating LSD1. Further studies are required to clarify whether the regulation of LSD1 acetylation is unique to Notch signaling or if it is broadly involved in other signaling pathways associated with stem cell regulation.

It is interesting to note that all four Notch receptors are surface markers, but only Notch3 seems to be functionally relevant for the regulation of LSD1 expression. This result might reflect discordance between surface expression of Notch receptors and the activity of Notch signaling pathways. Consistent with our observations, another study demonstrated that a rare population of $CD24^+ITGB4^+$ $Notch^{hi}$ cells that drive tumor propagation requires Notch3 for self-renewal in non–small cell lung cancer (45). Importantly, Notch3 seems to enhance self-renewal upon reactivation, highlighting the need to characterize the output of Notch signaling, instead of merely Notch expression. Such a discrepancy might be rooted in the availability of Notch ligands in the tumor microenvironment.

CAFs are major cellular components of the tumor microenvironment and promote tumor progression through

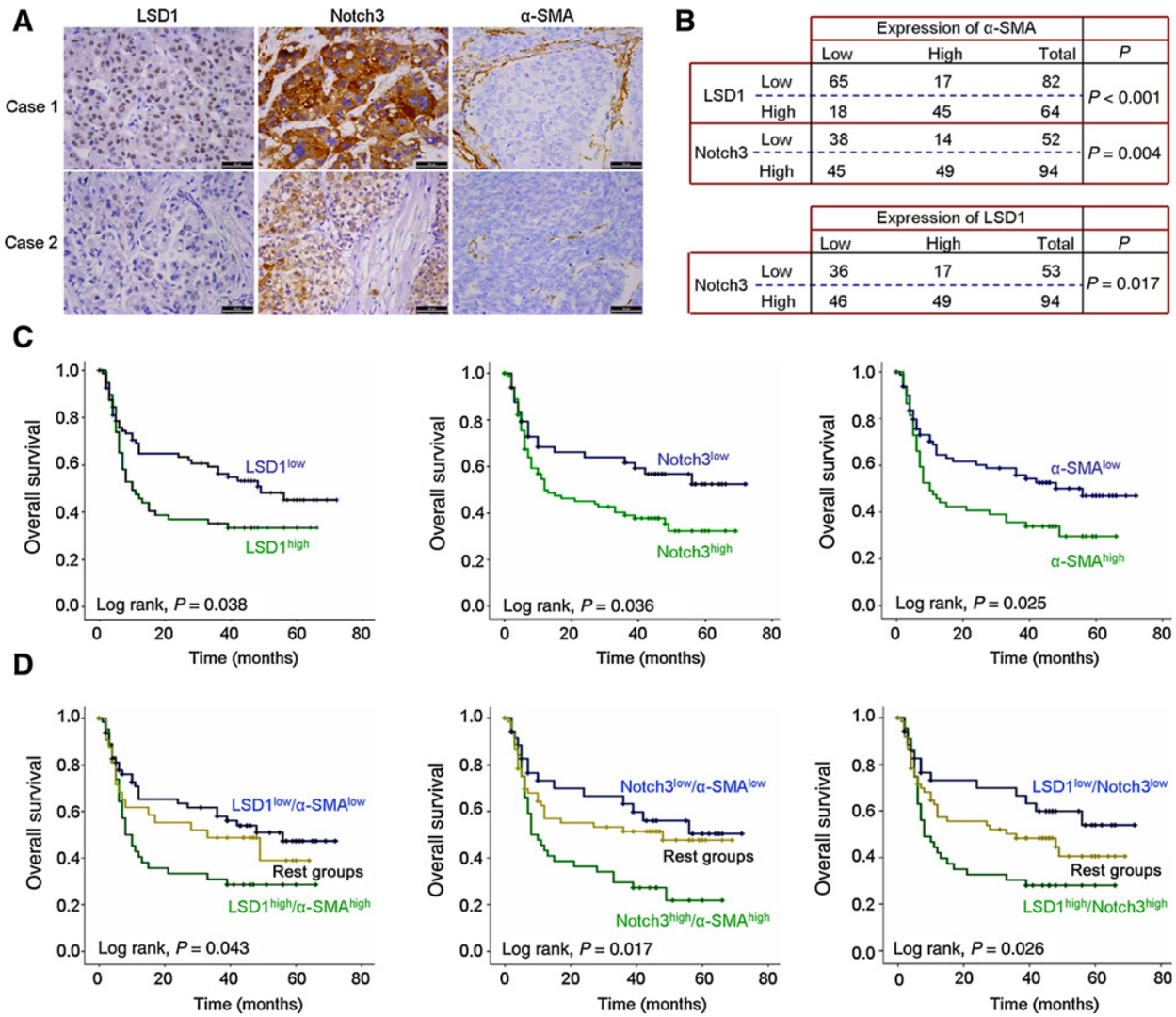


Figure 7.

Clinical impact of CAFs, Notch3, and LSD1 on HCC patient survival. **A,** IHC staining of HCC samples for LSD1, Notch3, and α-SMA. Representative images are shown. Scale bars, 50 μm. **B,** Correlations among of LSD1, Notch3, and α-SMA expression in HCC patients and statistical analysis of IHC staining. **C,** Kaplan–Meier curves showing overall survival of HCC patients based on LSD1 (left), Notch3 (middle), or α-SMA (right) expression. **D,** Kaplan–Meier curves showing overall survival based on LSD1/Notch3 (left), LSD1/α-SMA (middle), or Notch3/α-SMA (right) expression in HCC samples.

multiple mechanisms including increased tumor cell proliferation, angiogenesis, invasion, sustained stemness, and inhibition of tumor cell death (46, 47). We found that CAFs isolated from HCC tissues can efficiently inhibit liver CSC differentiation and promote liver non-CSC self-renewal. Inhibition of Notch3 signaling abolished the CAF-induced biological responses. Clinical analysis showed that stromal fibroblasts, LSD1, or Notch3 could predict overall survival in HCC patients, which is similar as previous observation (26, 39). However, we further demonstrated that high LSD1/Notch3, LSD1/α-SMA, and Notch3/α-SMA were associated with the poorest outcome, compared with that with the other possible combinations.

In summary, we demonstrated a novel mechanism of HCC self-renewal though SIRT1-mediated deacetylation of LSD1, which occurs via Notch signaling. This regulatory event is critical for liver CSC self-renewal and *in vivo* tumor propagation. Importantly, CAF-regulated and Notch3-mediated LSD1 expression in liver CSCs was shown to promote stemness. Thus, targeting LSD1 acetylation by inhibiting CAFs/Notch3 in liver CSCs might represent a potential strategy for liver cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: C. Liu, C. Qian
Development of methodology: L. Liu, X. Chen, J. Cheng, C. Zhang, J. Shen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Liu, J. Cheng, J. Shan

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Liu, J. Cheng
Writing, review, and/or revision of the manuscript: C. Liu, J. Cheng, C. Qian
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Zhang
Study supervision: C. Qian

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