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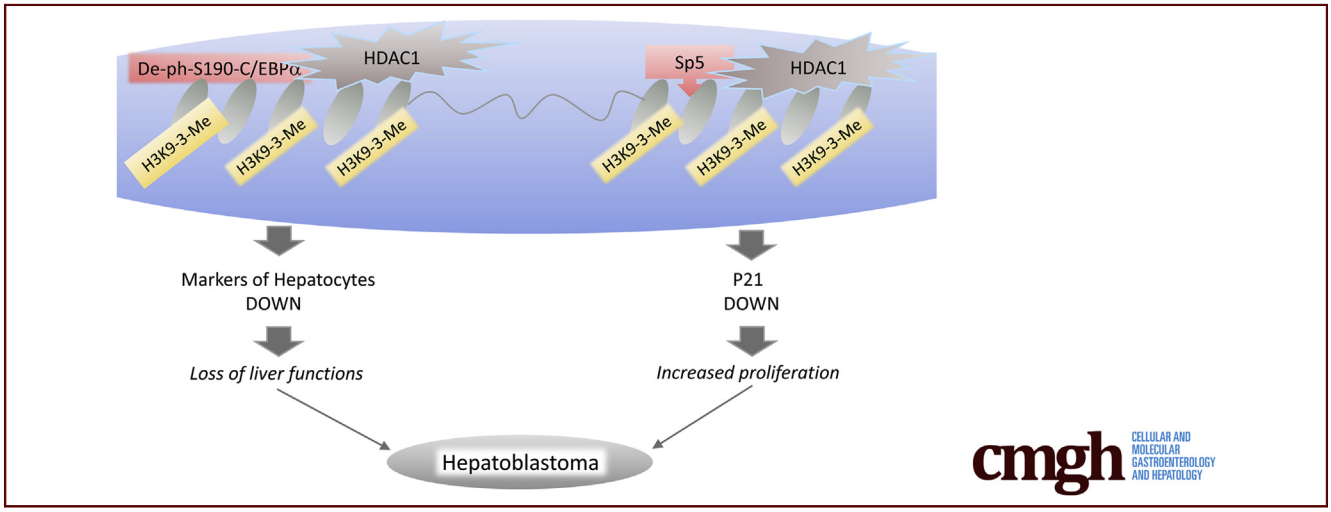
CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

HDAC1-Dependent Repression of Markers of Hepatocytes and P21 Is Involved in Development of Pediatric Liver Cancer

Maria Rivas,[1,3] Michael E. Johnston II,[1,2] Ruhi Gulati,[1] Meenasri Kumbaji,[1] Talita Ferreira Margues Aguiar,[3] Lubov Timchenko,[4] Ana Krepischi,[3] Soona Shin,[1,2] Alexander Bondoc,[1] Gregory Tiao,[1,2] James Geller,[5] and Nikolai Timchenko[1,2]

[1]Division of General and Thoracic Surgery, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio; [2] Department of Surgery, University of Cincinnati College of Medicine, University of Cincinnati, Cincinnati, Ohio; [3]Institute of Biosciences, University of São Paulo, São Paulo, Brazil; [4]Department of Neurology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio; [5]Department of Oncology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio



SUMMARY

Dedifferentiation of hepatocytes into cancer cells is a first step in the development of liver cancer. In this article, we show that the dedifferentiation of hepatocytes involves repression of markers of hepatocytes and the increase of liver proliferation, which are mediated by histone deacetylase 1–dependent repression of promoters of markers of hepatocytes and p21.

BACKGROUND & AIMS: Epigenetic regulation of gene expression plays a critical role in the development of liver cancer; however, the molecular mechanisms of epigenetic-driven liver cancers are not well understood. The aims of this study were to examine molecular mechanisms that cause the dedifferentiation of hepatocytes into cancer cells in aggressive hepatoblastoma and test if the inhibition of these mechanisms inhibits tumor growth.

METHODS: We have analyzed CCAAT/Enhancer Binding Protein alpha (C/EBPα), Transcription factor Sp5, and histone deacetylase (HDAC)1 pathways from a large biobank of fresh hepatoblastoma (HBL) samples using high-pressure liquid chromatography–based examination of protein–protein complexes and have examined chromatin remodeling on the promoters of markers of hepatocytes and p21. The HDAC1 activity was inhibited in patient-derived xenograft models of HBL and in cultured hepatoblastoma cells and expression of HDAC1-dependent markers of hepatocytes was examined.

RESULTS: Analyses of a biobank showed that a significant portion of HBL patients have increased levels of an oncogenic de-phosphorylated-S190-C/EBPα, Sp5, and HDAC1 compared with amounts of these proteins in adjacent regions. We found that the oncogenic de-phosphorylated-S190-C/EBPα is created in aggressive HBL by protein phosphatase 2A, which is increased within the nucleus and dephosphorylates C/EBPα at Ser190. C/EBPα–HDAC1 and Sp5–HDAC1 complexes are abundant in hepatocytes, which dedifferentiate into cancer cells. Studies in HBL cells have shown that C/EBPα–HDAC1 and Sp5–HDAC1 complexes reduce markers of hepatocytes and p21 via repression of their promoters. Pharmacologic inhibition of C/EBPα–HDAC1 and Sp5–HDAC1 complexes by Suberoylanilide hydroxamic acid (SAHA) and small interfering RNA–mediated inhibition of HDAC1 increase expression of hepatocyte markers, p21, and inhibit proliferation of cancer cells.

CONCLUSIONS: HDAC1-mediated repression of markers of hepatocytes is an essential step for the development of

HBL, providing background for generation of therapies for aggressive HBL by targeting HDAC1 activities. *(Cell Mol Gastroenterol Hepatol 2021;12:1669–1682; https://doi.org/10.1016/j.jcmgh.2021.06.026)*

Keywords: Hepatoblastoma; HDAC1; C/EBPα; Sp5; Epigenetic; Liver Cancer.

Liver cancer is the fifth most common cancer and the third most common cause of cancer-related death in the world.[1] It recently was established that the cellular origin of hepatocellular carcinoma (HCC) and hepatoblastoma (HBL) are hepatocytes that undergo dedifferentiation into cancer cells.[2–6] A portion of HBL patients have an aggressive form of HBL that is characterized by metastases, multiple nodules at diagnosis, vascular invasion, chemoresistance, and relapse.[7–9] Recent publications have shown that epigenetic silencing by histone deacetylases (HDACs) might be critical for the development of aggressive pediatric liver cancer.[10] In particular, an increase of HDAC1 and HDAC2 proteins was found in a large group of patients with HBL.[11] One of the key transcription factors that bring HDAC proteins to the promoters is CCAAT/Enhancer Binding Protein alpha (C/EBPα). C/EBPα is a tumor-suppressor protein that arrests cell proliferation by inhibiting cyclin dependent kinases 2 and 4 (cdk2/4) and Transcription factor that binds to E2 recognition site (E2F) signaling.[9,12] However, recent studies have shown that C/EBPα is increased in a group of patients with liver cancers and shows oncogenic activities.[5,6,13,14] The oncogenic activities of C/EBPα are associated with mutations or post-translational modifications occurring in the region of C/EBPα between amino acids 180 and 200.[14,15] This 20-amino acid region is rich for prolines and contains Ser190. Our studies have shown that, in adult cancers and in cancer cells, C/EBPα is dephosphorylated by protein phosphatase 2A (PP2A) at Ser190 and loses its growth-inhibitory activities.[11,12] Recent studies of pediatric liver cancer identified a group of patients with aggressive HBL who have high levels of C/EBPα, dephosphorylated at Ser190.[5,6] Normally, phosphorylated ph-S190-C/EBPα activates transcription of liver-specific genes via interactions with chromatin remodeling protein p300.[16,17] However, the de-phosphorylated-S190 C/EBPα does not interact with p300 and has reduced ability to activate target genes.[17]

In addition to C/EBP proteins, Sp family proteins also are involved in the regulation of biological processes in the liver including liver proliferation and cancer.[18] A critical target of the Sp family is cdk inhibitor p21. It has been shown that the promoter of p21 contains 6 Sp binding sites in very close proximity to the start of transcription and that Sp1/3 up-regulates the expression of p21.[19] However, if Sp1/3 are associated with HDAC1, they repress p21 promoter.[20,21] It also has been shown that inhibition of HDAC releases this repression.[20,22] In this article, we present evidence that another member of the Sp family, Sp5, is increased in patients with aggressive HBL, forms complexes with HDAC1, and down-regulates p21 by repression of the p21 promoter.

Results

PP2A-Oncogenic C/EBPα Pathway Is Activated in Patients With Aggressive HBL

It has been shown that the oncogenic C/EBPα and Sp5 are increased in patients with HBL.[4,6,23] Because both proteins interact with HDAC1, we first examined their levels in our fresh biobank (22 HBL samples obtained immediately after surgery). Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis showed that approximately 30% of the fresh biobank contains HBL samples with an increase of both C/EBPα and HDAC1. A high increase of Sp5 was observed in a larger portion of the fresh samples, including all samples with increased C/EBPα and HDAC1 (Figure 1*A*). The growth-inhibitory region of C/EBPα is located between amino acids 180 and 200. We found that the oncogenic form of C/EBPα is dephosphorylated at Ser190, which is located in a growth-inhibitory region and is surrounded by 12 prolines, 7 of which are located upstream of Ser190 and 5 are located downstream of Ser190 (Figure 1*B*). Mutations of Ser190 and amino acids of the surrounding region have been found previously in patients with HCC. Our studies of HCC and HBL have shown that C/EBPα is dephosphorylated at Ser190 in these cancers.[5,6,14,15] Therefore, we asked if C/EBPα is dephosphorylated at Ser190 in a fresh biobank. For these studies, we initially selected 4 HBL samples with the highest increase of C/EBPα and HDAC1 (Figure 1*A*). Examination of levels of total C/EBPα protein and levels of ph-S190-C/EBPα in these fresh HBL samples found that total C/EBPα is increased in HBL patients, but the ph-S90 isoform is reduced dramatically compared with background regions (Figure 1*C*). This result shows that the increased C/EBPα is dephosphorylated at Ser190. Because previous reports have shown that PP2A dephosphorylates C/EBPα at Ser190 in cancers,[6,14,15] we examined levels of a catalytic subunit of PP2Ac and found that it is increased in nuclear extracts of aggressive HBL, but it is reduced in cytoplasmic fractions of HBL. Examination of whole-cell extracts showed identical levels of PP2Ac in background and tumor regions of HBL patients, suggesting translocation of PP2A from cytoplasm to nucleus. Examination of HDAC1 showed that this protein also is increased in nuclear extracts from tumors of HBL patients. We next examined levels of the oncogene Gank, which degrades the

Abbreviations used in this paper: **ALCD, aggressive liver cancer domain; C/EBPα, CCAAT/Enhancer Binding Protein alpha; cdk2/4, cyclin dependent kinases 2 and 4; ChIP, chromatin immunoprecipitation; Co-IP, co-immunoprecipitation; HBL, hepatoblastoma; HCC, hepatocellular carcinoma; HDAC, histone deacetylase; HNF, Hepatocyte Nuclear Factor; mRNA, messenger RNA; MW, molecular weight; NRF2, Nuclear factor erythroid-2; OCT, organic cation transporter; Ola, Olaparib; PARP, Poly [ADP-ribose] polymerase; PDX, patient-derived xenograft; PP2A, protein phosphatase 2A; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; SEC, size exclusion chromatography; SLC, solute carrier.**

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2352-345X
https://doi.org/10.1016/j.jcmgh.2021.06.026

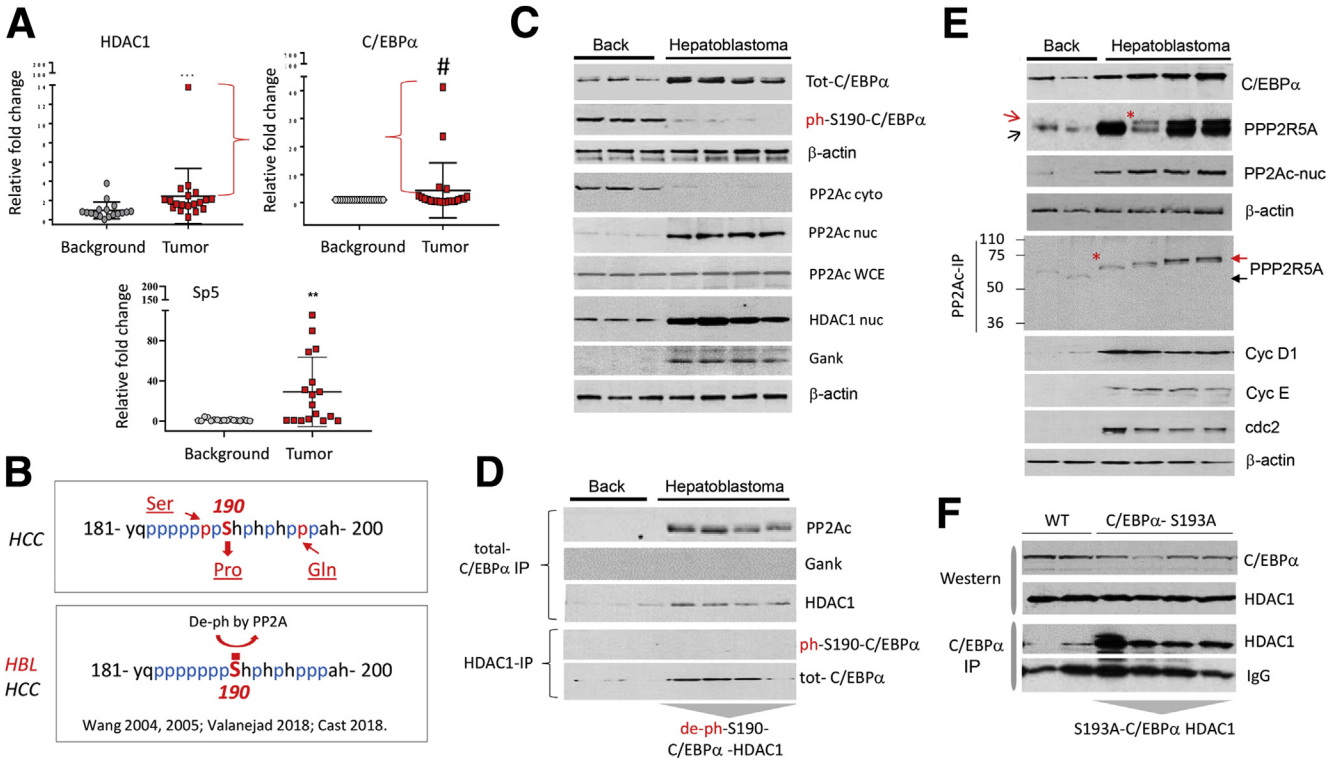

Figure 1. Oncogenic C/EBPα–HDAC1 pathway is activated in patients with aggressive HBL. (*A*) Levels of HDAC1, C/EBPα, and Sp5 mRNAs in fresh biobank HBL samples. Quantitation was performed using 22 HBL tumor samples and 22 corresponding background (adjacent) regions. *P* values are as follows: HDAC1 (***$P < .001$), C/EBPα ($^{\#}P < .05$), and Sp5 (**$P < .01$). (*B*) Cancer-related mutations and modifications in the growth-inhibitory region of C/EBPα. (*C*) Western blot showing expression of proteins in tumor sections and in background regions of patients with HBL. Representative results are shown for 4 HBL tumor sections and 3 background regions. Two to 3 technical replicates were performed for each biological sample. (*D*) Co-IP studies. *Upper*: IP with antibodies to total C/EBPα and Western blot with antibodies to PP2Ac, gankyrin, and HDAC1. *Bottom*: IP of HDAC1 and Western blot with antibodies to ph-S190-C/EBPα and to total C/EBPα. Two repeats with 4 samples were performed. (*E*) PPP2R5A interacts with catalytic subunit PP2Ac in nuclei of HBL patients. *Upper*: Western blot for proteins shown on the *right*. *Red arrows* show slow-migrating isoform of RRR2R5A, *black arrows* shows fast-migrating isoform. *Middle*: Co-IP; *bottom*: Western blot shows cell-cycle proteins. Two to 3 technical replicates were performed for each biological sample. (*F*) *Upper*: Western blot for C/EBPα and HDAC1. *Bottom*: Co-IP studies. C/EBPα was immunoprecipitated from nuclear extracts of livers of wild-type (WT) and C/EBPα-S193A mice, and HDAC1 was examined in these IPs. Two to 3 technical replicates were performed for each biological sample. IgG, signals of IgG.

phosphorylated ph-S190-C/EBPα isoform[4] and observed an increase of Gank in these aggressive HBL samples (Figure 1*C*). However, next co-immunoprecipitation (Co-IP) studies found that Gank does not interact with the de-phosphorylated-S190-C/EBPα, which explains why oncogenic C/EBPα is not degraded in HBL. Further Co-IP studies have shown that de-phosphorylated-S190-C/EBPα interacts with PP2A and HDAC1 (Figure 1*D*). We next examined if the increase of PP2Ac in the nucleus might be associated with translocation of PP2Ac from the cytoplasm to the nucleus. PP2A consists of 3 subunits: scaffold subunit A, catalytic subunit C, and regulatory subunits B.[24] The subunit B consists of several molecules with different functions.[25] Among these subunits, Protein phosphatase 2, regulatory subunit B (B56), alpha isoform (PPP2R5A) is increased in HCC and shuttles between the cytoplasm and nucleus, determining the intracellular location of the catalytic PP2Ac subunit.[26] Therefore, we asked if the PPP2R5A might translocate catalytic subunit PP2Ac to the nucleus in aggressive HBL patients. Western blot showed that, in background regions, PPP2R5A is detectable as a single band (isoform) and that this isoform is increased in tumor sections. Most importantly, in tumor sections, we detected the appearance of a slowly migrating immunoreactive PPP2R5A protein that strongly interacts with catalytic PP2Ac in nuclear extracts from tumors of aggressive HBL (Figure 1*E*). These studies suggest that the slowly migrating isoform of PPP2R5A is increased in patients with HBL and delivers catalytic PP2Ac to the nucleus. Western blot with cell-cycle proteins showed that cyclin D1, cyclin E, and cdc2 are increased in HBL samples compared with background regions, showing the increased proliferation in tumor sections (Figure 1*E*, bottom).

To obtain additional proof that de-phosphorylated-S190 C/EBPα has an increased ability to interact with HDAC1, we used genetically modified C/EBPα-S193A mice that mimic de-phosphorylated-S190- C/EBPα (mouse Ser193 is equivalent to human S190). In this animal model, the S193A mutant causes the development of liver cancer at a young age, which is characterized by the appearance of dedifferentiating hepatocytes.[6] Co-IP studies have shown that wild-

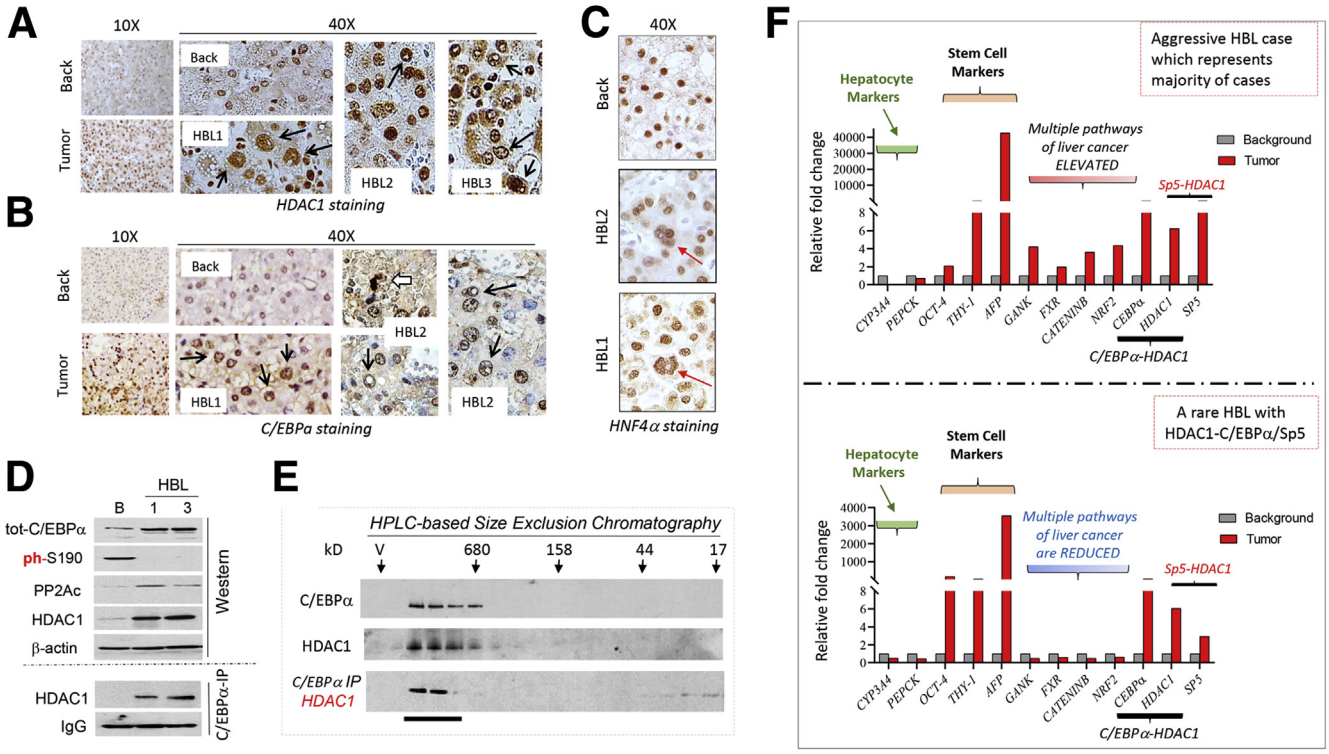

Figure 2. Oncogenic forms of C/EBPα and HDAC1 are observed in dedifferentiating hepatocytes. (*A–C*) Staining of HBL tumors with antibodies to HDAC1, C/EBPα, and HNF4α, respectively. The images with 10× and 40× magnification are shown. The 10× magnification reflects increased staining of proteins. Dedifferentiated hepatocytes with intranuclear inclusion are shown by *arrows* on 40× images. *Open arrow* shows a mitotic figure in a C/EBPα-positive hepatocyte. (*D*) Western blot shows an increase of the oncogenic form of C/EBPα and HDAC1 in tumor sections of HBL patients. *Bottom*: C/EBPα–HDAC1 complexes determined by Co-IP. Two to 3 technical replicates were performed for each biological sample. (*E*) Proteins were detected in fractions of SEC by Western blot. *Bottom*: Co-IP that showed the high MW C/EBPα–HDAC1 complexes. The SEC experiments were performed 1 time owing to limited amounts of the HBL tumor tissue. (*F*) Expression of mRNAs for hepatocyte markers, stem cell markers, and different pathways of liver cancer in the case typical for the majority of aggressive HBL patients (*upper*), and in a rare HBL patient who had a reduction of β-catenin, NRF2, and gankyrin, but an increase of C/EBPα-HDAC1 and Sp5-HDAC1. qRT-PCR was performed. HPLC, high-pressure liquid chromatography; tot, total.

type C/EBPα has a weak interaction with HDAC1; however, the mutant C/EBPα-S193A strongly interacts with HDAC1 (Figure 1*F*). Because the mutant C/EBPα-S193A cannot be phosphorylated at Ser193, these studies showed that the dephosphorylation of C/EBPα at Ser190/193 is critical for strong interactions with HDAC1. The increased interactions of the mutant C/EBPα-S193A with HDAC1 in this animal model also suggest that the mutations of human C/EBPα at Ser190 observed in HCC (Figure 1*B*) create a protein that might interact with HDAC1 in human liver cancers and possesses oncogenic activities.

HDAC1 and Oncogenic Form of C/EBPα Are Observed in Hepatocytes That Dedifferentiate to Cancer Cells

Because de-phosphorylated-S190-C/EBPα creates dedifferentiating hepatocytes with intranuclear inclusions,[6] we asked if the increased HDAC1 and C/EBPα are located in the dedifferentiating hepatocytes. Immunohistochemistry of 3 additional fresh HBL samples showed that HDAC1 is located in dedifferentiating hepatocytes that are a large size and have intranuclear inclusions (Figure 2*A*). Similar to HDAC1, C/EBPα also is abundant in nuclei of large dedifferentiating hepatocytes and in hepatocytes with mitotic figures (Figure 2*B*). Staining with antibodies to marker of hepatocytes Hepatocyte Nuclear Factor 4 Alpha, HNF4 alpha (HNF4α) confirmed that the large cells with intranuclear inclusions are hepatocytes (Figure 2*C*). Western blot with antibodies to total C/EBPα and to ph-S190-C/EBPα showed that de-phosphorylated-S190-C/EBPα is increased in these HBL patients (Figure 2*D*). HDAC1 protein also is increased in these HBLs. Co-IP studies and size exclusion chromatography (SEC) showed that the oncogenic C/EBPα and HDAC1 form complexes within these fresh HBL samples (Figure 2*D* and *E*). Thus, immunostaining and molecular investigations of HBL patients showed that HDAC1 and C/EBPα are located in dedifferentiating hepatocytes and form complexes. The case of HBL3 is a rare case, and is described later.

Identification of the Rare HBL Patient Who Has Activated HDAC1, C/EBPα, and Sp5, While Other Pathways of Liver Cancer Are Reduced

After resections, we performed characterization of HBL samples by qRT-PCR–based analyses of stem cell markers,

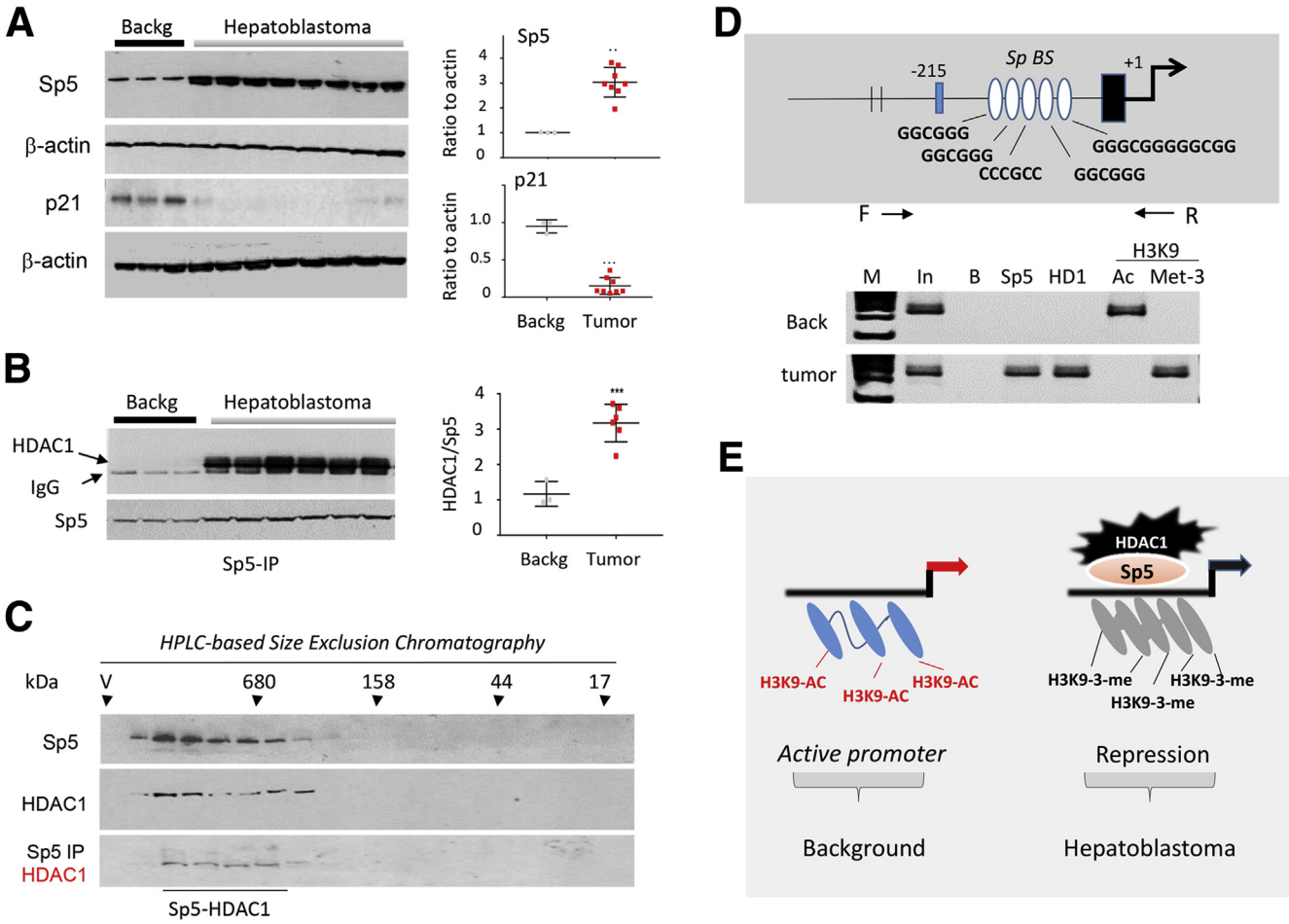

Figure 3. Sp5–HDAC1 complexes repress p21 promoter and expression of p21 protein in aggressive HBL. (*A*) Western blot shows expression of Sp5 and p21 in 8 fresh samples from HBL patients. Box plots show quantitation of Sp5 and p21 levels as ratios to β-actin. Three background (Backg) regions and 8 tumor sections were used for these calculations (Sp5: $^{**}P < .01$; p21: $^{***}P < .001$). (*B*) Co-IP studies show HDAC1–Sp5 complexes in aggressive HBL. Box plots show ratios of HDAC1 to Sp5 in Sp5 IPs ($^{***}P < .001$). (*C*) SEC analyses of nuclear proteins from a tumor of the rare HBL patient who had activation of the HDAC1–Sp5 pathway. *Bottom*: Co-IP that showed the Sp5–HDAC1 complexes. The SEC experiments were performed 1 time owing to limited amounts of the HBL tumor tissue. (*D*) The location of 6 Sp family binding sites (GC boxes) in the p21 promoter. *Bottom*: ChIP assay with background and tumor sections of the HBL patient. Two technical replicates were performed with the same samples. (*E*) A hypothesis for repression of p21 promoter in HBL tumors. HPLC, high-pressure liquid chromatography.

markers of hepatocytes, and major cancer pathways of HBL including β-catenin, Nuclear factor erythroid-2 (NRF2), and Gank. Figure 2*F* shows such qRT-PCR analysis of the HBL sample, which has an expression profile identical to those in other HBL samples, and analysis of the rare HBL3 sample, which has significant differences. Stem cell markers and oncogenic pathways, including β-catenin, NRF2, Gank, oncogenic C/EBPα, HDAC1, and Sp5, are increased in representative HBL samples. However, the rare sample HBL3, has a reduction of β-catenin, NRF2, and Gank, along with an increase of HDAC1-C/EBPα and HDAC1-Sp5 pathways (Figure 2*F*). This increase of the C/EBPα-HDAC1 pathway in this patient on protein levels is shown in Figure 2*D* and *E*. This suggests that the activation of HDAC1-dependent chromatin silencing is critical and, in some cases, might be the major contributor for the development of HBL.

Sp5 Forms Complexes With HDAC1 in HBL Patients

Given the increase of Sp5 messenger RNA (mRNA) in patients with HBL, we next examined protein levels of Sp5. Western blot analysis of 8 HBL samples found that protein levels of Sp5 are 2.5- to 3.5-fold increased in patients with HBL (Figure 3*A*). Examination of a target of Sp5-HDAC1 p21 showed that p21 is reduced (Figure 3*A*). Co-IP studies showed that Sp5 and HDAC1 form complexes in aggressive HBL (Figure 3*B*). We next asked if the Sp5–HDAC1 complexes were observed in our rare HBL3 case shown in Figure 2*F*. SEC showed that both HDAC1 and Sp5 are located in high molecular weight (MW) regions of SEC and form a complex (Figure 3*C*). To examine if HDAC1–Sp5 complexes repress the p21 promoter, we performed a chromatin immunoprecipitation (ChIP) assay. Because the p21 promoter contains 6 SP binding sites in close proximity to the start of

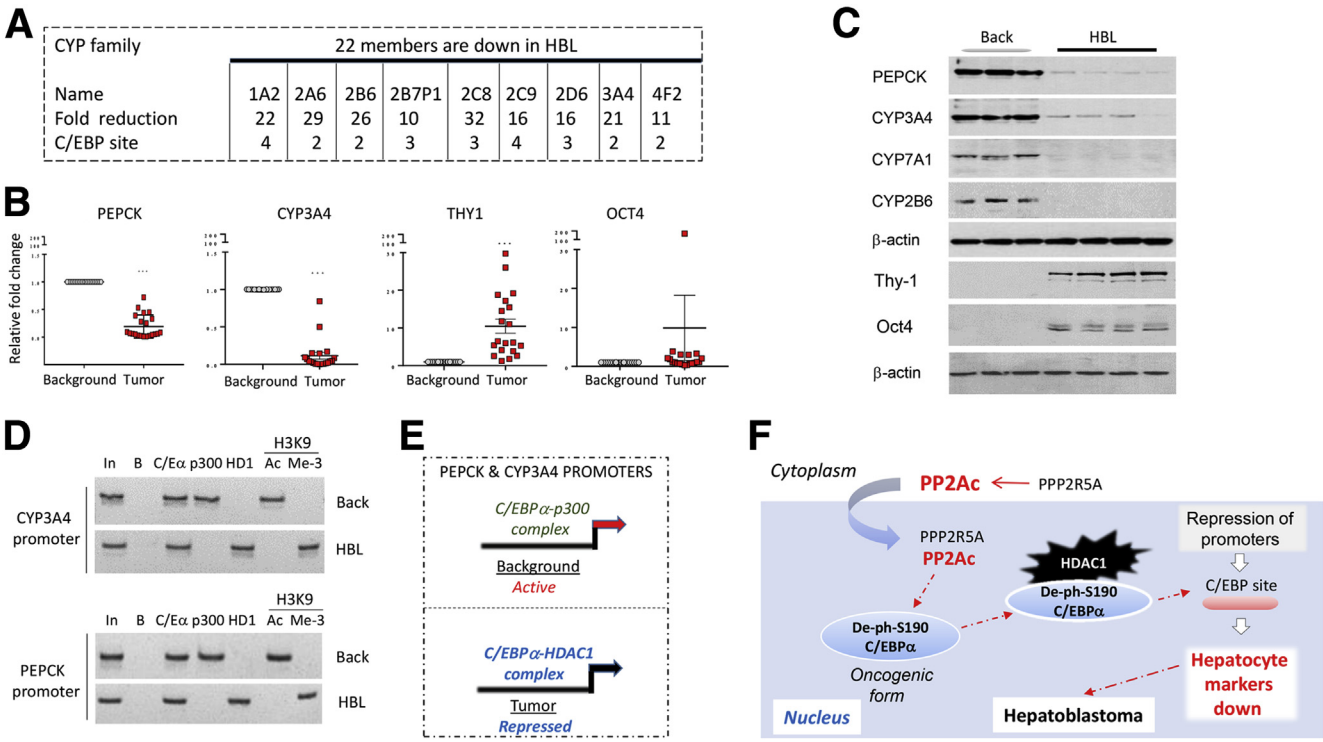

Figure 4. Oncogenic C/EBPα–HDAC1 complexes repress expression of markers of hepatocytes, CYP family proteins. (*A*) A summary of RNA sequencing results for the CYP family[5] and identification of C/EBPα sites in the promoters of CYP family members. A fold reduction in HBL and the number of C/EBPα sites in their promoters are shown. (*B*) Expression of PEPCK, CYP3A4, Thy-1, and Oct4 mRNAs in fresh biobank. qRT-PCR was performed with RNA isolated from background and tumor regions of 22 HBL patients. ***$P < .001$. (*C*) Western blot shows reduction of CYP family and PEPCK in patients with aggressive HBL, while stem cell markers are increased. The picture shows representative images from 3 background and 4 tumor sections of HBL patients. Two to 3 technical replicates were performed. (*D* and *E*) ChIP assay with CYP3A4 and PEPCK promoters. (*F*) A hypothesis for the role of PP2A–C/EBPα–HDAC1 pathways in aggressive pediatric liver cancer.

transcription[19] (Figure 3*D*), we used PCR primers that cover this region of the promoter. Figure 3*D* shows that, in background regions, HDAC1 and Sp5 are not bound to the p21 promoter. However, HDAC1 and Sp5 occupy the p21 promoter in HBL tumors, which correlates with methylation of H3K9, reflecting repression of the promoter. Because HDAC1 does not bind directly to DNA, these results suggest that Sp5 delivers HDAC1 to the p21 promoter. Taken together, these studies show that the Sp5–HDAC1 axis is activated in aggressive HBL and is involved in the repression of p21 (Figure 3*E*).

De-phosphorylated-S190-C/EBPα/HDAC1 Complex Occupies Promoters of PEPCK *and* CYP3A4 *Genes and Represses Their Expression in HBL Patients*

Our previous examination of a large cohort of HBL patients showed that a family of cytochrome genes (*CYP*), markers of hepatocytes, are reduced dramatically.[4] Therefore, we searched promoter regions of the *CYP* family of genes with higher levels of reduction in HBL and found that all of them contain 2–4 C/EBPα binding sites (Figure 4*A*), suggesting that they might be targets of C/EBPα–HDAC1 complexes. qRT-PCR analysis of a fresh biobank showed that mRNAs of *CYP3A4* and *PEPCK* (another marker of hepatocytes) are reduced in the fresh biobank of HBL samples, while markers of stem cells Thy1 and Oct4 are increased (Figure 4*B*). Therefore, we looked at the levels of the PEPCK, CYP3A4, and stem cell marker proteins and found that PEPCK, CYP3A4, CYP7A1, and CYP2B6 also are reduced in tumor sections of fresh aggressive HBL, while Thy1 and Oct 4 are increased (Figure 4*C*). ChIP assay showed that the promoters of *PEPCK* and *CYP3A4* genes are activated in background regions by C/EBPα–p300 complexes; however, these promoters are repressed in tumor sections by C/EBPα–HDAC1 complexes (Figure 4*D* and *E*). Based on these studies of tumor samples from HBL patients, we suggested that, in aggressive HBL, a regulatory PPP2R5A subunit translocates the catalytic subunit PP2Ac to the nucleus, where PP2Ac dephosphorylates C/EBPα at Ser190, leading to formation of C/EBPα–HDAC1 complexes. These complexes repress promoters of markers of hepatocytes CYP3A4 and PEPCK, resulting in reduction of these proteins (Figure 4*F*). We next confirmed this hypothesis in HBL cells.

Inhibition of PP2A in Cancer Cells Eliminates de-phosphorylated-S190-C/EBPα–HDAC1 Complexes

We first asked if the inhibition of an initial step of the oncogenic PP2A–C/EBPα pathway, PP2A activity, by pharmacologic approach reduces the oncogenic form of C/EBPα

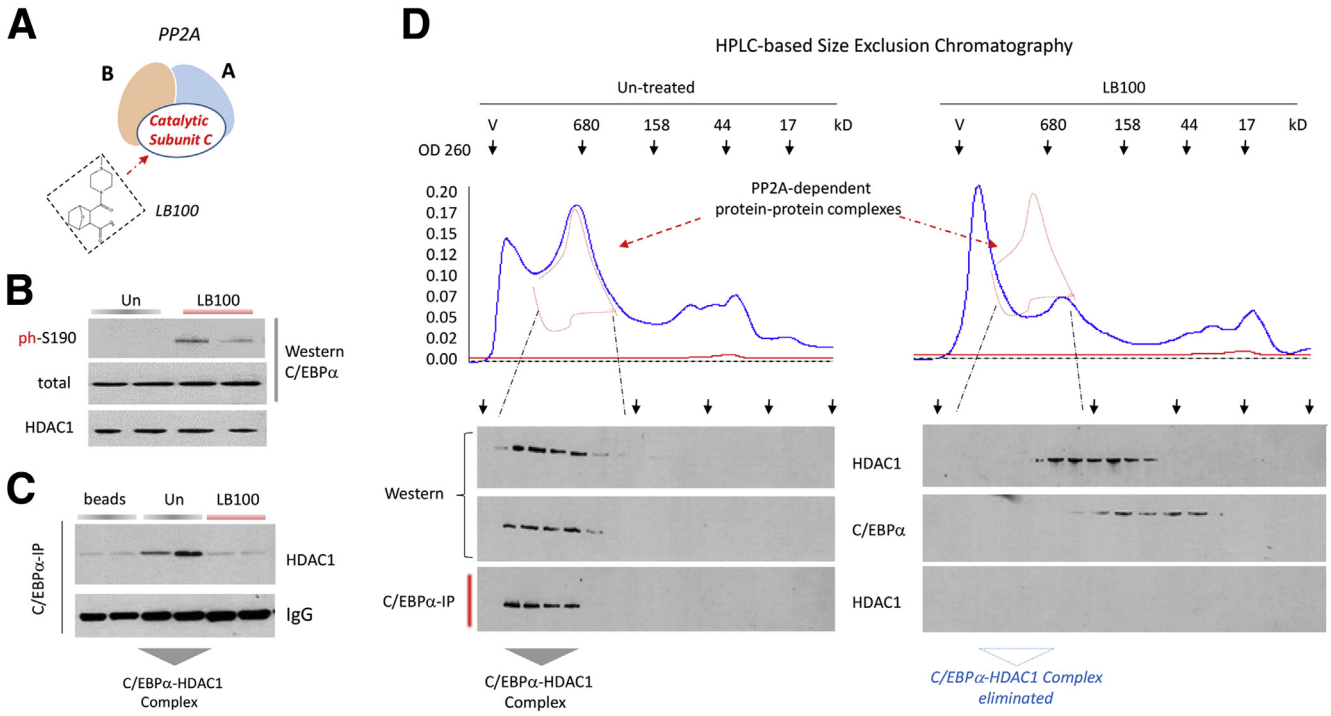

Figure 5. Inhibition of PP2A by LB100 eliminates C/EBPα–HDAC1 complexes in cancer cells. (*A*) Structure of PP2A. (*B*) Western blot showing that LB100 inhibits dephosphorylation of C/EBPα at Ser190 in Huh6 cells. Membranes were probed with antibodies to ph-S190 and total C/EBPα and to HDAC1. Three technical repeats were performed with 3 biological replicates. (*C*) Co-IP shows that LB100 reduces C/EBPα–HDAC1 complexes. Three technical repeats were performed with 2 biological replicates. (*D*) SEC of proteins from untreated and LB100-treated Huh6 cells. *Upper*: Optical density profiles of SEC runs. The region of SEC with high-MW complexes is shown. *Lower*: Western blot and Co-IP from SEC fractions. Three technical repeats were performed with 2 biological replicates.

and levels of C/EBPα–HDAC1 complexes. To inhibit PP2A activity, we used LB100, which is highly specific to PP2A[27] (Figure 5*A*). We found that the treatment of Huh6 cells with 5 μmol/L LB100 for 48 hours restores phosphorylation of C/EBPα at Ser190 (Figure 5*B*) and eliminates C/EBPα–HDAC1 complexes (Figure 5*C*). The SEC approach also was used and surprisingly showed a dramatic reduction of the large region of optical density in the region of high MW complexes of SEC (Figure 5*D*), suggesting that PP2A controls multiple high-MW protein–protein complexes. Examination of C/EBPα and HDAC1 in SEC fractions showed that, in untreated cells, both proteins are located in high-MW fractions and form a complex. The inhibition of PP2A by LB100 destroys the complexes and shifts the proteins to lower MW fractions.

Pharmacologic Inhibition of PP2A and HDAC1 Inhibits Proliferation of Cancer Cells

We next examined if Food and Drug Administration–approved inhibitors of HDAC1, SAHA (vorinostat), and LB100 might inhibit proliferation of Huh6 cells. Huh6 cells were treated with LB100, SAHA, or with the combination of SAHA + LB100. Figure 6*A* shows that total protein levels of C/EBPα and HDAC1 are increased slightly by LB100 and SAHA correspondingly. In addition, it shows that C/EBPα is phosphorylated at Ser190 in cells treated with LB100, or with the combination of LB100 and SAHA, but not in cells with SAHA alone. Interestingly, Western blot with antibodies to Poly [ADP-ribose] polymerase 1 (PARP1) showed the appearance of cleaved forms of PARP1, indicating apoptosis, mainly in cells treated with a combination of drugs (Figure 6*A*, red arrow). HDAC1–C/EBPα complexes are eliminated in cells treated with LB100 and with the combination of LB100 + SAHA (Figure 6*B*). Interestingly, the rescue of phosphorylation of C/EBPα at Ser190 restores its ability to form complexes with p300, which are activators of many C/EBPα-dependent genes.[16,17] Our further ChIP studies showed that the LB100-restored C/EBPα–p300 complexes are involved in activation of PEPCK and CYP3A4 (Figure 7*D*).

Examination of proliferation found that treatments of Huh6 cells with SAHA and LB100 reduce proliferation of the cells, along with combined treatments that lead to further inhibition of proliferation (Figure 6*C*). We found that untreated Huh6 cells form large clusters containing multiple cells, but treatment with SAHA or LB100 alone reduces the size of the cell clusters. Combined treatments resulted in an even smaller size of clusters (Figure 6*D*). The terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling assay found that LB100 or SAHA alone initiated apoptosis, but combined treatments lead to a higher degree of cell death (Figure 6*D*, bottom). Examination of cell-cycle proteins showed that cdk2, cyclin D1, cdc2, and cyclin E were

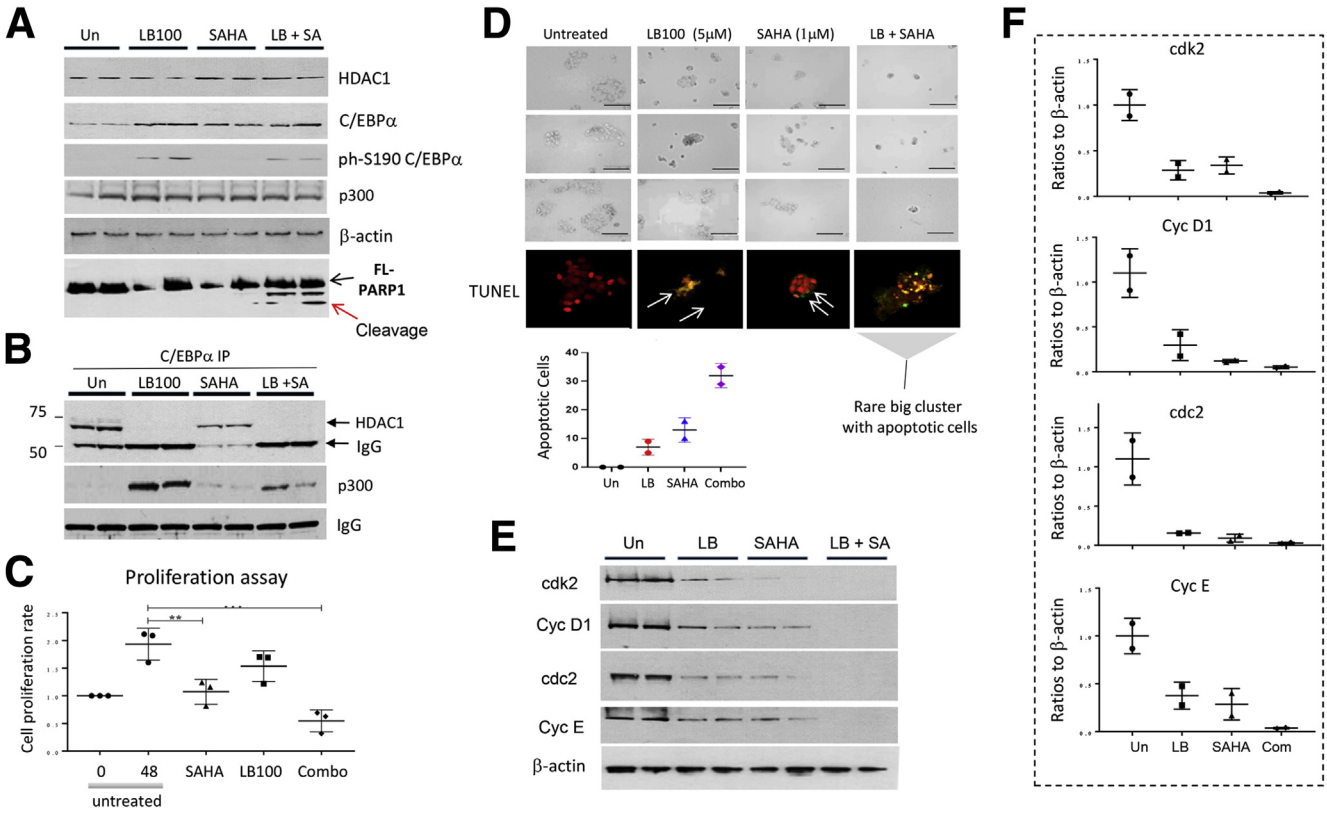

Figure 6. Inhibition of PP2A and HDAC1 by LB100 and SAHA reduces the proliferation of hepatoblastoma Huh6 cells. (*A*) Expression of C/EBPα and HDAC1 proteins after treatment of Huh6 cells with LB100, SAHA, and with a combination of these drugs. Three technical repeats were performed with 2 biological replicates of each treatment. (*B*) Co-IP shows C/EBPα–HDAC1 and C/EBPα–p300 complexes in untreated and treated cells. Two technical repeats were performed with 2 biological replicates. (*C*) Proliferation assay of cells treated with drugs. The proliferation assay was performed 3 times with 3 technical replicates. $^{**}P < .01$, $^{***}P < .001$. (*D*) Typical images of untreated and treated Huh6 cells. *Bottom*: Results of terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. A rare cluster of LB100 + SAHA–treated cells shows massive apoptosis within this cluster. Typical images from 3 independent treatments. The box plots show quantitation of apoptotic cells. (*E*) Western blot for cell-cycle proteins. Two technical replicates were performed with 2 treatments of cells. (*F*) Protein levels are shown as ratios to β-actin. Combo, combination of LB100 + SAHA; LB, LB100; Un, untreated cell.

reduced in cells treated with LB100 or SAHA alone, whereas combined treatments resulted in the strongest inhibition of these proteins (Figure 6*E* and *F*); thus showing that the inhibition of PP2A by LB100 destroys C/EBPα–HDAC1 complexes and inhibits proliferation of cancer cells.

Elimination of C/EBPα–HDAC1 Complexes Increases Levels of Markers of Hepatocytes via Removal of C/EBPα–HDAC1 Complexes From the Promoters

In addition to the CYP family, 35 members of another family of proteins, organic cation transporters (OCT or SLC) are reduced in HBL patients.[4,5] We analyzed promoters of the 7 members of the OCT family with the highest levels of reduction and found that examined *SLC* genes contained 1–3 C/EBPα sites in their promoters (Figure 7*A*). Figure 7*B* shows that, in hepatoblastoma HepG2 and Huh6 cell lines, the LB100-mediated elimination of C/EBPα–HDAC1 complexes leads to activation of 5 members of the CYP family: CYP2C8, CYP3A4, CYP1A2, CYP2A6, and CYP2B6. We observed a more complicated pattern of expression of *OCT* genes. The members of OCT family SLC22A7, SLC2A4, and SLC22A18 are increased in cells treated with LB100, whereas SAHA increased expression of *SLC22A1*, *SLC22A7*, *SLC2A2*, and *SLC2A4* genes. Examination of protein levels of CYP3A4, CYP2B6, and PEPCK showed that LB100 and SAHA increased the levels of these proteins (Figure 7*C*). ChIP assay showed that, in untreated HepG2 cells, C/EBPα and HDAC1 occupy and repress PEPCK and CYP3A4 promoters. However, the combined treatments of HepG2 cells with SAHA and LB100 remove these complexes and activate the promoters (Figure 7*D*). It is interesting that LB100 + SAHA treatments not only remove C/EBPα–HDAC1 complexes from the promoters, but also restore C/EBPα–p300 complexes that occupy the PEPCK and CYP3A4 promoters in treated cells (Figure 7*D*). This switch of the complexes is similar to the patterns observed in tumor and background sections of the HBL patients (Figure 4*D*).

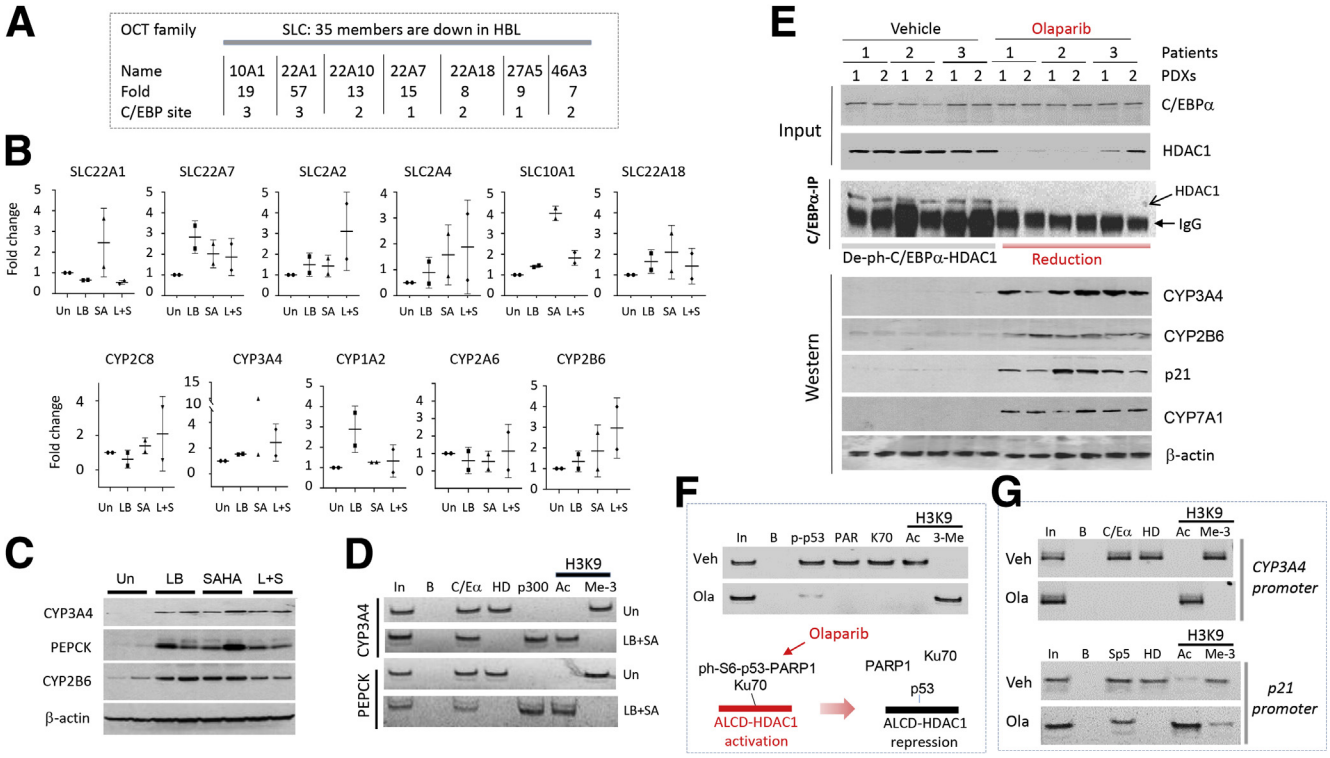

Figure 7. Elimination of C/EBPα–HDAC1 complexes increases the expression of markers of hepatocytes in Huh6 and HepG2 cells and in PDXs. (*A*) The summary of RNA sequencing data for OCT family in a large biobank is shown. Fold reduction and number of C/EBPα sites in promoters of these genes are shown. (*B*) mRNA levels of CYP and OCT family mRNAs in untreated and drug-treated Huh6 and HepG2 cells were determined by qRT-PCR. The box plots show a summary for 2 treatments in each cell line. (*C*) Western blot shows the increase of CYP3A4, PEPCK, and CYP2B6 in HepG2 cells treated with drugs. Three technical repeats were performed with 2 biological replicates. (*D*) ChIP assay with CYP3A4 and PEPCK promoters in untreated HepG2 cells and in cells treated with a combination of LB100 + SAHA. Two repeats of the ChIP assay were performed. (*E*) ChIP assay with ALCD within the *HDAC1* gene. *Bottom*: Olaparib removes the ph–S6–p53–PARP1 complex from the HDAC1 ALCD and represses this locus. (*F*) Treatments of HBL-PDXs with olaparib (Ola) reduces HDAC1–C/EBPα complexes and increases the expression of CYP family and p21 proteins. Six PDXs bearing HBL tumors from 3 patients were treated with vehicle (Veh) (control) and with olaparib. Input: Expression of C/EBPα and HDAC1 was examined by Western blot. Co-IP: C/EBPα–HDAC1 complexes were examined by Co-IP. The experiments were performed twice with 6 PDXs from 3 HBL patients. Western: Expression of CYP family proteins and p21 in olaparib-treated PDXs. Western blot was performed twice with 6 PDXs from 3 HBL patients. (*G*) ChIP assay with the CYP3A4 and p21 promoters. The ChIP assay was performed once owing to limited amounts of the tissues. IgG, signals of heavy chains of IgG. HD, HDAC1; LB, LB 100; Un, untreated cells.

Olaparib-Mediated Reduction of HDAC1 and Subsequent Reduction of C/EBPα–HDAC1 Complexes in Patient-Derived Xenografts Increases Expression of Markers of Hepatocytes

We previously showed that the increase of many oncogenes in HBL is mediated by activation of specific regions of the genome in what we refer to as aggressive liver cancer domains (ALCDs), and that PARP1 activates ALCDs in aggressive HBL.[5] We recently found that the inhibition of PARP1 by olaparib in patient-derived xenografts (PDXs) reduces levels of the ALCD-dependent oncogene, inhibits tumor growth, and increases expression of hepatocyte markers CYP3A4 and CYP2A6.[28] Because these *CYP* genes are direct targets of C/EBPα–HDAC1 complexes, we suggested that HDAC1 might be reduced in PDXs treated with Olaparib (Ola) by an ALCD-dependent pathway. Therefore, we carefully searched the *HDAC1* gene and identified an ALCD within the first intron with 90% homology to other ALCDs. This ALCDs contain the perfect binding site for the ph-S6-p53–PARP1 complexes. ChIP assay showed that the ph-S6-p53–PARP1 complexes occupy and activate the ALCDs in untreated PDXs and that olaparib removes the complexes and represses this locus of the *HDAC1* gene (Figure 7*E*). We next performed Western blot of 6 vehicle-treated and 6 Ola-treated PDXs and found that levels of HDAC1 are reduced in Ola-treated PDXs, while levels of C/EBPα were not changed (Figure 7*F*, input). Consistent with a reduction of HDAC1, C/EBPα–HDAC1 complexes also are reduced in Ola-treated PDXs. Western blot showed that targets of C/EBPα–HDAC1 complexes CYP3A4, CYP2B6, and CYP7A1 are increased in Ola-treated PDXs (Figure 7*F*, Western). We also detected an increase of p21 in Ola-treated PDXs (Figure 7*F*) because the inhibition of HDAC1 removes HDAC1–Sp5 complexes from the p21 promoter (Figure 7*G*). ChIP assay with a CYP3A4 promoter showed that, in control PDXs, the promoter is occupied and repressed by the C/EBPα–HDAC1 complex, while Ola

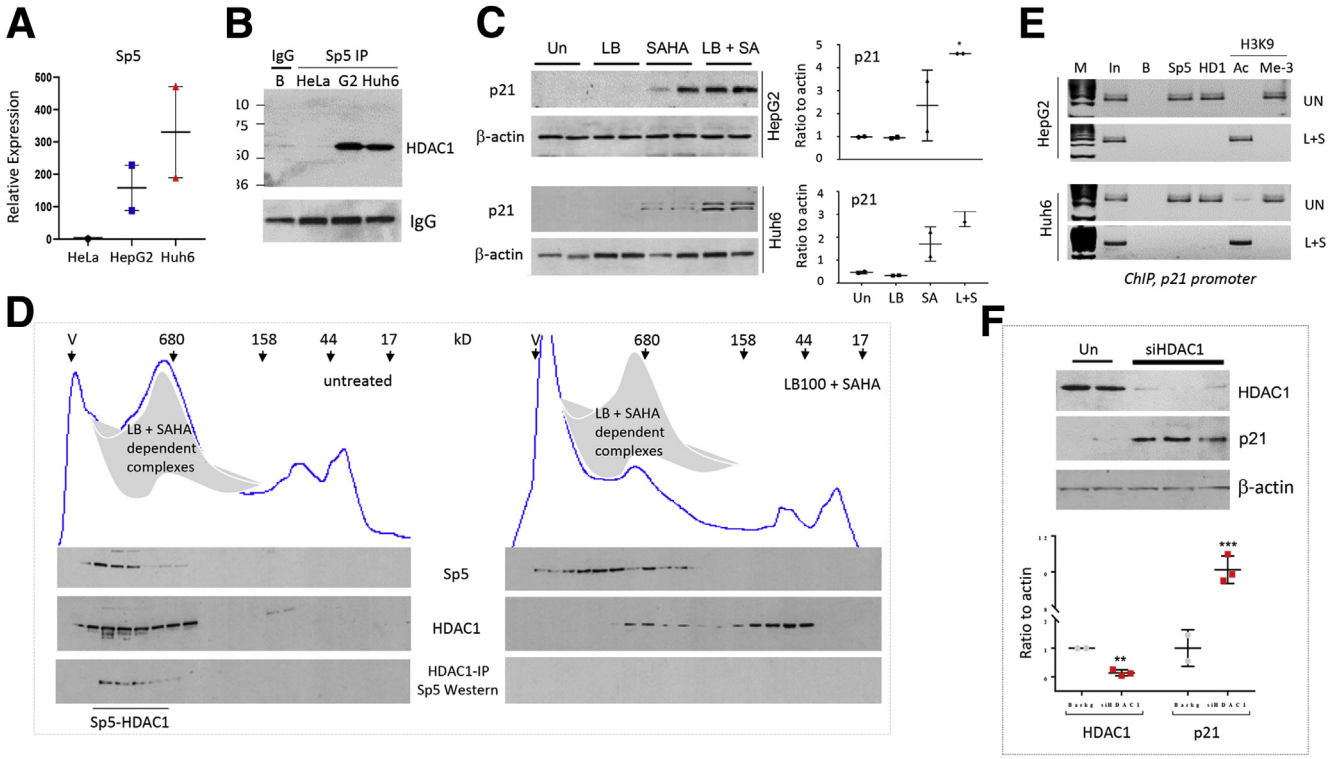

Figure 8. Reduction of Sp5–HDAC1 complexes by SAHA and inhibition of HDAC1 by small interfering RNA increases the expression of p21 protein. (*A*) Levels of Sp5 mRNA were determined by qRT-PCR in HeLa, HepG2, and Huh6 cells. (*B*) Co-IP studies show Sp5–HDAC1 complexes in HepG2 and Huh6 cells. Two repeats were performed. (*C*) Western blot shows that treatments of Huh6 and HepG2 cells with SAHA and LB100 + SAHA increase the expression of p21. Box plots show levels of p21 as ratios to β-actin. *$P < .05$. (*D*) Treatment of Huh6 cells with a combination of LB100 + SAHA destroys Sp5–HDAC1 complexes. The SEC approach was used as described in the Figure 5*D* legend. (*E*) ChIP assay with p21 promoter. (*F*) Western blot shows that the inhibition of HDAC1 by small interfering RNA increases the expression of p21 in HepG2 cells. Box plots show ratios of p21 to β-actin. The experiment was performed 3 times with 3 biological replicates. LB, LB100; Un, untreated cells.

removes the complexes from the promoter, leading to the opening of ALCD as H3K9 acetylation shows (Figure 7*G*). Taking studies in cultured cells and PDXs together, we conclude that the elimination of C/EBPα–HDAC1 complexes by LB100 + SAHA or by Ola activates promoters of PEPCK and CYP3A4, and that reduction of Sp5-HDAC1 activates the p21 promoter.

Inhibition of HDAC1 Activity by SAHA Removes Sp5–HDAC1–Mediated Repression of the p21 Promoter and Increases Levels of p21

Because HDAC1–Sp5 complexes repress p21 in HBL patients (Figure 3*D*), we examined if inhibition of HDAC1 in cancer cells might release this repression and restore expression of p21. qRT-PCR showed that levels of Sp5 in HBL cells HepG2 and Huh6 are 100–500 times higher than in non-HBL HeLa cells (Figure 8*A*). Co-IP studies showed that Sp5 forms complexes with HDAC1 in HepG2 and Huh6 cells (Figure 8*B*). We next asked if inhibition of HDAC1 by SAHA de-represses p21. Western blot showed that p21 is increased in cells treated with SAHA and with the combination of SAHA + LB100 (Figure 8*C*). The SEC approach showed that, similar to treatments with LB100 alone, the combination SAHA + LB100 leads to a reduction of optical density in the region of high-MW complexes (Figure 8*D*). Western blot and Co-IPs showed that treatments with LB100 + SAHA destroy the Sp5–HDAC1 complexes. ChIP assay found that treatments of HepG2 and Huh6 cells with LB100 + SAHA remove Sp5–HDAC1 complexes from the p21 promoter (Figure 8*E*). To examine if HDAC1 is responsible for repression of p21, we inhibited HDAC1 by specific small interfering RNA and found that p21 is increased in cells with reduced levels of HDAC1 (Figure 8*F*). Taken together, these studies showed that removal of the Sp5–HDAC1 complexes from the p21 promoter activates the promoter of p21 and increases protein levels of p21.

Discussion

The loss of hepatocyte markers is a critical step in the development of liver cancer. In this article, we identified mechanisms that are involved in the repression of hepatocyte markers and the p21 protein in hepatoblastoma. These occur on the levels of epigenetic repression of the promoters and are mediated by HDAC1 in complexes with the oncogenic form of C/EBPα and Sp5. The oncogenic form of

C/EBPα is created by dephosphorylation of Ser190 by PP2A.[5,14,15] Similar to C/EBPα, PP2A initially was identified as a tumor-suppressor protein.[25] Our data in this article strongly suggest that PP2A displays oncogenic activities in pediatric liver cancer. In support of our findings, several reports found that PP2A possesses oncogenic activities in other cancers.[24,25,29] For example, HCC associated with hepatitis C infection expresses high levels of PP2A, and treatment of this HCC with inhibitors of PP2A reduce proliferation within these cells.[29] Several studies have shown that an inhibitor of PP2A, cantharidin, has antitumor activity in many types of tumor cells.[25,30] Our studies of HBL patients showed that HDAC1 and the oncogenic forms of C/EBPα and Sp5 are increased in tumor sections of HBL patients. Immunostaining of tumor sections of HBL patients showed that HDAC1 and C/EBPα are accumulated in dedifferentiating hepatocytes with intranuclear inclusions, which have been shown previously to contain the stem cell marker Delta Like Non-Canonical Notch Ligand 1 (DLK1).[6] Further studies of patients with HBL showed that HDAC1 forms complexes with oncogenic C/EBPα and Sp5 and that these complexes occupy and repress promoters of hepatocyte markers CYP family proteins and the p21 promoter.

To test this hypothesis, we examined PP2A–C/EBPα–HDAC1 and Sp5–HDAC1 pathways in cancer cells derived from HBL patients: HepG2 and Huh6. The studies with inhibition of PP2A showed that PP2A is responsible for creating an oncogenic form of C/EBPα for formation of C/EBPα–HDAC1 complexes and subsequent repression of markers of hepatocytes. We also found that the olaparib-mediated reduction of HDAC1 and C/EBPα–HDAC1 complexes in PDX models leads to de-repression and increased expression of markers of hepatocytes. In tissue culture models, the inhibition of HDAC1 by SAHA leads to de-repression of the p21 promoter by removal of Sp5–HDAC1 complexes. Although SAHA inhibits activity of HDAC proteins, we surprisingly observed that SAHA also destroys Sp5–HDAC1 complexes and that Sp5 is not bound to p21 promoters after treatment. Thus, our studies of HBL patients, cells, and PDX models showed the critical role of HDAC1/C/EBPα/Sp5 in the development of pediatric liver cancer. In this regard, previous studies congruently have shown that the inhibition of PP2A additionally significantly inhibits the activity of HCC.[29,30] The PP2A–C/EBPα pathway seems to be a critical part of PP2A oncogenic activities in liver cancer because restoration of C/EBPα activities by ectopic expression of constitutively active C/EBPα-S193D inhibits proliferation of cancer cells.[15] In summary, given that LB100 is entering into clinical trials,[31] LB100, as well as the inhibitor of HDAC1 SAHA, have significant potential for translational and clinical applications in treating HCC and HBL.

Methods

Animals

Animal experiments were approved by the Institutional Animal Care and Use Committee at Cincinnati Children's Hospital (protocol IACUC2014-0042).

Pediatric HBL Patients

This study underwent review and was approved before undertaking by the Institutional Review Board at Cincinnati Children's Hospital Medical Center and University of Cincinnati Medical Center. Informed consent was obtained by each study patient before obtaining specimens. A significant portion of HBL samples were from patients who were faced with aggressive tumors that were characterized by multiple nodules at diagnosis, vascular invasion, chemoresistance, and relapse. These cases are referred to as *aggressive hepatoblastoma*.

Immunohistochemistry

Liver sections were fixed in 4% Paraphormaldehide (PFA), embedded in paraffin, and sectioned (6-μm sections). Immunohistochemistry for HDAC1, C/EBPα, and HNF4α were performed with fresh HBL samples. Antibodies used were as follows: HDAC1 (05-100-I; EMD Millipore, Santa Cruz, CA), C/EBPα (sc-61; Santa Cruz, CA), and HNF4α (PP-K9218-00; Perseus Proteomics, Tokyo, Japan).

Real-Time qRT-PCR

Total RNA was isolated from human livers and from HepG2 and Huh6 cells as described.[5,6] Complementary DNA was synthesized with 2 μg total RNA using a High-Capacity Complementary DNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA). Complementary DNA was diluted 5 times and subsequently used for RT-PCR assays with the TaqMan Gene Expression system (Applied Biosystems). Gene expression analysis was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Levels of all mRNAs were normalized to 18S ribosomal RNA. Data in this article present a summary of 3 technical replicates with 3–8 biological replicates. For qRT-PCR analyses of each gene, we used 22 background and 22 tumors sections of HBL patients.

Antibodies, Protein Isolation, Western Blot, Co-IP

Protein extracts were prepared as previously described.[5,6] Nuclear extracts (50 μg) were loaded on 4%–20% gradient gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes (Bio-Rad). For the investigations of PP2Ac, cytoplasmic nuclear and whole-cell extracts were used. Because C/EBPα is detected mainly in nuclei, Co-IP was performed with nuclear extracts using an improved Trueblot (Rockland Immunochemical, Limerick, PA) protocol as described.[5] Antibodies used for Western blot and Co-IP were as follows: PP2Ac (clone7A6; Millipore Sigma, Burlington, MA), PPP2R5A (ab89621; Abcam, Cambridge, MA), PARP1 (Santa Cruz), cyclin D1 (sc-753; Santa Cruz), Gank (12985S; Cell Signaling Technologies, Danvers, MA), cdc2 (sc-954; Santa Cruz), CY3PA4 (sc-30621; Santa Cruz), CYP2B6 (sc-55924; Santa Cruz), CYP7A1 (E-10; Santa Cruz), PEPCK (F-3; Santa Cruz), HDAC1 (05-100-I; EMD Millipore), C/EBPα (sc-61; Santa Cruz), p21 (sc-6246; Santa Cruz), HNF4α (PP-K9218-00; Perseus Proteomics), Sp5 (ab36593; Abcam), and β-actin (A5316; Sigma). Antibodies

to ph-S190-C/EBPα have been described in our previous article.[5] Western blot and Co-IP experiments were performed 3 times which each biological replicate.

High-Pressure Liquid Chromatography–Based SEC

To determine the size of C/EBPα–HDAC1 and Sp5–HDAC1 complexes, protein extracts were separated on high-pressure liquid chromatography SEC column SEC600 (Bio-Rad). A total of 30 μL from SEC fractions were loaded on denaturing 4%–20% acrylamide gel and examined by Western blot with antibodies to HDAC1, C/EBPα, and Sp5. For detection of protein–protein complexes in SEC fractions, C/EBPα or Sp5 were immunoprecipitated and HDAC1 was examined by Western blot using the improved Trueblot protocol.[5] A detailed procedure of SEC has been described in our previous articles.[5,6]

ChIP Assay

ChIP assay was performed as described in our previous articles.[5,6] Briefly, chromatin solutions were isolated from background and tumor sections of livers of HBL patients. For detection of C/EBPα complexes, C/EBPα, HDAC1, p300, histone H3K9-Ac, and histone H3K9-trimethylated were immunoprecipitated and the IPs were examined by PCR with primers to CYP3A4 and PEPCK promoters. For detection of Sp5–HDAC1 complexes, Sp5, HDAC1, H3K9Ac, and H3K9-trimethylated were immunoprecipitated from chromatin solutions. The IPs were examined by PCR with primers to p21 promoter, which cover 6 Sp binding sites (Figure 3*D*). The sequences of the primers have been described in previous articles.[5,17,19] This article shows representative results of 2–3 repeats of ChIP assays.

Proliferation Assay

Huh6 cells were seeded in 96-well plates at 5×10^4. Cells were treated with LB100 (5 μmol/L), SAHA (1 μmol/L), and by a combination of these drugs. The Chokcystokinin, CCK-8 reagent (Sigma) was added into each well and incubated for 4 hours, followed by reading at 450 nm on a microplate reader. Each experiment had 3 repeats per treatment and were repeated 3 times. The proliferation rate was calculated by comparing the absorbance of the treated and nontreated wells after 48 hours with the 0-hour nontreated well.

Terminal Deoxynucleotidyl Transferase–Mediated Deoxyuridine Triphosphate Nick-End Labeling Assay

A tunnel assay (ab66108; Abcam) was conducted with Huh6 cells treated with LB100, SAHA, and with a combination of LB100 + SAHA. Untreated and treated Huh6 cells were collected after 48 hours of treatment. After treatment, cells were harvested and fixed in 1% paraformaldehyde. Cells were resuspended in the DNA labeling solution consisting of reaction buffer, Terminal deoxynucleotidyl Transferase (TdT) enzyme, fluorescein isothiocyanate–deoxyuridine triphosphate, and ddH_2O and incubated in the DNA labeling solution for 60 minutes at 37°C. Cells were counterstained with propidium iodide/RNase A. The number of apoptotic cells was counted per each plate and presented as a box plot (Figure 6).

Statistical Analysis

All values are presented as means ± SEM. An unpaired Student t test was applied for comparison of normally distributed data. Two-way analysis of variance was used with a Bonferroni test for multiple comparisons between different time points if the P value was less than .05. Statistical significance was defined as follows: $P < .05$, $P < .01$, $P < .001$, and $P < .0001$. All statistical analyses were performed using GraphPad Prism 6.0 (San Diego, CA).

All authors had access to the study data and approved the final manuscript.

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Received May 17, 2021. Accepted June 30, 2021.

Correspondence
Address correspondence to: Nikolai Timchenko, PhD, Liver Tumor Program, Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, Ohio 45229. e-mail: Nikolai.Timchenko@cchmc.org; fax: (513) 636-4200.

Acknowledgements
The authors thank Ashley Cast for help with immunostaining HBL livers.

CRediT Authorship Contributions
Maria Rivas (Conceptualization: Lead; Data curation: Lead; Formal analysis: Lead; Software: Lead; Validation: Lead; Writing – original draft: Supporting)
Michael Johnston (Conceptualization: Supporting; Data curation: Equal; Formal analysis: Equal; Writing – original draft: Supporting)
Ruhi Gulati (Conceptualization: Supporting; Data curation: Supporting; Formal analysis: Equal; Software: Equal; Writing – original draft: Supporting)
Meenasri Kumbaji (Data curation: Equal; Formal analysis: Equal)

Talita Ferreira Margues Aguiar (Data curation: Supporting; Formal analysis: Supporting; Software: Supporting)

Lubov Timchenko (Data curation: Supporting; Formal analysis: Supporting; Validation: Supporting; Writing – original draft: Supporting)

Ana Krepischi (Conceptualization: Equal; Formal analysis: Supporting; Software: Supporting; Supervision: Supporting)

Soona Shin (Conceptualization: Supporting; Formal analysis: Supporting; Writing – review & editing: Equal)

Alexander Bondoc (Conceptualization: Supporting; Formal analysis: Supporting; Validation: Supporting)

Gregory Tiao (Conceptualization: Supporting; Formal analysis: Supporting; Funding acquisition: Equal; Writing – review & editing: Equal)

James Geller (Conceptualization: Supporting; Formal analysis: Supporting; Validation: Supporting)

Nikolai Timchenko, PhD (Conceptualization: Lead; Data curation: Equal; Formal analysis: Lead; Funding acquisition: Lead; Methodology: Lead; Validation: Lead; Writing – original draft: Lead)

Conflicts of interest

The authors disclose no conflicts.

Funding

This work is supported by National Institutes of Health grants R01DK102597 and R01CA159942 (Nikolai Timchenko), Internal Development Funds from Cincinnati Children's Hospital Medical Center (Nikolai Timchenko), Fibrolamellar Cancer Foundation grant FCF-0015 (Nikolai Timchenko), National Institutes of Health grant P30 DK078392, and by the William M and Ann K Grace Charitable Foundation.