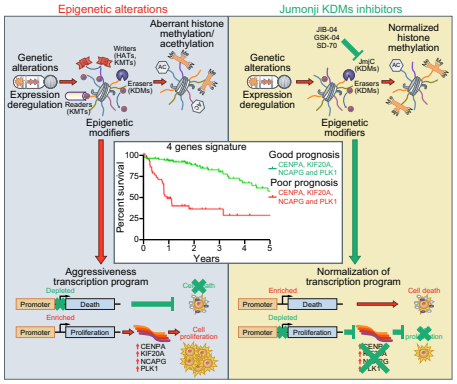
Research Article
Cancer

JOURNAL
OF HEPATOLOGY

A comprehensive study of epigenetic alterations in hepatocellular carcinoma identifies potential therapeutic targets

Graphical abstract



Highlights

- Mutations and expression alterations of epigenetic modifiers are frequent in HCC.
- Jumonji lysine demethylase inhibitors normalize aggressive transcription programs in HCC.
- *CENPA, KIF20A, NCAPG* and *PLK1* gene expression signature defines prognosis in HCC.
- Epigenetic inhibitors are a potential new therapeutic tool for HCC.

Authors

Juan Bayo, Esteban J. Fiore, Luciana M. Dominguez, ..., Josepmaria Argemi, Elisabeth D. Martinez, Guillermo D. Mazzolini

Correspondence

gmazzoli@austral.edu.ar
(G.D. Mazzolini)

Lay summary

In this study, we found that mutations and changes in expression of epigenetic modifiers are common events in human hepatocellular carcinoma, leading to an aggressive gene expression program and poor clinical prognosis. The transcriptional program can be reversed by pharmacological inhibition of Jumonji enzymes. This inhibition blocks hepatocellular carcinoma progression, providing a novel potential therapeutic strategy.

http://dx.doi.org/10.1016/j.jhep.2019.03.007

Research Article
Cancer

JOURNAL OF HEPATOLOGY

A comprehensive study of epigenetic alterations in hepatocellular carcinoma identifies potential therapeutic targets

Juan Bayo[1], Esteban J. Fiore[1], Luciana M. Dominguez[1], Alejandrina Real[1], Mariana Malvicini[1], Manglio Rizzo[1], Catalina Atorrasagasti[1], Mariana G. García[1], Josepmaria Argemi[2], Elisabeth D. Martinez[3,4], Guillermo D. Mazzolini[1,5,*]

[1]*Gene Therapy Laboratory, Instituto de Investigaciones en Medicina Traslacional, Facultad de Ciencias Biomédicas, CONICET, Universidad Austral, Derqui-Pilar, Argentina;* [2]*Center for Liver Diseases, Pittsburgh Research Center, University of Pittsburgh Medical Center, Pittsburgh, PA, USA;* [3]*Department of Pharmacology, UT Southwestern Medical Center, Dallas, TX, USA;* [4]*Hamon Center for Therapeutic Oncology Research, UT Southwestern Medical Center, Dallas, TX, USA;* [5]*Liver Unit, Hospital Universitario Austral, Derqui-Pilar, Argentina*

Background & Aims: A causal link has recently been established between epigenetic alterations and hepatocarcinogenesis, indicating that epigenetic inhibition may have therapeutic potential. We aimed to identify and target epigenetic modifiers that show molecular alterations in hepatocellular carcinoma (HCC).

Methods: We studied the molecular-clinical correlations of epigenetic modifiers including bromodomains, histone acetyltransferases, lysine methyltransferases and lysine demethylases in HCC using The Cancer Genome Atlas (TCGA) data of 365 patients with HCC. The therapeutic potential of epigenetic inhibitors was evaluated *in vitro* and *in vivo*. RNA sequencing analysis and its correlation with expression and clinical data in the TCGA dataset were used to identify expression programs normalized by Jumonji lysine demethylase (JmjC) inhibitors.

Results: Genetic alterations, aberrant expression, and correlation between tumor expression and poor patient prognosis of epigenetic enzymes are common events in HCC. Epigenetic inhibitors that target bromodomain (JQ-1), lysine methyltransferases (BIX-1294 and LLY-507) and JmjC lysine demethylases (JIB-04, GSK-J4 and SD-70) reduce HCC aggressiveness. The pan-JmjC inhibitor JIB-04 had a potent antitumor effect in tumor bearing mice. HCC cells treated with JmjC inhibitors showed overlapping changes in expression programs related with inhibition of cell proliferation and induction of cell death. JmjC inhibition reverses an aggressive HCC gene expression program that is also altered in patients with HCC. Several genes downregulated by JmjC inhibitors are highly expressed in tumor *vs.* non-tumor parenchyma, and their high expression correlates with a poor prognosis. We identified and validated a 4-gene expression prognostic signature consisting of *CENPA, KIF20A, PLK1,* and *NCAPG*.

Conclusions: The epigenetic alterations identified in HCC can be used to predict prognosis and to define a subgroup of high-risk patients that would potentially benefit from JmjC inhibitor therapy.

Lay summary: In this study, we found that mutations and changes in expression of epigenetic modifiers are common events in human hepatocellular carcinoma, leading to an aggressive gene expression program and poor clinical prognosis. The transcriptional program can be reversed by pharmacological inhibition of Jumonji enzymes. This inhibition blocks hepatocellular carcinoma progression, providing a novel potential therapeutic strategy.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and usually occurs in patients with cirrhosis.[1] HCC is the sixth most frequent solid tumor and the second leading cause of cancer-related death worldwide and, unfortunately, its incidence and mortality are steadily increasing in Western countries.[1] Liver resection, transplantation, and tumor ablation are considered curative options although they are applied in only 30–40% of patients. The multikinase inhibitors sorafenib, as first line therapy, and regorafenib, as second line therapy, have been approved for advanced HCC, yet they have modest impact on patient survival.[1,2] Thus, there is an urgent need for new effective therapies.

Epigenetic mechanisms that affect DNA based processes, such as transcription, DNA repair and replication through changes in the chromatin conformation and ultimately in the cell state are common in human cancers.[3] Among epigenetic mechanisms, histone post-translational modifications (HPTMs) are a set generally reversible marks including phosphorylation, acetylation, methylation and Ubiquitination.[3] In particular, histone acetylation and methylation have emerged as key regulators of gene transcription that can dynamically modify gene expression.[4] The governance of chromatin structure through changes in HPTMs involves the action of writers, readers and erasers. Writers, including histone acetyltransferases (HATs) and lysine methyltransferases (KMTs), are enzymes that add

Keywords: Human hepatocellular carcinoma; Epigenetic; Histone demethylases; Lysine demethylases; Jumonji C demethylases; Histone methyltransferases; Histone acetyltransferases; Bromodomains; Epigenetic inhibitors; Gene expression signature; Patient survival.
Received 6 July 2018; received in revised form 26 February 2019; accepted 6 March 2019; available online 15 March 2019

* Corresponding author. Address: Gene Therapy Laboratory, Instituto de Investigaciones en Medicina Traslacional, Facultad de Ciencias Biomédicas, CONICET-Universidad Austral, Av. Pte. Perón1500 (B1629AHJ) Derqui-Pilar, Buenos Aires, Argentina. Tel.: +54 2304387408.
E-mail address: gmazzoli@austral.edu.ar (G.D. Mazzolini).

the post-translational modifications. On the other hand, erasers such as lysine demethylases (KDMs) and histone deacetylases (HDACs) remove the HPTMs. Finally, readers are a set of proteins that recognize specific HPTMs allowing the binding of other proteins to specific chromatin locations.[5] Among readers, bromodomains (BRDs) recognize chromatin regions that have acetylated histones.[5]

Cancer hallmarks are a set of modifications acquired by cancer cells during tumor growth and development;[6] it has been reported that epigenetic plasticity can affect many of these cancer cell traits. For instance, changes to DNA packaging allow tumor cells to resist cell death through tumor suppressor silencing or oncogene activation. Similarly, changes in gene expression patterns allow invasion and metastasis via epithelial-mesenchymal transition.[6] In human HCC, it has been reported that several lysine methyltransferases (*EZH-2, SETDB1* and *EHMT2/G9a*) and lysine demethylases (*KDM3A, KDM4B, KDM5B* and *KDM1A*) are frequently upregulated.[7–13] In addition, high levels of these enzymes in HCC have been associated with poor prognosis in patients, and their knockdown decreased HCC cell proliferation and tumorigenicity in experimental models.[7–13] Thus, given the importance of HPTMs on HCC development, growth and metastasis, pharmacologic inhibition of these epigenetic pathways could emerge as a new therapeutic strategy.[5] Recently, 2 HDAC inhibitors, belinostat and reminostat, have been tested in phase I/II clinical trials in patients with HCC, showing that these targeted therapies have potential efficacy.[14]

In recent years, strong efforts have been made to develop new generation epigenetic inhibitors using innovative technologies to target the epigenetic enzymes involved in cancer growth and progression.[5,15–31] Herein, we sought to comprehensively investigate alterations in epigenetic readers, writers and erasers in HCC and to preclinically assess the therapeutic potential of their pharmacological inhibition.

Materials and methods

TCGA analysis

Clinical information, gene expression and mutations data (RNA sequencing [RNA-Seq]) for the indicated genes were obtained from The Cancer Genome Atlas (TCGA) for patients with HCC (n = 365) using the FIREBROWSE portal (http://firebrowse.org). Overall survival of high and low expression groups was compared with the Log-rank test using GraphPad prism program. For tumor (T) *vs.* adjacent non-tumoral (NT) tissue comparison, a sub-cohort of 50 cases of paired samples from both types of tissues were used to calculate the individual fold changes.

Cell viability assay

Standard MTS assays were performed on cells treated during 4 days with increasing doses of epigenetic inhibitors. Dose response curves were plotted using a non-linear regression model and IC_{50} values were determined from the fitted curves using GraphPad prism.

Cell cycle and apoptosis assays

Floy cytometry (BD FACSCalibur) was used to analyze the cell cycle (with propidium iodide) and apoptosis (with Annexin-V).

In vivo experiment

Animal experiments were carried out under approved IACUC protocols (protocol number: APN-2017-001) and followed the requirement of the state authority for animal care procedures. Animals were housed in open polycarbonate cages with standard mouse chow and a water bottle. The mice were maintained in a temperature controlled environment under a 12 h/12 h light/dark cycle. Six-to-eight-week-old male C3H/HeJ mice were purchased from Comisión Nacional de Energía Atómica, Ezeiza, Buenos Aires, Argentina. Orthotopic tumors were established by subcapsular inoculation of 1.25×10^5 Hepa129 cells into the left liver lobe of C3H/HeJ mice by laparotomy. Seven days after tumor implantations a group of mice received JIB-04 (at indicated doses) or vehicle intraperitoneally or by oral gavage. Mice were sacrificed at 2 weeks and tumor size and number of HCC satellites were evaluated.

Histone post-translational modifications analysis

HuH7 were treated with JIB-04, GSK-J4, SD-70 (2 times IC_{50} concentration) or DMSO for 24 h. Then, cells were harvested, histones extracted and the levels of different HPTMs were evaluated using the EpiQuik Histone H3 Modification Multiplex Assay Kit (cat# P-3100-96) and western blot.

Histone demethylase activity assay

For histone demethylase activity determination, HuH7 cells treated as above were harvested, cell extracts prepared, and demethylase activity assayed using the Epigentek kit P-3081 for H3K9me3 demethylation or P-3084 for H3K27me3 demethylation.

Transcriptome analysis

The RNA-Seq analysis was performed on HuH7 cells treated as above. Genes were considered differentially expressed when log2 (fold change) was greater than 0.3 or lower than −0.3 with a false discovery rate (FDR) <0.05. Gene ontology and pathway analysis were done using the ToppGene suite.[32] Results were validated by quantitative reverse transcription PCR (qRT-PCR) in Hep3B, SK-Hep-1 and PLC/PRF/5 cell lines.

Hierarchical clustering

Using our gene signature (*CENPA*, *KIF20A*, *NCAPG* and *PLK1*), unsupervised hierarchical clustering was performed to segregate the TCGA and the GSE14520 cohorts of patients with HCC using the "R" software.

Statistical analyses

Statistical analysis was performed using IBM SPSS version 17 and PRISM 6.0 software package. p <0.05 was considered as statistically significant.

For further details regarding the materials used, please refer to the CTAT table and supplementary information.

Results

Identification of epigenetic modifiers with altered expression in human HCC

Epigenetic reprogramming is considered a key event during carcinogenesis and, therefore, a target for cancer therapy.[5] We wondered whether epigenetic modifier alterations are common events during hepatocarcinogenesis and whether they could be a therapeutic target for HCC. To address this, we analyzed HCC data (n = 365) from TCGA to determine the mutation frequencies of BRDs (n = 8), HATs (n = 18), KMTs (n = 35) and KDMs (n = 29). Here we report that 75% of patients present a somatic

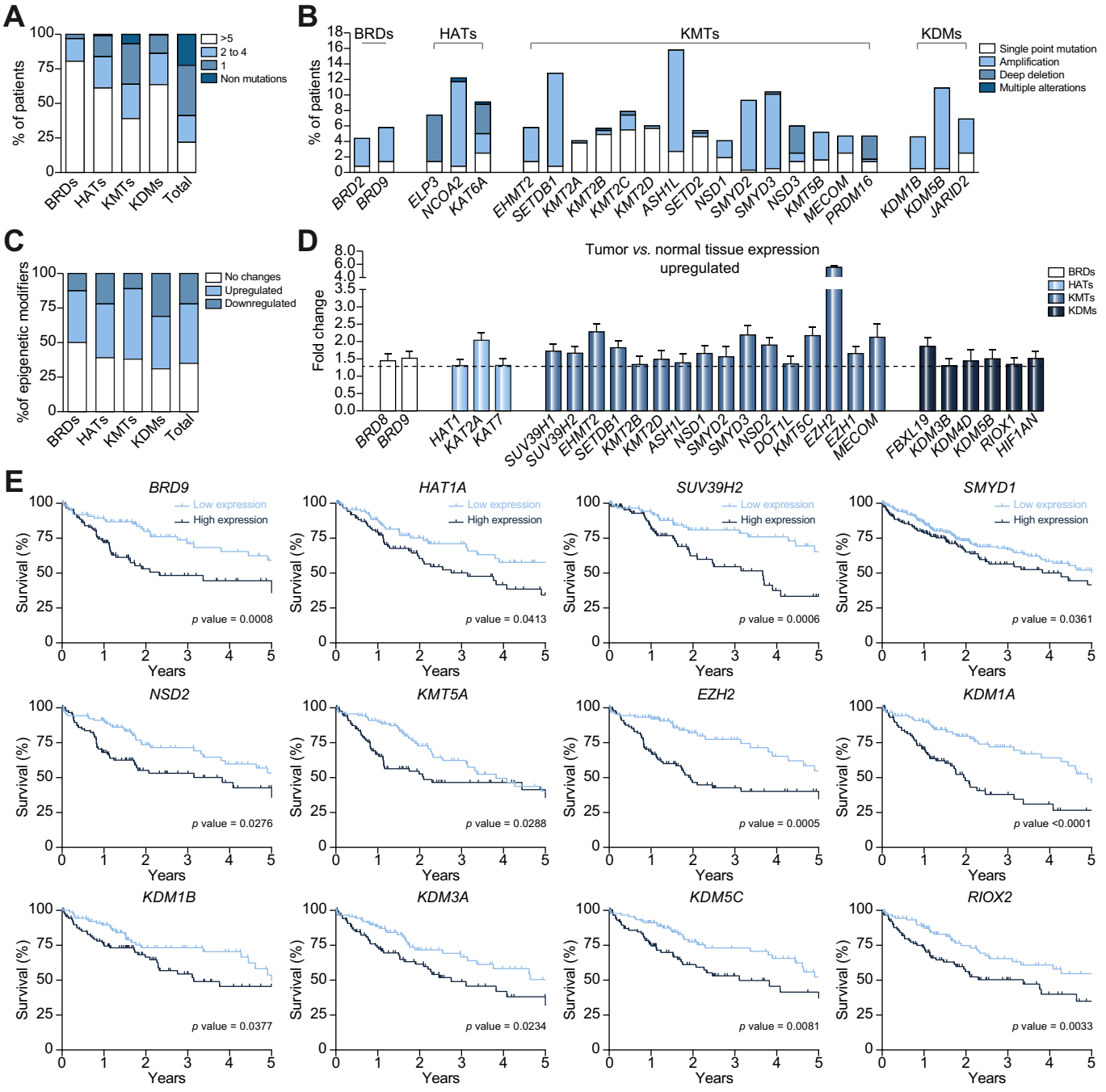


Fig. 1. Alteration of epigenetic modifiers is a common event in HCC and their expression correlates with poor patient prognosis in TCGA. A) Somatic mutation data of patients with HCC (TCGA data, n = 365) was analyzed and the percentage of patients with 1, 2–4 or >5 genes mutated in the BRD, HAT, KMT or KDM families individually or altogether (Total) are shown. B) Epigenetic modifiers (BRDs, HATs, KMTs and KDMs) that present mutations including genetic amplification, genetic deletion, single point mutation (missense mutations and truncating mutations) and multiple alterations (combination of above described) in more than 4% of the patients. C-D) HCC RNA-Seq data of TCGA was analyzed and upregulated or downregulated gene expression of epigenetic modifiers (paired *t* test, $p < 0.05$) between matched samples of tumor and non-tumor adjacent tissue (n = 50) were determined. C) Graph represent % of BRDs, HATs, KMTs, KDMs families or all together (Total) that are up-, downregulated or did not present changes when tumor *vs.* non-tumor expression was compared. D) Epigenetic modifiers that present a fold change higher than 1.3 and a $p < 0.001$ (paired *t* test) when evaluated in matched tumor *vs.* non-tumor patient samples. E) Patients with high expression of *BRD9*; *HAT1*; KMTs including: *SUV39H2, SMYD1, NSD2, KMT5A, EZH-2*; and KMDs including: *KDM1A, KDM1B, KDM3A, KDM5C* and *RIOX2*, analyzed by RNA-Seq (TCGA data, n = 365) have significantly worse prognosis than those expressing low levels. High (n = 92) *vs.* low (n = 92) levels were defined by top and bottom quartiles, as described in Methods. The association between expression in HCC and the survival time of selected patients was analyzed with Kaplan-Meier survival analysis and *p* values (Cox-regression) are indicated in the fig. BRD, bromodomains; HAT, histone acetyl transferases; HCC, hepatocellular carcinoma; KDM, lysine demethylases; KMT, lysine methyl transferases; RNA-Seq, RNA sequencing; TCGA, The Cancer Genome Atlas.

mutation in at least 1 of the epigenetic modifiers studied. Furthermore, 20% of them have more than 5 epigenetic modifiers mutated (Fig. 1A). In addition, we observed that the frequency of patients with HCC and at least 1 somatic mutation is 20% for BRDs, 38% for HATs, 57% for KMTs, and 35% for KDMs (Fig. 1A). Analyzing individual modifiers, 23 of the 90 epigenetic modifiers

studied are mutated in at least 4% of patients with HCC (Fig. 1B). Among them, the genes *BRD9, NCOA2, SETDB1, ASH1L, SMYD2, SMYD3,* and *KDM5B* are highly amplified in patients with HCC. We then determined the relationship between the presence of mutations in the epigenetic modifiers studied here and clinical-pathological features such as alpha-fetoprotein (AFP levels: high >100, low <100), HCC risk factors, cirrhosis, and massive macrotrabecular architecture. It should be noted that the presence of genetic lesions in any of the 90 epigenetic modifiers and the clinical-pathological features are independent (Table S1). Similarly, there is no significant correlation between mutations in most of the epigenetic modifiers studied and largely representative HCC mutations such as *TP53, CTNNB1, TERT, AXIN1, HNF1A, RPSKA3, PIK3CA, ATM, FGF9* and *TSC1/TSC2*.[33] Interestingly, we found that several epigenetic modifier mutations co-segregate with other HCC mutations (Table S1). It could be hypothesized that this co-segregation is due to the close proximity of the genes in the chromosome. For instance, we found a co-segregation between mutations of: *BRD9* with *TERT* both located at chromosome 5p15.33 region; *CREBBP* with *AXIN1* both located at chromosome 16p13.3 region; either *PHF8* (located at chromosome Xp11.22 region) or *KDM6A* (located at chromosome Xp11.3 region) with *RPS6KA3* (located at Chromosome Xp22.12 region); and *KDM2A* (located at chromosome 11q13.2 region), and *FGF19* (located at chromosome 11q13.3 region). Meanwhile, we also found 2 examples where the mutations co-segregate but are on different chromosomes. These cases are *FGF19* and *KDM7A* located on chromosome 11q13.3 region, and chromosome 7q34 region, respectively, and *KDM5C* and *TSC1/TSC2* located on chromosome Xp11.22 region, 16p13.3 region, and 9q34.13 region, respectively.

Then, we investigated whether the events of amplification observed above could correlate with the expression levels of those epigenetic modifiers when comparing T *vs.* NT tissues in matched samples using TCGA RNA-Seq data (Table S1, n = 50). Here we found that 37% of BRDs (3/8), 38% of HATs (7/18), 51% of KMTs (18/35) and 38% of KDMs (11/29) are upregulated in T in comparison with matched NT tissue (Table S1, Fig. 1C). Moreover, when analyzed in aggregate 43% and 22% of the epigenetic modifiers are upregulated or downregulated, respectively. Particularly, among the genetically amplified epigenetic modifiers, *BRD9, EHMT-2, ASH1L, SMYD2, SMYD3,* and *KDM5B* were upregulated in HCC (Table S1, Fig. 1C). Nevertheless, it should be noted that most of the cases that have T and NT paired samples do not have genetic alterations in these genes, indicating that the main driver of the deregulated expression is not genetic. (Fig. S1A). Furthermore, *BRD8, HAT1A, KAT2A, SUV39H1, SUV39H2, SETDB1, KMT2B, KMT2D, NSD1, NSD2, DOT1L, KMT5C, EZH2, EZH1, MECOM, FBXL19, KDM3B, KDM4D, RIOX1, HIF1AN* are enriched in T *vs.* NT tissue by at least 1.3-fold in patients with HCC (Fig. 1D). Importantly, BRD9, EHMT-2, SMYD2, EZH-2 and KDM5B are potentially druggable targets for which pharmacological inhibitors have been developed (Table S2).

Taking these results into consideration, we wondered whether the expression levels of these upregulated genes correlated with the clinical prognosis of patients (n = 365). Previously, it has been reported that patients with high tumor expression levels of *EZH-2, EHMT2/G9a, KDM3A, KDM4B,* and *KDM1A* have a significantly worst prognosis. Here we found that in addition, higher levels of *BRD9, HAT1A*; the KMTs: *SUV39H2, SMYD1, NSD2 and KMT5A*; and the KDMs: *KDM1A, KDM1B, KDM3A, KDM5C* and *RIOX2* correlated with poor prognosis among patients with HCC (Table S1, Fig. 1E). However, we did not confirm previous reports for *KDM5B* and *SETDB1* (Fig. S1B). These analyses also showed that high expression of the KDMs, *KDM8* and *PHF8*, which are downregulated in T *vs.* NT tissue (Table S1), correlate with a better prognosis (Fig. S1C). Interestingly, epigenetic inhibitors are commercially available for several of these KDMs (Table S2). Taken together, these results could define subgroups of patients that may benefit from therapeutic epigenetic modulation with different families of inhibitors, if tumors are actually driven by these modifiers.

Epigenetic compounds have antitumor effects against HCC *in vitro*

We first tested the antiproliferative activity of a set of epigenetic small-molecules targeting the epigenetic modifiers described on a panel of HCC cell lines (Table S2). High to moderate antiproliferative effect after 4 days of drug exposure was observed for the inhibitors of the Jumonji C family of KDMs (JmjCs) JIB-04, GSK-J4, SD-70 and ML-324; the KMT inhibitors BIX-1294 and LLY-507; and the BRD4 inhibitor JQ-1 (Fig. 2A, Fig. S2, Table S3). Interestingly, the range of IC_{50} values for these inhibitors were similar for those observed for belinostat, an HDAC inhibitor already tested in patients with advanced HCC (Fig. 2A, Fig. S2, Table S3).[14] Meanwhile, middle to low antiproliferative activity was observed for another set of JmjC inhibitors (PBIT, CPI-455, NCDM-32B and KDM5-C70), KDM1A demethylase inhibitors (GSK-LSD1 and GSK-2879552), EZH-2 methyltransferase inhibitors (EPZ-6438 and CPI-1205), bromodomain inhibitors (BI-9654 and BI-7273), the SMARCA bromodomain inhibitor (PFI3), and the WDR5/KMT2A interaction inhibitor OICR-9429 (Fig. 2A, Fig. S2, Table S3). We next evaluated if the presence of mutations or differences in the expression levels of the targets of the inhibitors would explain the difference observed in the IC_{50} values across HCC lines. To test this, genetic and expression data of HuH7, Hep3B, HepG2, SK-Hep-1 and PLC/PRF/5 cells were obtained from the Cancer Cell Line Encyclopedia.[34] We observed that *KDM2A* is amplified in HuH7 and Hep3B cells; *KDM4C* is deleted in PLC/PRF/5 cells, and that *RIOX1* is deleted in HepG2 cells. The analysis of the expression levels showed that there are no significant differences in the expression of the different targets of our panel of epigenetic inhibitors (Fig. S3A). Furthermore, we also correlated the IC_{50} values of the inhibitors with the expression of their respective targets (Table S4). This analysis showed significant correlation only between the expression of *KDM3B* and *KDM5B*, and the IC_{50} values for JIB-04 and PBIT, respectively (Fig. S3B). Strikingly, in both cases the correlation was inversely proportional, suggesting that tumors with high expression of these 2 epigenetic modifiers will potentially be more sensitive to JIB-04 or PBIT inhibitors. These results are encouraging since both *KDM3B* and *KDM5B* are overexpressed in T *vs.* NT tissues (Fig. 1D).

In vitro characterization of the antitumoral effect of KDM and KMT inhibitors on HCC

To study the mechanisms involved in the anticancer activity of the most effective inhibitors, we evaluated their effect on cell cycle progression and apoptosis. Cell cycle analysis by FACs revealed that while BIX-01294 and JQ-1 induce a cell cycle arrest in G1 phase, LLY-507 arrests the cells in S/G2 phases (Fig. 2B and Fig. S4A). JmjC inhibitors induced a consistent cell cycle arrest in the G1/S interphase in different HCC cell lines

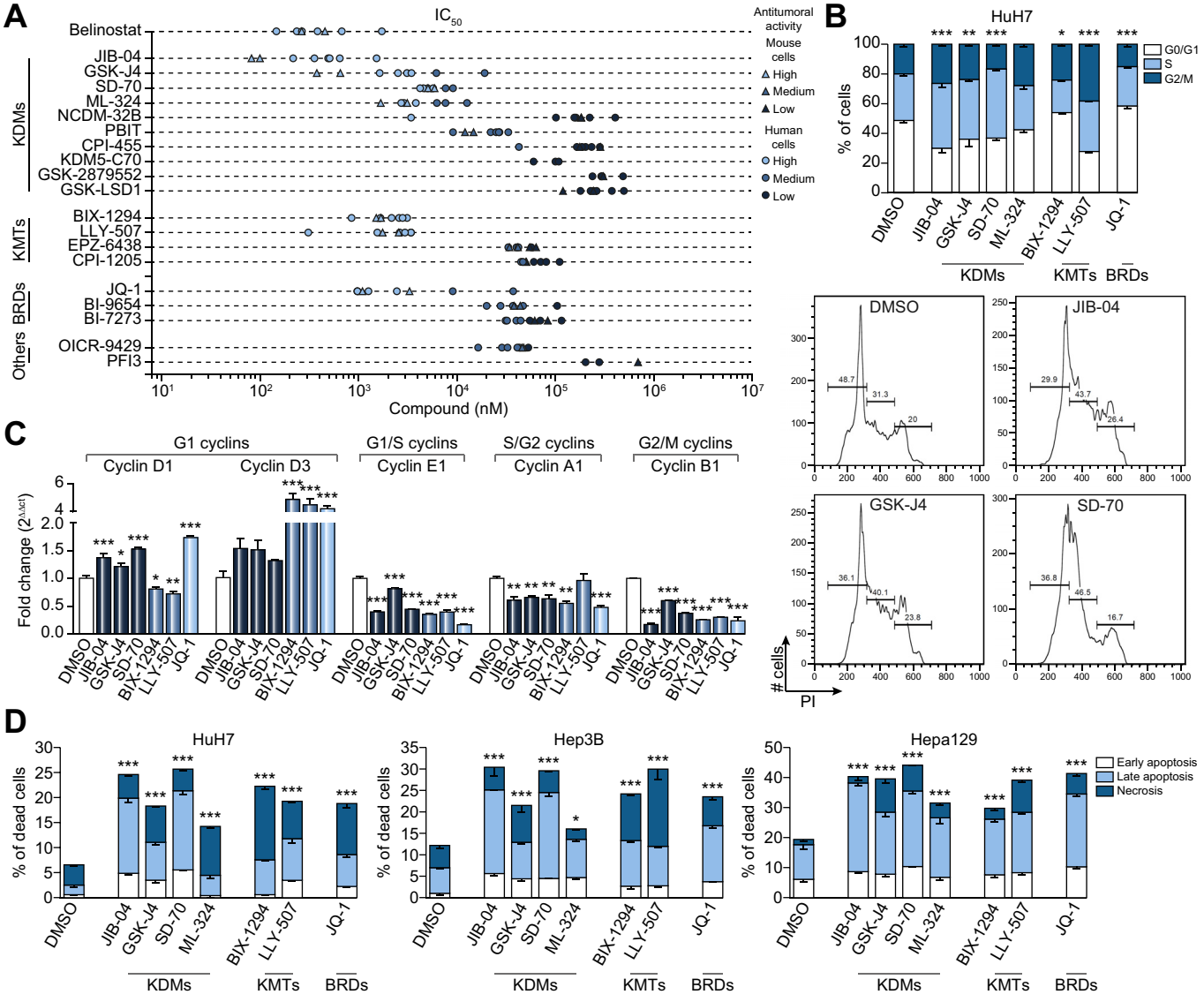


Fig. 2. Inhibitors of KDMs, KMTs, and BRDs potently decrease HCC cell survival *in vitro* by inducing cell cycle arrest and apoptosis. A) Log scale representation of cell viability IC_{50} values comparing the sensitivities of mouse (Hepa129 and BNL, ▲) and human (HuH7, Hep3B, HepG2, SK-Hep1, PLC/PRF/5 and FOCUS, ●) HCC cell lines (each dot represents one cell line) treated with a set of epigenetic inhibitors (indicated in panels) over 4 days evaluated by standard MTS assay. IC_{50} values were determined by averaging the values obtained from the fitted curves using GraphPad prism of 2-3 independent assays, each containing 4 replicates. B) HuH7 cell cycle arrest induced by inhibitors of JmjC-KDMs (JIB-04, GSK-J4, SD-70 and ML-324), EHMT2 (BIX-1294), SMYD2 (LLY-507) or BRD4 (JQ-1) after 24 h of treatment. Cells were treated with 2 × IC_{50} concentration. Bars represent % ± SEM (n = 3) of cells that are in G0/G1 phase, S phase or G2/M phase. $^{*}p$ <0.05, $^{**}p$ <0.01 and $^{***}p$ <0.001 *vs.* DMSO, by Kruskal-Wallis. Representative histograms of HuH7 cells treated with DMSO, JIB-04, GSK-J4 or SD-70. C) mRNA expression levels of cyclins determined by qRT-PCR in HuH7 cells treated as in B. Bars represent mean ± SEM. $^{*}p$ <0.05, $^{**}p$ <0.01 and $^{***}p$ <0.001 *vs.* DMSO, by ANOVA. D) HCC cell death induced by epigenetic inhibitors as in B. Bars represent % ± SEM (n = 3) of cells that are in early apoptosis, late apoptosis or necrosis. $^{*}p$ <0.05 and $^{***}p$ <0.001 *vs.* DMSO, by Kruskal-Wallis. BRD, bromodomains; HCC, hepatocellular carcinoma; KDM, lysine demethylases; KMT, lysine methyl transferases; qRT-PCR, quantitative reverse transcription PCR.

(Fig. 2B and Fig. S4B). To complement these studies, we evaluated the expression levels of a set of cyclin genes after treatment with the epigenetic inhibitors. HCC cells treated with JmjC inhibitors, BIX-1294 or JQ-1 express high levels of G1 cyclins, and low levels of cyclins that regulate the progression through the other cell cycle phases (Fig. 2C, Fig. S4C). LLY-507 treatment results in reduced levels of multiple cyclins, yet S to G2 transition cyclins are unaltered and cells accumulate at this transition. In addition, Annexin-V FITC staining showed that this subset of epigenetic inhibitors was able to induce apoptosis in HCC cells after 24 h of exposure (Fig. 2D, Fig. S5A-C). Since inhibitors of JmjC demethylases showed consistently low IC_{50} and were able to induce a strong pro-apoptotic effect and cell cycle arrest *in vitro,* we further characterized the anticancer mechanisms of action of JIB-04, GSK-J4 and SD-70 epigenetically and transcriptionally in HCC cells.

JmjC inhibitor-treated HCC cells exhibit robust changes in histone 3 global methylation levels

Recently, it was demonstrated that cell death induced by genotoxic compounds, such as doxorubicin or paclitaxel, leads to an increase in H3K27me3, H3K4me3 and H3K9me2 and a decrease in global acetylation.[35] To assess if the anticancer effect of JmjC inhibitors was on-target, we investigated differences in global

post-translational modification after treatment with JIB-04, GSK-J4 and SD-70 in HuH7 cells by ELISA (24 h treatment, Fig. 3A) or western blot (24 h and 48 h treatment, Fig. 3B). Both JIB-04 and SD-70 treated cells showed increased levels of H3K4me3, H3K9me3, H3K27me3 and H3K36me3. GSK-J4 treated cells accumulated H3K27me3 and all 3 inhibitors decreased H3K27ac levels. No changes were observed in the levels of acetylation of lysines that are not modified by methylation, such as H3K14 and H3K56 (Fig. 3A). To corroborate that the increase in levels of histone methylation was due to JmjC inhibition we measured H3K9me3 and H3K27me3 enzymatic demethylase activity in treated cell lysates on exogenous histone substrates. As observed in Fig. 3C, H3K9me3 demethylase activity was decreased by 24 h or 48 h treatment with JIB-04 or SD-70 but not by GSK-J4 treatment. H3K27me3 demethylase activity was blocked by all 3 JmjC inhibitors (Fig. 3D). Similar results were observed in Hep3B cells (Fig. S6). Taken together, these results indicate that the increased histone methylation observed is the direct result of inhibition of JmjC demethylases.

JmjC inhibitors reverse an aggressive gene transcription program of cell proliferation, cell death evasion and WNT pathway signaling in HCC

To investigate the transcriptional changes induced by the inhibition of JmjC activity leading to cell cycle arrest and induction of apoptosis we performed global gene expression profiling in HuH7 HCC cells by RNA-Seq. After 24 h of treatment, we identified 3,358 genes with log2(fold change) of 0.3 or more triggered by JIB-04, 1,693 by GSK-J4 and 2,842 by SD70 (p <0.05, Table S5). In addition, 3,708, 2,018 and 3,089 genes were downregulated by these compounds, respectively (log2(fold change) <−0.3, p <0.05, Fig. S7A, Table S5). Comparison of the differentially expressed genes (DEGs) in HuH7 cells treated with JIB-04, GSK-J4 and SD-70, showed that the 3 inhibitors induced overlapping gene expression signatures (Fig. S7B, Table S5). Consistent with their effect on cell cycle, gene ontology functional enrichment (GO) analysis indicated that 205 of these DEGs modulated either by JIB-04, GSK-J4 or SD70 are related to cell cycle progression (Fig. S7B). Likewise, pathway enrichment analysis showed that genes related to gene expression, the cell cycle, and mitosis were also modulated by the 3 inhibitors (Fig. S7B). Analysis of overlapping genes showed that 904 genes were commonly depleted, and 728 genes were commonly enriched, after exposure to these JmjC inhibitors (Fig. 4A, Table S5). In addition, 1,730 downregulated genes and 1,684 upregulated genes were commonly modulated by JIB-04 and SD-70 but not by GSK-J4. GO analysis showed that downregulated common genes were related to "positive regulation of cell proliferation", "negative regulation of apoptosis" and "WNT pathway" among other GO terms (Fig. 4A, Table S5). The upregulated common DEGs included GO terms such as "apoptotic process", "positive regulation of cell death", "positive regulation of programed cell death" and "negative regulation of cell proliferation" (Fig. 4A, Table S5). To gain insight into the role of these

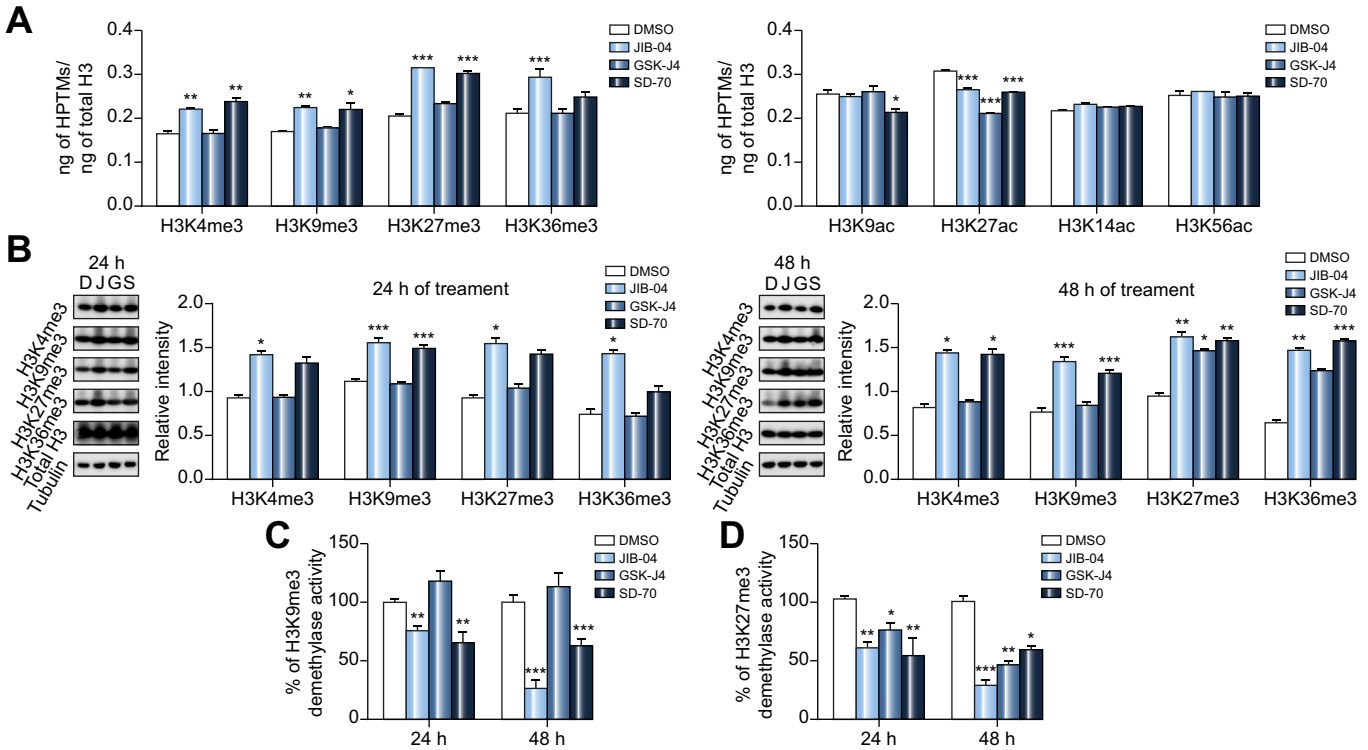
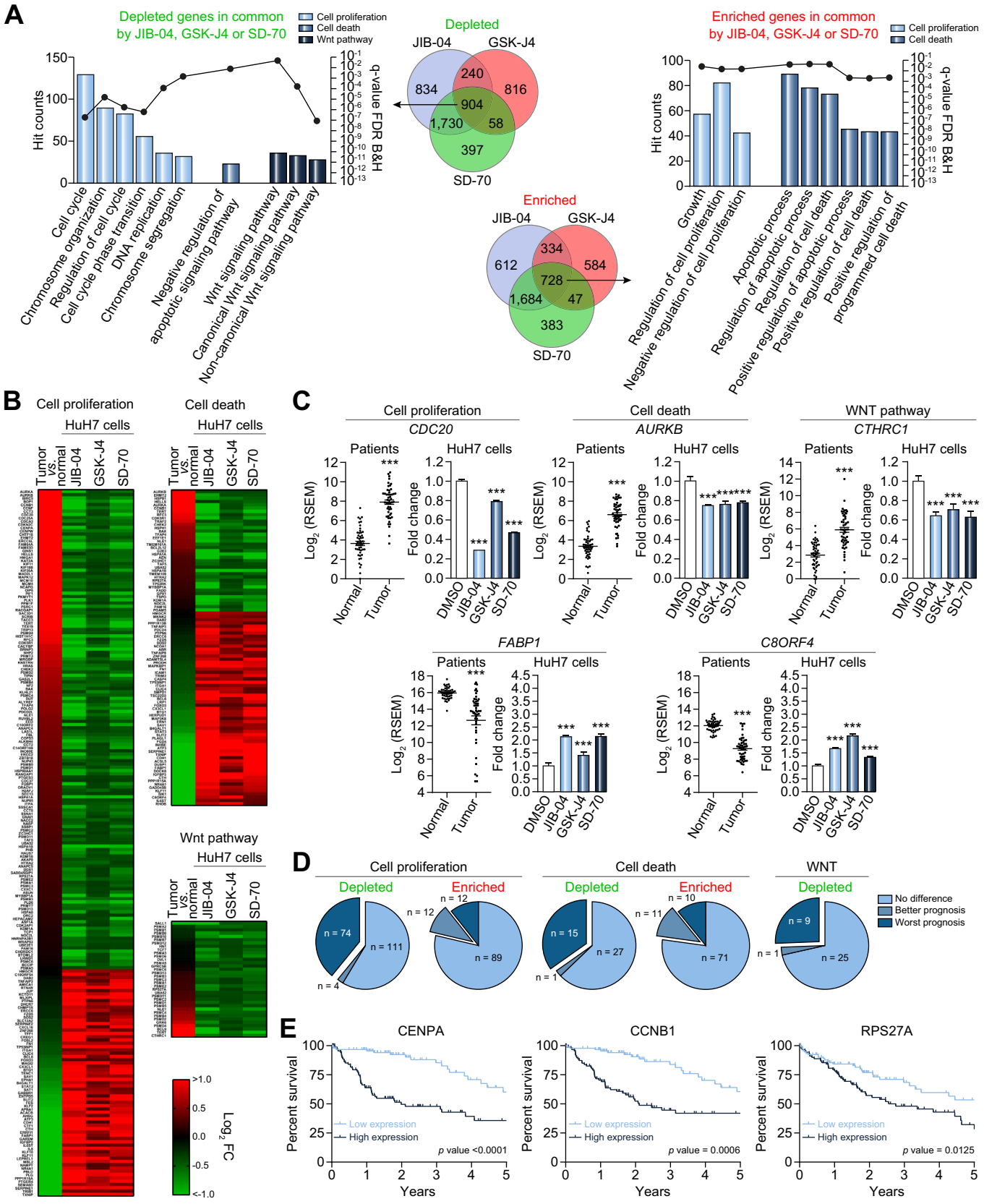


Fig. 3. JmjC inhibitors induce global changes in HPTMs through specific impairment of demethylase activity. A) ELISA analysis for different HPTMs of histone extracts from HuH7 cells treated for 24 h with JIB-04, GSK-J4 or SD-70 at 2 times the IC_{50} doses. Bars represent the average ratios of HPTMs ng and total H3 ng ± SEM. *p <0.05, **p <0.01 and *p <0.001 *vs.* DMSO (Kruskal-Wallis). B) Western blot validation of H3K4me3, H3K9me3, H3K27me3 and H3K36me3 levels in HuH7 cells treated during 24 or 48 h with the different JmjC-KDM inhibitors. D (DMSO, control), J (JIB-04), G (GSK-J4) and S (SD-70). The immunoblot data from 3 independent experiments were quantified and expressed as the average ratio HPTMs signal/total H3 + SEM. *p <0.05 and ***p <0.001 *vs.* DMSO, by Kruskal-Wallis. C-D) Demethylase activity of H3K9me3 (C) and H3K27me3 (D) measured on HuH7 cells treated during 24 or 48 h with JmjC-KDM inhibitors. Values are expressed as % ± SEM of DMSO-treated cells in 3 independent experiments. *p <0.05, **p <0.01 and ***p <0.001 *vs.* DMSO (Kruskal-Wallis). H3, histone 3; HPTMs, histone post-translational modifications; KDM, lysine demethylases.


A
Depleted genes in common by JIB-04, GSK-J4 or SD-70
Cell proliferation
Cell death
Wnt pathway
Hit counts
q-value FDR B&H
Cell cycle
Chromosome organization
Regulation of cell cycle
Cell cycle phase transition
DNA replication
Chromosome segregation
Negative regulation of apoptotic signaling pathway
Wnt signaling pathway
Canonical Wnt signaling pathway
Non-canonical Wnt signaling pathway
Depleted
JIB-04
GSK-J4
SD-70
834
240
816
904
1,730
58
397
Enriched
612
334
584
728
1,684
47
383
Enriched genes in common by JIB-04, GSK-J4 or SD-70
Growth
Regulation of cell proliferation
Negative regulation of cell proliferation
Apoptotic process
Regulation of apoptotic process
Regulation of cell death
Positive regulation of apoptotic process
Positive regulation of cell death
Positive regulation of programmed cell death
B
Cell proliferation
HuH7 cells
Cell death
Wnt pathway
Tumor vs. normal
JIB-04
GSK-J4
SD-70
Log2 FC
C
Cell proliferation
Cell death
WNT pathway
CDC20
AURKB
CTHRC1
FABP1
C8ORF4
Patients
HuH7 cells
Log2 (RSEM)
Fold change
Normal
Tumor
DMSO
D
Cell proliferation
Cell death
WNT
Depleted
Enriched
n = 74
n = 111
n = 4
n = 12
n = 89
n = 15
n = 27
n = 1
n = 10
n = 71
n = 9
n = 25
No difference
Better prognosis
Worst prognosis
E
CENPA
CCNB1
RPS27A
Percent survival
Low expression
High expression
p value <0.0001
p value = 0.0006
p value = 0.0125
Years

genes in the development and progression of HCC, we investigated if the DEGs modulated by either JIB-04, GSK-J4 or SD-70 are part of an expression program required by HCC. We compared the expression of these genes between NT and T in patients with HCC using TCGA data. It should be noted that among the genes commonly depleted by JmjC inhibitors, 55% (104/189) of cell proliferation genes, 46% (20/43) of cell death genes and 35% (12/35) of WNT pathway genes were upregulated in T *vs.* NT samples (Fig. 4B, Table S6). Likewise, the analysis of the commonly enriched genes by JmjC inhibitors showed that 44% (50/113) of the negative regulation of cell proliferation genes and 49% (45/92) of cell death DEGs were downregulated in T *vs.* NT tissue (Fig. 4B, Table S6). Examples of these genes are shown in Fig. 4C. These data indicate that JmjC inhibitors can partially normalize the aggressive gene expression pattern characteristic of HCC tissue. We also found a small group of genes associated with "cell proliferation" or "cell death" GO terms that were down- or upregulated, respectively, in both HCC treated cells and human HCC samples (Fig. S8A, Table S6). GO analysis of these subgroup of genes indicates an anti-proliferative and pro-apoptotic phenotype (Fig. S8B). To validate the results observed by RNA-Seq on HuH7 cell line, we performed qRT-PCR of 9 representative genes in Hep3B, SK-Hep-1, and PLC/PRF/5 cells treated with the JmjC inhibitors and observed similar gene expression profiles. These results confirm that JmjC inhibitors generally reverse malignant HCC transcriptional patterns (Fig. S9).

Next, we evaluated if the common DEGs correlate with the prognosis of patients with HCC. This analysis showed that high expression of 39% (74/189) of cell proliferation genes, 35% (15/43) of anti-cell death genes and 25% (9/35) of WNT pathway genes, depleted by inhibitor treatment, correlated with a worst prognosis in patients with HCC (Fig. 4D, Table S6). In addition, high expression of JmjC inhibitor enhanced genes correlates with a better prognosis (Fig. 4D, Table S6). Fig. 4E shows representative genes that correlate with poor patient prognosis when highly expressed and that are commonly decreased by the inhibitors.

A 4-gene signature identifies a group of patients with poor prognosis that might potentially benefit from JmjC inhibitor therapy

Considering the strong modulatory effect achieved of JmjC inhibitors on HCC cells we wondered whether we could identify a group of patients who could eventually benefit from treatment with these drugs. Fifty-eight genes, which are upregulated in HCC and whose high expression correlates with poor prognosis, were depleted by JmjC inhibitors (Fig. 5A). Meanwhile, 18 genes, which are downregulated in HCC and whose low expression correlates with poor prognosis, were enriched after JmjC inhibition (Fig. 5B). Further stringent filtering identified the genes that were altered in HCC T *vs.* NT tissues with the most significant hazard ratios. This allowed us to identify a 4-gene signature composed of *CENPA, KIF20A, PLK1* and *NCAPG*. Based on our 4-gene signature, unsupervised hierarchical clustering separated the patients with HCC into 4 major groups that have increased expression levels of *KIF20A, CENPA, PLK1* and *NCAPG* (Group 1 < Group 2 < Group 3 < Group 4) (Fig. 5C, Table S7). There is no clear correlation between amplifications in any 1 of the genes (*CENPA*, *KIF20A, PLK1* and *NCAPG*) and the 4 subgroups of patients ($p = 0.1239$ by Fisher's exact test). This raises the possibility that the differences observed in *CENPA, KIF20A, PLK1,* or *NCAPG* expression levels between the different groups could be due to epigenetic deregulation. Importantly, our signature defines a group with good prognosis, with a median survival of 5.8 years (Group 1), and a group with poor prognosis, with a median survival of 0.95 years (Group 4, Fig. 5D). In addition, the univariate analysis showed that the performance status, TNM staging, positive margin resection, systemic adjuvant treatment and signature groups were all associated with overall survival. However, the multivariate analysis presented the signature groups and performance status as the only independent predictors of overall survival (Table 1 and Table S8).

To further characterize the groups defined by our signature we evaluated correlations within the different groups for clinicopathological and molecular features characteristic of HCC. We found that Group 4 and Group 3 were associated with high levels of AFP, a massive macrotrabecular histoarchitecture, and mutations in TP53 (Table 2). In addition, while Group 4 is associated with mutations of *TSC1/TSC2*, Group 3 is associated with wild-type genotype of *CTNNB1* and *TERT*. On the other hand, Group 1 and Group 2 are associated with low levels of AFP, and while Group 1 is associated with non-alcoholic fatty liver disease (NAFLD), Group 2 does not. At the molecular level, while Group 1 is associated with mutations in *CTNNB1* and *HNF1A* and with wild-type *TP53, TERT, FGF19* and *TSC1/TSC2* status, Group 2 is associated with *TERT* mutations (Table 2).

To validate the 4-gene signature in an independent cohort, we performed clustering analysis using the microarray data from 221 HCC samples from patients chronically infected with hepatitis virus B (HVB) from the GSE14520 dataset.[36] Strikingly, the clinical relevance of this molecular signature is highlighted due its capability to clearly separate patients of the GSE14520 dataset into 2 groups, with good *vs.* poor prognosis

Fig. 4. JmjC inhibitors reverse a pro-proliferative and anti-apoptotic expression program in human HCC cells. A) Venn diagram of down- or upregulated genes (FDR = 0.05, Log2(fold change) = ±0.3) and gene ontology analysis of commonly depleted (left panel) or enriched (right panel) genes in HuH7 after JmjC inhibitor treatments (JIB-04, GSK-J4 or SD-70) during 24 h at 2 × IC_{50} doses. Graph shows number of genes (right axis, column bars) and q-value FDR B&H (Left axis, dots). B) Heatmap of genes regulating "cell proliferation", "cell death" and "WNT pathway" that are differentially expressed in tumor *vs.* non-tumor human samples from TCGA (n = 50) and are normalized in HuH7 cells treated with JmjC inhibitors during 24 h at 2 × IC_{50} doses. C) Top up- or downregulated genes in tumor *vs.* non-tumor tissue (TCGA data) that are normalized by JmjC inhibitors and regulate "cell proliferation" (CDC20 and FABP1), "cell death" (AURKB, negative regulator, and C8ORF4. positive regulator) and "WNT pathway signaling" (CTHRC1). Patient RNA-Seq data is graphed as Log2(RSEM) ± SEM. ***p <0.001 *vs.* normal, paired t test. HuH7 cells data represent the fold change ± SEM obtained from 3 independent biological replicates analyzed by RNA-Seq. ***p <0.001 *vs.* DMSO, FDR by B&H. D) Pie charts showing number of genes depleted or enriched in HuH7 cells by JmjC inhibitor treatment regulating "cell proliferation", "cell death" and "WNT pathway signaling" that correlate with patient prognosis when highly expressed in patients with HCC. E) Representatives Kaplan-Meier survival analysis of the top genes related with regulation of "cell proliferation" (CENPA), "cell death" (CCNB1) and "WNT pathway" (RPS27A) that correlate with a poor clinical prognosis when highly expressed in human HCC (p <0.001). B&H, Benjamini and Hochberg's procedure; FDR, false discovery rate; HCC, hepatocellular carcinoma; RNA-Seq, RNA sequencing; TCGA, The Cancer Genome Atlas.

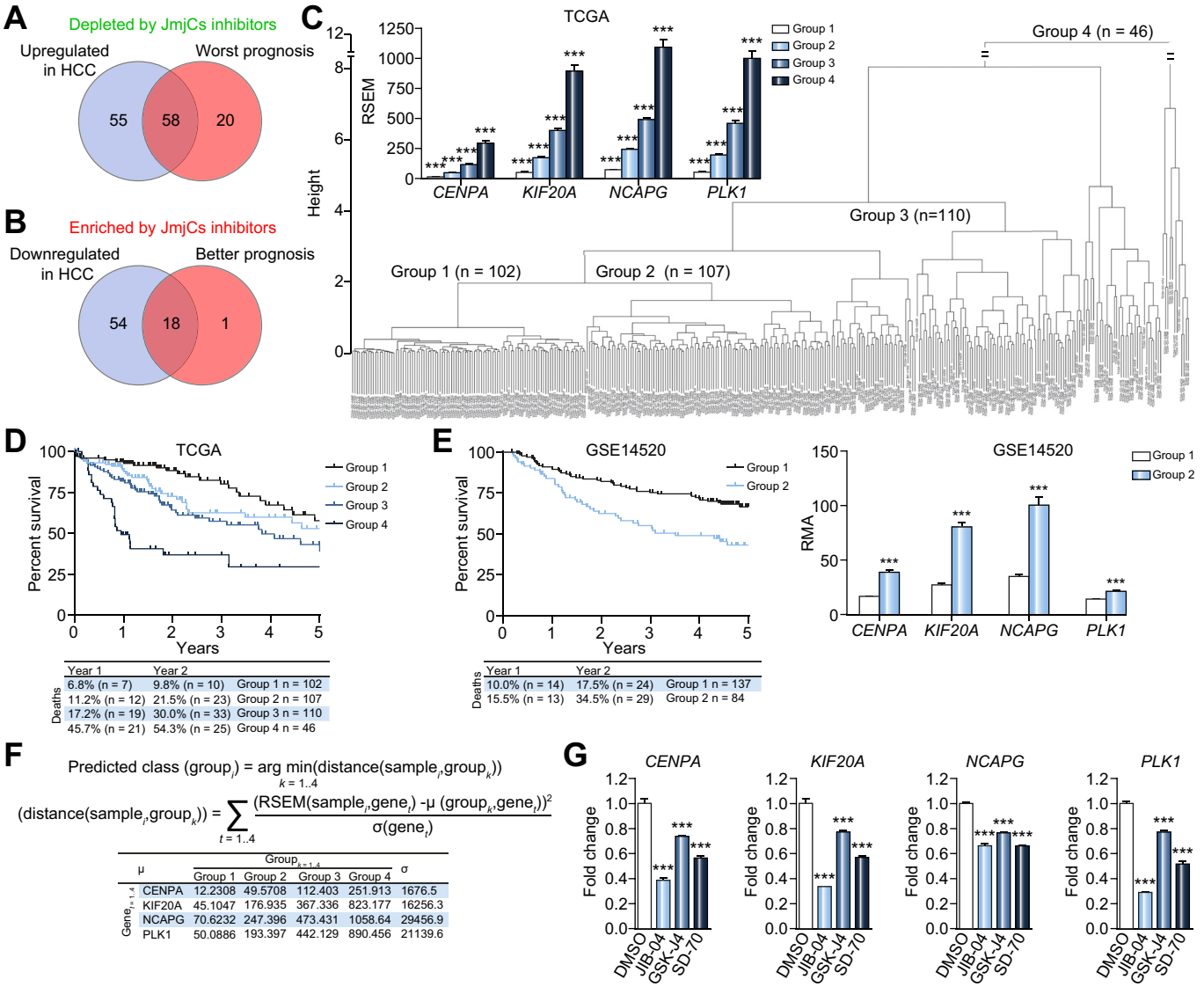


Fig. 5. The gene signature formed by *CENPA*, *KIF20A*, *NCAPG* and *PLK1* defines a group of patients with poor survival that could benefit from JmjC inhibitor therapy. A) Venn diagram showing overlap of genes related with "cell proliferation", "cell death" and "WNT pathway" that are depleted by JmjC inhibitors in HCC cells, are upregulated in tumor *vs.* normal tissues and correlate with a poor prognosis when are highly expressed in tumors. B) Venn diagram showing overlap of genes related with regulation of "cell proliferation", "cell death" and "WNT pathway" that are enriched by JmjC inhibitors in HCC cells, are downregulated in tumor *vs.* normal tissues and correlate with a better prognosis when highly expressed in tumors. C) Using RNA-Seq expression of 4 genes (*CENPA*, *KIF20A, NCAPG* and *PLK1*) obtained from TCGA, unsupervised hierarchical clustering of HCC patients (n = 365) segregated the patients into 4 major groups with increasing expression of *CENPA, KIF20A, NCAPG* and *PLK1*. Graph bar shows average expression levels ± SEM of *CENPA, KIF20A, NCAPG* and *PLK1* genes in the 4 groups of patients identified. ***p <0.001 *vs.* the other groups. D) Kaplan-Meier survival analysis of the 4 groups of patients with HCC revealed significant differences in overall survival (p <0.001). E) Using microarray expression values of the 4 genes signature obtained from the GSE14520 dataset, unsupervised hierarchical clustering of patients with HCC (n = 221) segregated the patients into 2 major groups with increasing expression of *CENPA, KIF20A, NCAPG* and *PLK1*. Kaplan-Meier survival analysis revealed significant differences in overall survival across groups (p <0.001, left panel). Right panel graph bar shows average expression levels ± SEM of *CENPA, KIF20A, NCAPG* and *PLK1* genes in the 2 groups of patients identified. ***p <0.001 *vs.* the Group 1. F) Predictor of HCC classification using the RSEM values obtained by RNA-Seq generated by a Diagonal Linear Discriminant Analysis. The formula used for Group membership prediction and the parameters for each gene and for each Group used in the formula are shown. G) Analysis of RNA-Seq expression data of *CENPA, KIF20A, NCAPG* and *PLK1* in HuH7 cells after JmjC inhibitors treatment during 24 h at twice the IC_{50} doses. Bars represent the fold change ± SEM obtained from 3 independent biological replicates analyzed by RNA-Seq. ***p <0.001 *vs.* DMSO, FDR by Benjamini and Hochberg's procedure. FDR, false discovery rate; HCC, hepatocellular carcinoma; RNA-Seq, RNA sequencing; TCGA, The Cancer Genome Atlas.

(Fig. 5E, Fig. S10A). Analysis of patients with hepatitis B infection in the TCGA database yielded survival curves similar to those obtained for the GSE14520 dataset (Fig. 5E and Fig. S10B-D). Therefore, the differences in the number of groups identified by the gene signature in the TCGA *vs.* GSE14520 datasets is likely driven by different characteristics in these patient populations. While TCGA includes a heterogeneous population representing multiple risk factors including hepatitis B virus, hepatitis C virus and NAFLD, the GSE14520 population is homogenous and formed by patients with hepatitis B virus only. To facilitate the future classification of new patients we defined a gene predictor using a Diagonal Linear Discriminant Analysis (Fig. 5F). The classifier generated here correctly classifies 94.5% of the patients from the TCGA database.

Table 1. Uni- and multivariate analysis of overall survival TCGA and GSE14520 datasets.

Dataset	TCGA							
Analysis type	Univariate analysis				Multivariate analysis*			
Variable	HR	95.0% CI		p value	HR	95.0% CI		p value
		Inferior	Superior			Inferior	Superior	
Sex	1.104			0.293				
Age								
< *vs.* > 65 years	1.933			0.164				
Performance status								
0-1 *vs.* ≥ 2	4.32	2.67	7	<0.001****				0.642
TNM Stage								
Stage I	RV			<0.001****				0.159
Stage II	1.42	0.87	2.31	0.16				0.834
Stage III	2.67	1.75	4.07	<0.0001****				0.084
Vascular invasion								
Negative				0.077	RV			0.007**
Microscopic				0.633	1.348	0.612	2.969	0.458
Macroscopic				0.03*	5.306	1.889	14.905	0.002**
Surgical margin resection				0.327	0.848			0.357
Systemic adjuvant treatment	0.669	0.450	0.994	0.047*	0.076			0.783
Signature								
Group 1	RV			<0.0001****	RV			0.006**
Group 2	1.361	0.803	2.306	0.252	1.641	0.605	4.450	0.331
Group 3	2.001	1.225	3.266	0.006**	2.231	0.882	5.640	0.090
Group 4	4.307	2.468	7.517	<0.0001****	6.930	2.268	21.176	0.001***
Dataset	**GSE14520**							
Analysis type	**Univariate analysis**				**Multivariate analysis***			
Variable	**HR**	**95.0% CI**		**p value**	**HR**	**95.0% CI**		**p value**
		Inferior	**Superior**			**Inferior**	**Superior**	
Sex	2.085			0.149				
Age								
< *vs.* > 65 years	2.153			0.142				
TNM Stage								
Stage I	RV			<0.0001****	RV			<0.0001****
Stage II	2.15	1.24	3.73	0.007**	2.022	1.163	3.516	0.013*
Stage III	5.21	2.97	9.14	<0.0001****	4.701	2.664	8.297	<0.0001****
Signature								
Group 1 *vs.* 2	2.069	1.349	3.174	0.001**	1.825	1.179	2.824	0.007**

RV: reference value; TNM stage: Neoplasm Disease Stage American Joint Committee on Cancer; TCGA (n = 181), GSE14520 (n = 218).

Table 2. Analysis of clinical, biological and histological features for the 4-gene signature identified groups.

Feature	p value	B&H	Group 1		Group 2		Group 3		Group 4	
			Feature	p value	Feature	p value	Feature	p value	Feature	p value
AFP (high/low)	6.3E-08***	6.4E-07***	Low	2.9E-04***	Low	0.0068**	High	7.2E-05***	High	1.5E-03***
Risk Factor (yes/no)	0.4800	0.6400								
HBV (yes/no)	0.0750	0.1154								
HCV (yes/no)	0.7430	0.7694								
Alcohol (yes/no)	0.6130	0.7000								
NAFLD (yes/no)	0.0250*	0.0500*	Yes	0.0160*	No	0.0240	NA	0.6270	NA	0.2420
Macrotrabecular-massive	4.3E-06***	2.9E-07***	No	2.5E-05***	NA	0.5340	Yes	0.0400*	Yes	1.0E-03***
TP53	4.4E-11***	9.0E-10***	WT	9.5E-08***	NA	0.2663	Mut	0.0370*	Mut	1.2E-07***
CTNNB1	0.0060**	0.0171*	Mut	0.0245*	NA	0.1541	WT	0.0095**	NA	0.1548
TERT	0.0005***	0.0027**	WT	0.0319*	Mut	0.0014**	WT	0.0190*	NA	0.1894
AXIN1	0.6300	0.7000								
HNF1A	0.0090**	0.0224*	Mut	0.0164*	NA	0.2930	NA	0.0623	NA	0.3164
RPS6KA3	0.5996	0.7000								
PIK3CA	0.4713	0.6400								
ATM	0.7694	0.7694								
FGF19	0.0349*	0.0636	WT	0.0319*	NA	0.4857	NA	0.0634	NA	0.1894
TSC1/TSC2	0.0100**	0.0223*	WT	0.0132*	NA	0.6886	NA	1.0000	Mut	0.0229*

*p <0.05, **p <0.01 and ***p <0.001. AFP, alpha-fetoprotein; B&H, Benjamini and Hochberg's procedure; HBV, hepatitis B virus; HCV, hepatitis C virus; Mut, mutant; NA, Not applicable; NAFLD, non-alcoholic fatty liver disease; WT, wild-type.

Importantly, considering that the analysis of the RNA-Seq data and the qRT-PCR validation showed that JmjC inhibitor treatment strongly deplete the expression of *CENPA, KIF20A, PLK1,* and *NCAPG* in HCC cells, our results suggest that patients from Group 4, who have the worst prognosis, could highly benefit from JmjC inhibitor therapy when it becomes clinically available (Fig. 5G).

JIB-04 exert a potent antitumor effect *in vivo*

To test the therapeutic potential of JmjC inhibition on HCC tumors *in vivo*, we evaluated JIB-04 efficacy in an orthotopic animal model of HCC established in fibrotic livers (Fig. 6A).[37] Both oral and systemic JIB-04 therapy inhibited HCC tumor growth, satellite nodule development, and carcinomatous ascites (Fig. 6B-D), confirming the potential of JmjC inhibitors for HCC treatment.

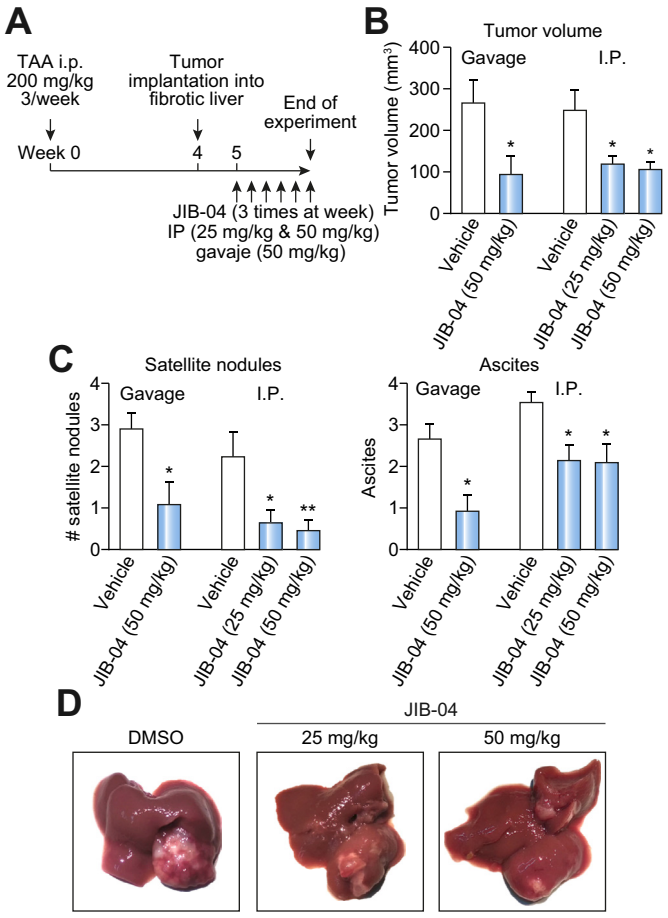


Fig. 6. The JmjC inhibitor JIB-04 exerts a potent antitumor effect on HCC *in vivo*. A) Experimental protocol. Fibrosis was induced by TAA intraperitoneal injections 3 times a week for 6 weeks. At week 4 orthotopic tumors were generated by intrahepatic injection of Hepa129 cell by laparotomy; 1 week later the mice were treated 3 times per week for a total of 6 doses with vehicle (gavage n = 7, intraperitoneal n = 11), JIB-04 either 25 mg/kg (intraperitoneal n = 11) or 50 mg/kg (gavage n = 6, intraperitoneal n = 11). Graph represents the average ± SEM tumor volume (B), # number of satellite nodules (C) and the degree of ascites (C). $^{*}p$ <0.05 and $^{**}p$ <0.01 *vs.* vehicle-treated mice by *t* test for gavage or Kruskal-Wallis for intraperitoneal administration respectively. D) Representative photographs of livers of mice bearing Hepa129 orthotopic tumors treated with intraperitoneal injection of vehicle or JIB-04 at the indicated doses. HCC, hepatocellular carcinoma. (This figure appears in colour on the web.)

Discussion

Accumulated evidence has unmasked the linkage between aberrant gene expression and the hepatocarcinogenesis process.[8] Our data revealed that deregulation and somatic mutations of multiple HPTMs writers (HATS and KMTs), readers (BRDs) or erasers (KDMs) are a striking hallmark of human HCC. In this study, we found not only that more than 75% of patients have at least 1 mutated epigenetic modifier, but also that *NCOA2, SETDB1, ASH1L* and *KDM5B* are mutated in more than 10% of patients. These 4 genes are added to other epigenetic modifiers that are frequently mutated in HCC such as the members of the SWI/SNF chromatin remodeling complexes *ARID1A* (4%-17%) and *ARID2* (3%–18%). Nevertheless, it should be noted that according to their role as tumor suppressors ARID1A and ARID2 present mostly deleterious mutations.[33] However, amplifications are more frequent in the epigenetic modifiers studied here. We found that 43% of the 90 epigenetic modifiers evaluated are upregulated in HCC T tissue compared to NT tissue. In addition, high expression of 12 of these 90 epigenetic modifiers correlates with a worse prognosis in patients with HCC. Supporting our data, similar results have been reported for some of these genes, including *EZH-2, SETDB1, SUV39H1, KMT1C, KDM1A, KDM3A, KDM5B* and *KDM5C*.[7–13] Our data suggests that although some of the epigenetic enzymes could impair HCC growth, most have a pro-tumoral effect. Further functional studies are necessary to confirm this.

Interestingly, the presence of alterations is more frequent in KMTs and KMDs than in HATs or BRDs. In addition, contrary to what is observed in the other families of epigenetic modifiers in which upregulation is more frequent than downregulation, the percentage of KDMs that are up- and downregulated is similar. One possible explanation for this observation is that histone acetylation is always an activation mark of chromatin, whereas the effect of histone methylation on chromatin depends on its localization. For example, trimethylation at H3K4 or H3K36 are marks of active chromatin, but di- or trimethylation at H3K9 or H3K27 correlate with repressed chromatin.[38] Thus, alterations in the methylation levels at different lysines by the same enzyme could activate some genes and repress others allowing complex expression programs. For instance, *KDM4B* or *KDM4D*, both upregulated in HCC, could remove both the active mark H3K36me3 and the inactive mark H3K9me3.[38]

In the last years, several targeted therapeutic strategies, such as sorafenib or regorafenib, have been developed to inhibit individual pathways that are altered in HCC.[2] Nevertheless, even when these therapies improved overall survival of patients with HCC they do not meet the curative clinical expectations.[2] Here we propose that therapies based on JmjC inhibitors could normalize several expression programs activated during hepatocarcinogenesis. Thus, epigenetic inhibitors that act as single agents could impact on multiple genes and pathways maximizing the clinical benefit. Recently, modest results have been reported in clinical trials using 2 HDAC inhibitors (belinostat and reminostat) for HCC treatment.[14] However, these studies highlight the current interest in epigenetic targeted therapies for this tumor type. By testing a panel of epigenetic inhibitors on HCC cell lines, we have determined that JmjC inhibitors JIB-04, GSK-J4, SD-70 and ML-324, the lysine methyltransferase inhibitors BIX-1294 and LLY-507, and the BRD4 inhibitor JQ-1 had a potent effect on cell survival at nM/low-μM doses. Particularly, JIB-04, a JmjC family pan inhibitor, had an IC_{50} in the nM range for most of the cell lines tested. Conversely, EZH-2 and KDM1A

inhibitors had a poor effect on HCC cell survival. This latest result was unexpected as previous experiments have highlighted the role of EZH-2 or KDM1A in HCC cell growth, apoptosis evasion and sorafenib resistance.[39,40] Nevertheless, recent reports showed that strategies targeting EZH-2 facilitate HCC eradication by natural killer cells, while those targeting KDM1A re-sensitized sorafenib-resistant tumors.[41,42] Taken together, these data indicate that therapies based on epigenetic modifiers could induce antitumor effects not only through their antiproliferative effect on HCC cells but also by stimulating the immune system. KDM1A and EZH-2 inhibitors are being tested in clinical trials.[5] Here we showed that JIB-04 exerted antitumoral effects in orthotopic xenografts when administered both by gavage or intraperitoneal injection. Consistently, analogous observations have also been made in other cancer models including breast, lung and colorectal cancer.[15,43,44]

It has been reported that carcinomatosis in HCC depends on uncontrolled cell division and is related to overexpression of cell cycle related genes.[45] In addition, the WNT pathway is highly relevant not only during hepatocarcinogenesis but also in HCC cancer stem cells.[46,47] Our data showed that most of the cell proliferation, anti-cell death, and WNT pathway genes depleted by the JmjC inhibitors are overexpressed on HCC T *vs.* NT tissues. The enriched DEGs in treated cells are downregulated in the T *vs.* NT tissues. These results indicate that JmjC inhibitors reverse transcriptional programs that are activated during hepatocarcinogenesis, restoring the cellular signaling balance.

Several reports have suggested that *CENPA, KIF20A, PLK1* and *NCAPG* genes could have potential as therapeutic targets for HCC since their knockdown affects HCC cell proliferation.[48–51] These genes constitute a signature we identified, which correlates with overall survival, and can be used to cluster patients into 4 prognostic groups. Group 4 has the worst prognosis and has similar clinicopathological and molecular features (high AFP, massive macrotrabecular, and mutant *TP53* and *TSC1/TSC2*) as those in the G3 transcriptomic class defined by Boyault *et al.*[49,50] The properties of Group 1 patients are similar to the G4, G5 and G5 transcriptomic classification (low AFP, NAFLD and *CTNNB1* mutations). Unlike other tumors, therapy in patients with HCC is decided on the basis of performance status, tumor stage and liver function but not based on molecular signatures.[1] On the contrary, breast cancer treatment decisions are based, at least in part, on the Oncotype DX molecular test.[52] In this scenario, our signature defines a subgroup of patients who have an upregulated proliferation program that could strongly benefit from JmjC inhibitor treatment. Since HCC diagnosis is based on imaging techniques and is not usually accompanied by liver biopsy,[1] research samples are mostly from patients with early stage disease. However, the subgroup of patients identified by our signature shows a poor prognosis independently of the tumor stage.

In summary, the present work highlights the importance of epigenetic alterations in HCC development and in patient prognosis. We propose that aberrant histone methylation is a major event during tumor growth based on the high number of KMTs and KDMs overexpressed and mutated in HCC. A combined *in-silico* and *in vivo* strategy was applied to identify therapeutic strategies to target epigenetic mechanisms, and to inhibit protumoral expression programs activated during hepatocarcinogenesis. Furthermore, in this study we developed a gene signature based on *CENPA, KIF20, PLK1,* and *NCAPG* gene expression and defined a subgroup of patients that have an upregulated proliferative expression program that is susceptible to JmjC inhibition. Finally, given the increasing number of small compounds that target epigenetic enzymes, the data provided here support the development of HCC treatments targeting these families of epigenetic modulators.

Financial support

This work was partly supported by The Welch Foundation (I-1878 to EDM), by the NCI (R01 CA125269 to EDM), by a DoD Concept award (W81XWH-16-1-0129 to EDM), by CPRIT (RP160493 to EDM) and by ANPCyT (PICTO-2016-0101, PICT-2015-2630 to GM).

Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

J.B. performed experiments, analyzed data and wrote the manuscript; E.J.F and L.M.D. performed experiments and analyzed data; A.R helped with TCGA analysis; M.M analyzed data and helped to write the manuscript; J.A., M.R. and C.A. analyzed data; M.G.G. helped to write the manuscript. E.D.M. helped to guide the work and wrote the manuscript; G.M. guided the work, analyzed data and wrote the manuscript.

Acknowledgements

We are especially grateful with the Bunge & Born Foundation for its support. We also thank Guillermo Gaston for technical assistance, and members of the Martinez's lab for insights. This work was partly supported by The Welch Foundation (I-1878 to EDM), by the NCI (R01 CA125269 to EDM), by a DoD Concept award (W81XWH-16-1-0129 to EDM), by CPRIT (RP160493 to EDM) and by ANPCyT (PICTO-2016-0101 and PICT-2015-2630 to GM).

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2019.03.007.

References

Author names in bold designate shared co-first authorship

[1] Forner A, Reig M, Bruix J. Hepatocellular carcinoma. Lancet 2018;391:1301–1314.
[2] Reig M, da Fonseca LG, Faivre S. New trials and results in systemic treatment of HCC. J Hepatol 2018;69:525–533.
[3] Dawson MA, Kouzarides T. Cancer epigenetics: from mechanism to therapy. Cell 2012;150:12–27.
[4] Venkatesh S, Workman JL. Histone exchange, chromatin structure and the regulation of transcription. Nat Rev Mol Cell Biol 2015;16:178–189.
[5] **Bayo J**, **Dalvi MP**, Martinez ED. Successful strategies in the discovery of small-molecule epigenetic modulators with anticancer potential. Future Med Chem 2015;7:2243–2261.
[6] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–674.
[7] Au SL, Ng IO, Wong CM. Epigenetic dysregulation in hepatocellular carcinoma: focus on polycomb group proteins. Front Med 2013;7:231–241.

[8] **Wong CM**, **Wei L**, Law CT, Ho DW, Tsang FH, Au SL, et al. Up-regulation of histone methyltransferase SETDB1 by multiple mechanisms in hepatocellular carcinoma promotes cancer metastasis. Hepatology 2016;63:474–487.

[9] Wei L, Chiu DK, Tsang FH, Law CT, Cheng CL, Au SL, et al. Histone methyltransferase G9a promotes liver cancer development by epigenetic silencing of tumor suppressor gene RARRES3. J Hepatol 2017;67:758–769.

[10] Yamada D, Kobayashi S, Yamamoto H, Tomimaru Y, Noda T, Uemura M, et al. Role of the hypoxia-related gene, JMJD1A, in hepatocellular carcinoma: clinical impact on recurrence after hepatic resection. Ann Surg Oncol 2012;19:S355–364.

[11] **Chen Z**, **Wang X**, Liu R, Chen L, Yi J, Qi B, et al. KDM4B-mediated epigenetic silencing of miRNA-615-5p augments RAB24 to facilitate malignancy of hepatoma cells. Oncotarget 2017;8:17712–17725.

[12] **Wu BH**, **Chen H**, **Cai CM**, Fang JZ, Wu CC, Huang LY, et al. Epigenetic silencing of JMJD5 promotes the proliferation of hepatocellular carcinoma cells by down-regulating the transcription of CDKN1A 686. Oncotarget 2016;7:6847–6863.

[13] Zhao ZK, Yu HF, Wang DR, Dong P, Chen L, Wu WG, et al. Overexpression of lysine specific demethylase 1 predicts worse prognosis in primary hepatocellular carcinoma patients. World J Gastroenterol 2012;18:6651–6656.

[14] Bitzer M, Horger M, Giannini EG, Ganten TM, Worns MA, Siveke JT, et al. Resminostat plus sorafenib as second-line therapy of advanced hepatocellular carcinoma - The SHELTER study. J Hepatol 2016;65:280–288.

[15] Wang L, Chang J, Varghese D, Dellinger M, Kumar S, Best AM, et al. A small molecule modulates Jumonji histone demethylase activity and selectively inhibits cancer growth. Nat Commun 2013;4:2035.

[16] Kruidenier L, Chung CW, Cheng Z, Liddle J, Che K, Joberty G, et al. A selective jumonji H3K27 demethylase inhibitor modulates the proinflammatory macrophage response. Nature 2012;488:404–408.

[17] Jin C, Yang L, Xie M, Lin C, Merkurjev D, Yang JC, et al. Chem-seq permits identification of genomic targets of drugs against androgen receptor regulation selected by functional phenotypic screens. Proc Natl Acad Sci USA 2014;111:9235–9240.

[18] G. Rai, A. Kawamura, A. Tumber, Y. Liang, J.L. Vogel, J.H. Arbuckle, et al., Discovery of ML324, a JMJD2 demethylase inhibitor with demonstrated antiviral activity, Probe Reports from the NIH Molecular Libraries Program. Bethesda (MD), 2010.

[19] Hamada S, Suzuki T, Mino K, Koseki K, Oehme F, Flamme I, et al. Design, synthesis, enzyme-inhibitory activity, and effect on human cancer cells of a novel series of jumonji domain-containing protein 2 histone demethylase inhibitors. J Med Chem 2010;53:5629–5638.

[20] Sayegh J, Cao J, Zou MR, Morales A, Blair LP, Norcia M, et al. Identification of small molecule inhibitors of Jumonji AT-rich interactive domain 1B (JARID1B) histone demethylase by a sensitive high throughput screen. J Biol Chem 2013;288:9408–9417.

[21] **Vinogradova M**, **Gehling VS**, Gustafson A, Arora S, Tindell CA, Wilson C, et al. An inhibitor of KDM5 demethylases reduces survival of drug-tolerant cancer cells. Nat Chem Biol 2016;12:531–538.

[22] Johansson C, Velupillai S, Tumber A, Szykowska A, Hookway ES, Nowak RP, et al. Structural analysis of human KDM5B guides histone demethylase inhibitor development. Nat Chem Biol 2016;12:539–545.

[23] Mohammad HP, Smitheman KN, Kamat CD, Soong D, Federowicz KE, Van Aller GS, et al. A DNA hypomethylation signature predicts antitumor activity of LSD1 inhibitors in SCLC. Cancer Cell 2015;28:57–69.

[24] Kubicek S, O'Sullivan RJ, August EM, Hickey ER, Zhang Q, Teodoro ML, et al. Reversal of H3K9me2 by a small-molecule inhibitor for the G9a histone methyltransferase. Mol Cell 2007;25:473–481.

[25] Nguyen H, Allali-Hassani A, Antonysamy S, Chang S, Chen LH, Curtis C, et al. LLY-507, a cell-active, potent, and selective inhibitor of protein-lysine methyltransferase SMYD2. J Biol Chem 2015;290:13641–13653.

[26] **Knutson SK**, **Warholic NM**, Wigle TJ, Klaus CR, Allain CJ, Raimondi A, et al. Durable tumor regression in genetically altered malignant rhabdoid tumors by inhibition of methyltransferase EZH2. Proc Natl Acad Sci USA 2013;110:7922–7927.

[27] Vaswani RG, Gehling VS, Dakin LA, Cook AS, Nasveschuk CG, Duplessis M, et al. Identification of (R)-N-((4-methoxy-6-methyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-2-methyl-1-(1-(1 -(2,2,2-trifluoroethyl)piperidin-4-yl)ethyl)-1H-indole-3-carboxamide (CPI-1205), a potent and selective inhibitor of histone methyltransferase EZH2, suitable for phase I clinical trials for B-cell lymphomas. J Med Chem 2016;59:9928–9941.

[28] **Filippakopoulos P**, **Qi J**, **Picaud S**, Shen Y, Smith WB, Fedorov O, et al. Selective inhibition of BET bromodomains. Nature 2010;468:1067–1073.

[29] Martin LJ, Koegl M, Bader G, Cockcroft XL, Fedorov O, Fiegen D, et al. Structure-based design of an in vivo active selective BRD9 inhibitor. J Med Chem 2016;59:4462–4475.

[30] **Grebien F**, **Vedadi M**, **Getlik M**, Giambruno R, Grover A, Avellino R, et al. Pharmacological targeting of the Wdr5-MLL interaction in C/EBPalpha N-terminal leukemia. Nat Chem Biol 2015;11:571–578.

[31] Vangamudi B, **Paul TA**, **Shah PK**, Kost-Alimova M, Nottebaum L, Shi X, et al. The SMARCA2/4 ATPase domain surpasses the bromodomain as a drug target in SWI/SNF-mutant cancers: insights from cDNA rescue and PFI-3 inhibitor studies. Cancer Res 2015;75:3865–3878.

[32] Chen J, Bardes EE, Aronow BJ, Jegga AG. ToppGene suite for gene list enrichment analysis and candidate gene prioritization. Nucleic Acids Res 2009;37:W305–311.

[33] Zucman-Rossi J, Villanueva A, Nault JC, Llovet JM. Genetic landscape and biomarkers of hepatocellular carcinoma. Gastroenterology 2015;149:1226–1239, e1224.

[34] **Barretina J**, **Caponigro G**, **Stransky N**, **Venkatesan K**, **Margolin AA**, Kim S, et al. The cancer cell line encyclopedia enables predictive modelling of anticancer drug sensitivity. Nature 2012;483:603–607.

[35] **Hatch SB**, **Yapp C**, Montenegro RC, Savitsky P, Gamble V, Tumber A, et al. Assessing histone demethylase inhibitors in cells: lessons learned. Epigenetics Chromatin 2017;10:9.

[36] Roessler S, Jia HL, Budhu A, Forgues M, Ye QH, Lee JS, et al. A unique metastasis gene signature enables prediction of tumor relapse in early-stage hepatocellular carcinoma patients. Cancer Res 2010;70: 10202–10212.

[37] Forner A, Llovet JM, Bruix J. Hepatocellular carcinoma. Lancet 2012;379:1245–1255.

[38] Kimura H. Histone modifications for human epigenome analysis. J Hum Genet 2013;58:439–445.

[39] **Lei ZJ**, **Wang J**, Xiao HL, Guo Y, Wang T, Li Q, et al. Lysine-specific demethylase 1 promotes the stemness and chemoresistance of Lgr5+ liver cancer initiating cells by suppressing negative regulators of beta-catenin signaling. Oncogene 2015;34:3214.

[40] Feng H, Yu Z, Tian Y, Lee YY, Li MS, Go MY, et al. A CCRK-EZH2 epigenetic circuitry drives hepatocarcinogenesis and associates with tumor recurrence and poor survival of patients. J Hepatol 2015;62:1100–1111.

[41] **Huang M**, **Chen C**, Geng J, Han D, Wang T, Xie T, et al. Targeting KDM1A attenuates Wnt/beta-catenin signaling pathway to eliminate sorafenib-resistant stem-like cells in hepatocellular carcinoma. Cancer Lett 2017;398:12–21.

[42] Bugide S, Green MR, Wajapeyee N. Inhibition of Enhancer of zeste homolog 2 (EZH2) induces natural killer cell-mediated eradication of hepatocellular carcinoma cells. Proc Natl Acad Sci USA 2018;115:E3509–E3518.

[43] Dalvi MP, Wang L, Zhong R, Kollipara RK, Park H, Bayo J, et al. Taxane-platin-resistant lung cancers co-develop hypersensitivity to JumonjiC demethylase inhibitors. Cell Rep 2017;19:1669–1684.

[44] Kim MS, Cho HI, Yoon HJ, Ahn YH, Park EJ, Jin YH, et al. JIB-04, a small molecule histone demethylase inhibitor, selectively targets colorectal cancer stem cells by inhibiting the Wnt/beta-catenin signaling pathway. Sci Rep 2018;8:6611.

[45] **Yan H**, **Li Z**, Shen Q, Wang Q, Tian J, Jiang Q, et al. Aberrant expression of cell cycle and material metabolism related genes contributes to hepatocellular carcinoma occurrence. Pathol Res Pract 2017;213:316–321.

[46] **Liu LJ**, **Xie SX**, Chen YT, Xue JL, Zhang CJ, Zhu F. Aberrant regulation of Wnt signaling in hepatocellular carcinoma. World J Gastroenterol 2016;22:7486–7499.

[47] **Lei ZJ**, **Wang J**, Xiao HL, Guo Y, Wang T, Li Q, et al. Lysine-specific demethylase 1 promotes the stemness and chemoresistance of Lgr5(+) liver cancer initiating cells by suppressing negative regulators of beta-catenin signaling. Oncogene 2015;34:3188–3198.

[48] Dietrich P, Freese K, Mahli A, Thasler WE, Hellerbrand C, Bosserhoff AK. Combined effects of PLK1 and RAS in hepatocellular carcinoma reveal rigosertib as promising novel therapeutic "dual-hit" option. Oncotarget 2018;9:3605–3618.

[49] Zhang Q, Su R, Shan C, Gao C, Wu P. Non-SMC condensin I complex, subunit G (NCAPG) is a novel mitotic gene required for hepatocellular cancer cell proliferation and migration. Oncol Res 2018;26:269–276.

[50] Shi C, Huang D, Lu N, Chen D, Zhang M, Yan Y, et al. Aberrantly activated Gli2-KIF20A axis is crucial for growth of hepatocellular carcinoma and predicts poor prognosis. Oncotarget 2016;7:26206–26219.

[51] **Li Y**, **Zhu Z**, **Zhang S**, Yu D, Yu H, Liu L, et al. ShRNA-targeted centromere protein A inhibits hepatocellular carcinoma growth. PLoS One 2011;6: e17794.

[52] Xin L, Liu YH, Martin TA, Jiang WG. The era of multigene panels comes? The clinical utility of oncotype DX and MammaPrint. World J Oncol 2017;8:34–40.