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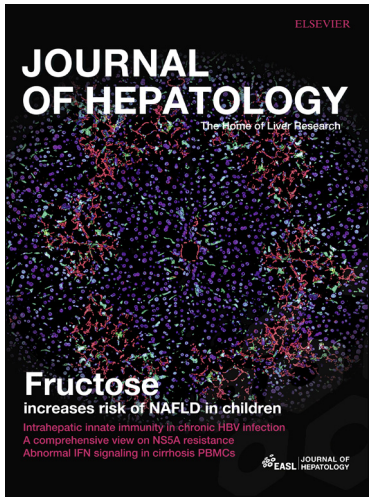

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A comprehensive study of epigenetic alterations in hepatocellular carcinoma identifies potential therapeutic targets.

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Keywords: human hepatocellular carcinoma, epigenetic, histone demethylases, lysine demethylases, Jumonji C demethylases, histone methyltransferases, histone acetyltransferases, bromodomains, epigenetic inhibitors, gene expression signature, patient survival.

Abbreviations:

Hepatocellular carcinoma (HCC), lysine demethylases (KDMs), lysine methyltransferases (KMTs), histone acetyltransferases (HATs), bromodomains (BRDs), histone post-translational modifications (HPTMs), histone deacetylases (HDAC),The Cancer Genome Atlas (TCGA), tumor (T), non-tumor (NT), Jumonji C family of KDMs (JmjCs), differential expressed genes (DEGs), gene ontology functional enrichment (GO), quantitative Real-Time PCR (qRT-PCR), centromere protein A (CENPA), kinesin family member 20A (KIF20A), polo-like kinase (PLK1), non-SMC condensin I complex and subunit G (NCAPG), hepatitis virus B (HVB), Diagonal Linear Discriminant Analysis (DLDA).

Abstract

Background and 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 HCC. The therapeutic potential of epigenetic inhibitors was evaluated *in vitro* and *in vivo*. RNA-Seq analysis and its correlation with expression and clinical data in the TCGA dataset were used to identify expression programs normalized by Jumonji lysine demethylases (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 KDMs (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 reverts an HCC aggressive gene expression program that is also altered in HCC patients. 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 for prognosis prediction and to define a sub-group of high-risk patients that potentially may 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 HCC, leading to an aggressive gene expression program and poor clinical prognosis. The transcriptional program can be reversed by pharmacological inhibiton of Jumonji enzymes blocking HCC, 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 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 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 on DNA packaging allows 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 tumors has 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 new therapeutic strategy (5). Recently, two histone deacetylase (HDAC) inhibitors, belinostat and reminostat, have been tested in phase I/II clinical trials on HCC patients 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). Here 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-Seq) for the indicated genes were obtained from The Cancer Genome Atlas (TCGA) for hepatocellular carcinoma (HCC) patients (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 T vs NT 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}s were determined from the fitted curves using GraphPad prism.

Cell cycle and apoptosis assays

Cell cycle using propidium iodide and apoptosis by Annexin-V were analyzed by flow cytometry (BD FACSCalibur).

***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 wire lid holding standard mouse chow and water bottle and maintained in a temperature controlled under a 12 hr light - 12 hr 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 by i.p. or gavage. Mice were sacrificed at 2 weeks and tumor size and number of HCC satellites evaluated.

Histone post-translational modifications analysis

HuH7 were treated with JIB-04, GSK-J4, SD-70 (2 times IC_{50} concentration) or DMSO for 24h. 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 and a FDR<0.05. Gene ontology and pathway analysis were done using the ToppGene suite (32). Results were validated by 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 HCC patients 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 see Supplementary methods , and refer to the CTAT table .

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 to define them as potential therapeutic target for HCC. To address this, we queried The Cancer Genome Atlas (TCGA) for HCC data (n=365) to determine the mutations frequencies of bromodomains (BRDs, n=8), histone acetyltransferases (HATs, n=18), lysine methyltransferases (KMTs, n=35) and lysine demethylases (KDMs, n=29). Here we report that 75% of patients present a somatic mutation in at least one of the epigenetic modifiers studied. Furthermore, 20% of them have more than 5 epigenetic modifiers mutated (Figure 1A). In addition, we observed that the frequency of HCC patients with at least one somatic mutation is 20% for BRDs, 38% for HATs, 57% for KMTs, and 35% for KDMs (Figure 1A). Analyzing individually, 23 of the 90 epigenetic modifiers studied are mutated in at least 4% of HCC patients (Figure 1B). Among them, the genes BRD9, NCOA2, SETDB1, ASH1L, SMYD2, SMYD3, and KDM5B are highly amplified in HCC patients. We then determined the relationship between the presence of mutations in the epigenetic modifiers studied here and clinical-pathological features such as 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 the epigenetic modifiers and largely representative HCC mutations such as TP53, CTNNB1, TERT, AXIN1, HNF1A, RPSKA3, PIK3CA, ATM, FGF9 and TSC1/TSC2 was performed (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 a close location 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). On the other hand, we also found 2 examples were the mutations co-segregate, but they 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 tumor (T) vs non-tumoral adjacent tissues (NT) 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 up-regulated in T in comparison with matched NT (Table S1, Figure 1C). Even more, when analyzed in aggregate 43% and 22% of the epigenetic modifiers are up or down-regulated, respectively. Particularly, among the genetically amplified epigenetic modifiers, BRD9, EHMT-

2, ASH1L, SMYD2, SMYD3, and KDM5B were upregulated in HCC (Table S1, Figure 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. (Figure 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 in HCC patients by at least 1.3-fold (Figure 1D). Importantly, BRD9, EHMT-2, SMYD2, EZH-2 and KDM5B are potential 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 up-regulated genes correlated with 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 bromodomain 9 (BRD9), histone acetyltransferase 1A (HAT1A), the histone methyltransferases SUV39H2, SMYD1, NSD2 and KMT5A, the histone lysine demethylases KDM1A, KDM1B, KDM3A, KDM5C and RIOX2 correlated with poor prognosis among patients with HCC (Table S1, Figure 1E). On the other hand, we didn't confirm previous the previous reports for KDM5B and SETDB1 (Figure S1B). These analyses also showed that high expression of the histone demethylases KDM8 and PHF8, which are down-regulated in T vs NT (Table S1), correlate with a better prognosis (Figure 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 ML324; the methyltransferase (KMTs) inhibitors BIX 01294 and LLY-507; and the BRD4 inhibitor JQ-1 (Figure 2A, Figure 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 (Figure 2A, Figure S2, Table S3)(14). On the other hand, 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), methyltransferase EZH-2 inhibitors (EPZ-6438 and CPI-1205), bromodomain inhibitors (BI-9654 and BI-7273), the SMARCA bromodomain inhibitor (PFI-3), and the WDR5/KMT2A interaction inhibitor OICR-9429 (Figure 2A, Figure 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 IC50s 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 amplificated in HuH-7 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 (Figure 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}s for JIB-04 and PBIT, respectively (Figure S3B). Strikingly, in both cases the correlation was inversely proportional suggesting that tumors with high expression of these two 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 (Figure 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 (Figure 2B and Figure S4A). JmjC inhibitors induced a consistent cell cycle arrest in the G1/S interphase in different HCC cell lines (Figure 2B and Figure 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, with BIX-01294 or with JQ-1 express high levels of G1 cyclins, and low levels of cyclins that regulate the progression through the other cell cycle phases (Figure 2C, Figure 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 (Figure 2D, Figure 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 lines.

JmjCs inhibitor-treated HCC cells have robust changes in histone 3 global methylation levels.

Recently, it was demonstrated that cell death induced by genotoxic compounds as doxorubicin or paclitaxel lead to an increase in H3K27me3, H3K4me3 and H3K9me2 and a decrease in global acetylation (35). To assess if the anti-cancer 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 (24h treatment, Figure 3A) or western blot (24h and 48h treatment, Figure 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 three inhibitors decreased H3K27ac levels. No changes were observed on levels of acetylation of lysines that are not modified by methylation such as H3K14, and H3K56 (Figure 3A). To corroborate that the increase in levels of histone methylation was due to JmjCs inhibition we measured H3K9me3 and H3K27me3 enzymatic demethylase activity in treated cell lysates on exogenous histone substrates. As observed in Figure 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 three JmjC inhibitors (Figure 3D). Similar results were observed in Hep3B cells (Figure S6). Taken together, these results indicate that the increased histone methylation observed is the direct result of inhibition of JmjC demethylases.

JmjCs inhibitors revert 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 3358 genes with log2(FC) of 0.3 or more triggered by JIB-04, 1693 by GSK-J4 and 2842 by SD70, ($p<0.05$, Figure S7A, Table S4). In addition, 3708, 2018 and 3089 genes were down-regulated by these compounds, respectively (log2(FC)<-0.3, $p<0.05$, Figure 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 three inhibitors induced overlapping gene expression signatures (Figure 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 (Figure S7B). Likewise, pathway enrichment analysis showed that genes related with gene expression, cell cycle, and mitosis were also modulated by the three inhibitors (Figure S7B). Analysis of overlapping genes showed that 904 genes were commonly depleted, and 728 genes were commonly enriched, after exposure to these JmjC inhibitors (Figure 4A, Table S5). In addition, 1730 down-regulated genes and 1684 up-regulated genes were commonly modulated by JIB-04 and SD-70 but not by GSK-J4. GO analysis showed that down-regulated common genes were related to "positive regulation of cell proliferation", "negative regulation of apoptosis" and "WNT pathway" among other GO terms (Figure 4A, Table S5). The up-regulated 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" (Figure 4A, Table S5). To gain insight into the role of these genes in the development and progression of HCC, we investigated if the DEGs modulate 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 HCC patients using the 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 up-regulated in T vs NT samples (Figure 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 HCC tumors compared to normal tissues (Figure 4B, Table S6). Examples of these genes are shown in in Figure 4C. These data indicate that JmjC inhibitors can partially normalize the aggressive gene expression pattern characteristic of HCC tumor vs normal tissues. We also found a small group of genes associated with "cell proliferation" or "cell death" GO terms that were down- or up-regulated, respectively, in both HCC treated cells and human HCC samples (Figure S8A, Table S6). GO analysis of these sub-group of genes indicates an anti-proliferative and pro-apoptotic phenotype (Figure S8B). To validate the results observed by RNA-Seq on HuH7 cell line, we performed quantitative real-time PCR (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 revert malignant HCC transcriptional patterns (Figure S9).

Next, we evaluated if the commonly DEGs correlate with the prognosis of HCC patients. 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 (Figure 4D, Table S6). On the other hand, high expression of JmjC inhibitor enhanced genes correlates with a better prognosis (Figure 4D, Table S6). Figure 4E shows representatives genes that correlate with poor patient prognosis when are 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.

Taking in consideration the strong modulatory effect achieved by JmjC inhibitors on HCC cells we wondered whether we could identify a group of patients who could eventually benefit from treatment with these drugs. Venn diagrams show 58 genes are depleted by JmjC inhibitors, up-regulated in HCC, and whose high expression correlates with poor prognosis (Figure 5A). On the other hand, 18 genes are enriched after JmjC inhibition, are down-regulated in HCC, and their low expression correlates with poor prognosis (Figure 5B). Further stringent filtering identifying genes that are highly altered in HCC T vs NT and have the most significant hazard ratios allowed us to identify a 4-gene signature composed by centromere protein A (CENPA), kinesin family member 20A (KIF20A), polo-like kinase (PLK1), non-SMC condensin I complex and subunit G (NCAPG). Based on our 4-genes signature, unsupervised hierarchical clustering separated the HCC patients into 4 mayor groups that have increased expression levels of KIF20A, CENPA, PLK1 and NCAPG (Group 1 < Group 2 < Group 3 < Group 4) (Figure 5C, Table S7). There is no clear correlation between amplifications in any one 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 (Group 1) and a group with poor prognosis with a median survival of 0.95 years (Group 4, Figure 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 facilitate the future classification of new patients we defined a gene predictor using a Diagonal Linear Discriminant

Analysis (Figure 5E). The classifier generated here correctly classifies 94.5% of the patients of the TCGA database.

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 are 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 NAFLD, Group 2 does not segregate with the NAFLD phenotype. 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 (Figure 5F, Figure S10A). Analysis of patients with HVB infection in the TCGA database yielded survival curves similar to those obtained for the GSE14520 dataset (Figure 5F and Figure 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 HBV, HCV and NAFLD, the GSE14520 population is homogenous and formed by HBV patients only.

Importantly, considering that the analysis of the RNA-Seq data and the qRT-PCR validation showed that JmjC inhibitors 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.

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 (Figure 6A) (37). Both oral and systemic JIB-04 therapy inhibited HCC tumor growth, satellite nodule development, and carcinomatous ascites (Figure 6B-D) confirming the potential of JmjC inhibitors for HCC treatment.

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 one of these epigenetic modifiers mutated, 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 suppressor ARID1A and ARID2 present mostly deleterious mutations (33). On the other hand, amplifications are more frequent in the epigenetic modifiers here studied. We found that 43% of the 90 epigenetic modifiers evaluated are up-regulated when comparing HCC T vs NT tissues. In addition, high expression of 12 of these 90 epigenetic modifiers correlates with a worst prognosis in HCC patients. 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. To establish this further functional studies are necessary.

Interestingly, the presence of alterations is more frequent in histone lysine methyltransferases and demethylases than in HATs or BRDs. In addition, contrarily to what is observed in the other families of epigenetic modifiers in which up-regulation is more frequent than down-regulation, the percentage of KDMs that is up- and down-regulated is similar. One possible explanation for this observation is that histone acetylation is always an activation mark of

chromatin, whereas histone methylation's effect 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 have been developed to inhibit individual pathways that are altered in HCC such as sorafenib or regorafenib (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 two HDACs inhibitors (belinostat and reminostat) for HCC treatment (14). However, these studies highlight the current interest on 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 ML324, the lysine methyltransferase inhibitors BIX-01294 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, showed the IC_{50} in the nM range for most of the cell lines tested. On the other hand, EZH-2 and KDM1A inhibitors showed poor effect on HCC cells survival. This latest result was unexpected since overexpression and knock down experiments of EZH-2 or KDM1A have shown their role on HCC cell growth, apoptosis evasion and sorafenib resistance (39, 40). Nevertheless, recent reports showed strategies targeting EZH-2 to facilitate HCC

eradication by natural killer cells or KDM1A as treatment of HCC sorafenib-resistant tumors (41, 42). Taking together, these data indicate that epigenetic-based therapies could induce antitumor effect not only due to their antiproliferative effect on HCC cells but also due to the stimulation of 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 cancer, lung cancer and colorectal carcinoma (15, 43, 44).

It has been reported that carcinomatosis in HCC depends of 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 process but also in HCC cancer stem cells (46, 47). Our data showed that most of 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 the hepatocarcinogenesis process, 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 affect HCC cell proliferation (48-51). These genes constitute a signature we identified which is present in HCC vs normal tissue and correlates with overall survival, clustering patients into four groups. Group 4 has 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, on the other hand, are similar to the G4, G5 and G5 transcriptomic classification (low AFP, NAFLD and CTNNB1 mutations).

Unlike other tumors, therapy in HCC patients 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 decision is based, at least in part, using the Oncotype DX molecular test (52). In this scenario, our signature defines a subgroup of patients who have an up-regulated proliferation program that could strongly benefit from JmjC inhibitor treatment. Since HCC diagnosis is based on imaging techniques and usually is not 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 pro-tumoral 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 sub-group of patients that have an up-regulated proliferative expression program vulnerable to JmjC inhibition therapy. 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.

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Figure Legends.

Figure 1. Alteration of epigenetic modifiers is a common event in HCC and their expression correlates with poor patient prognosis in the TCGA. A) Somatic mutation data of patients with HCC (TCGA data, n=365) was analyzed and percentage of patients with 1, 2-4 or more than 5 genes mutated in the bromodomains (BRD), histone acetyl transferases (HAT), lysine methyl transferases (KMT) or lysine demethylases (KDM) families individually or altogether (Total) are showed. B) Epigenetic modifiers (BRDs, HATs, KMT 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 up-regulated or down-regulated 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-, down-regulated or do not present changes when compared tumor vs non-tumor expression. D) Epigenetic modifiers that present a fold change higher than 1.3 and a $p<0.001$ (paired t-test) when evaluated matched tumor vs non-tumor patient samples. E) Patients with high expression of BRD9; HAT 1; lysine methyl transferases including: SUV39H2, SMYD1, NSD2, KMT5A, EZH-2; and lysine demethylases including: KDM1A, KDM1B, KDM3A, KDM5C and RIOX2, analyzed by RNA-Seq (TCGA

data, n=365) have significant 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 figure.

Figure 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}s were determined averaging the values obtained from the fitted curves using GraphPad prism of two 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 x IC_{50} concentration. Bars represent % $\pm$ 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 $\pm$ 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 % $\pm$ 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.

Figure 3. JmjC inhibitors induce global changes in histone postranslational modifications (HPTMs) through specific impairment of demethylase activity. A) ELISA analysis for different HPTMs of histone extracts from HuH7 cells treated during 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 histone 3 (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 three 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 three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 vs DMSO (Kruskal-Wallis).

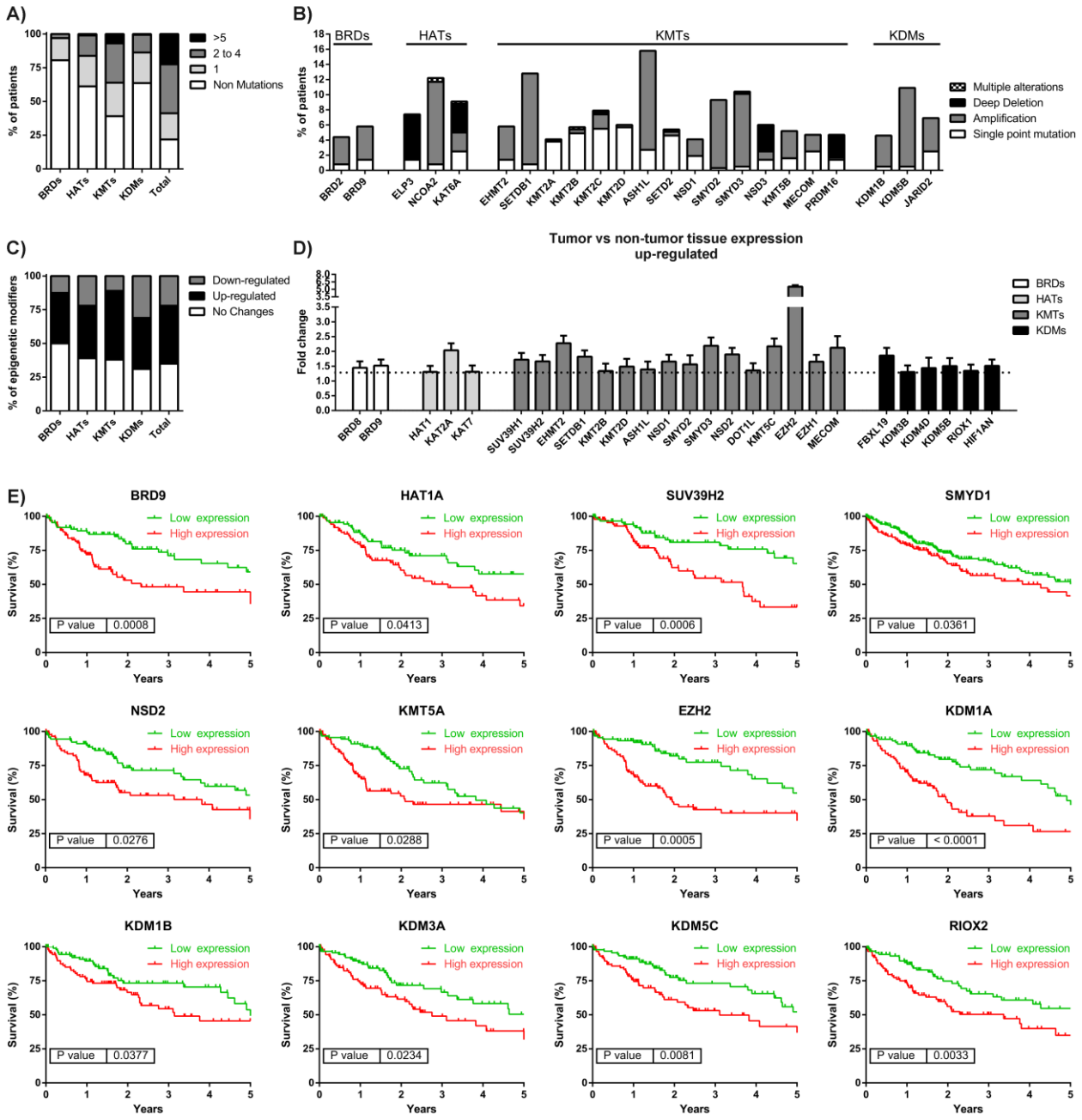
Figure 4. JmjC inhibitors reverse a pro-proliferative and anti-apoptotic expression program in human HCC cells. A) Venn diagram of down- or up-regulated genes (FDR = 0.05, Log2(FC)=±0.3) and gene ontology analysis of commonly depleted (left panel) or depleted (right panel) genes in HuH7 after JmjC inhibitor treatments (JIB-04, GSK-J4 or SD-70) during 24 h at 2 x 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 x IC_{50} doses. C) Top up- or down- regulated 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 three independent biological

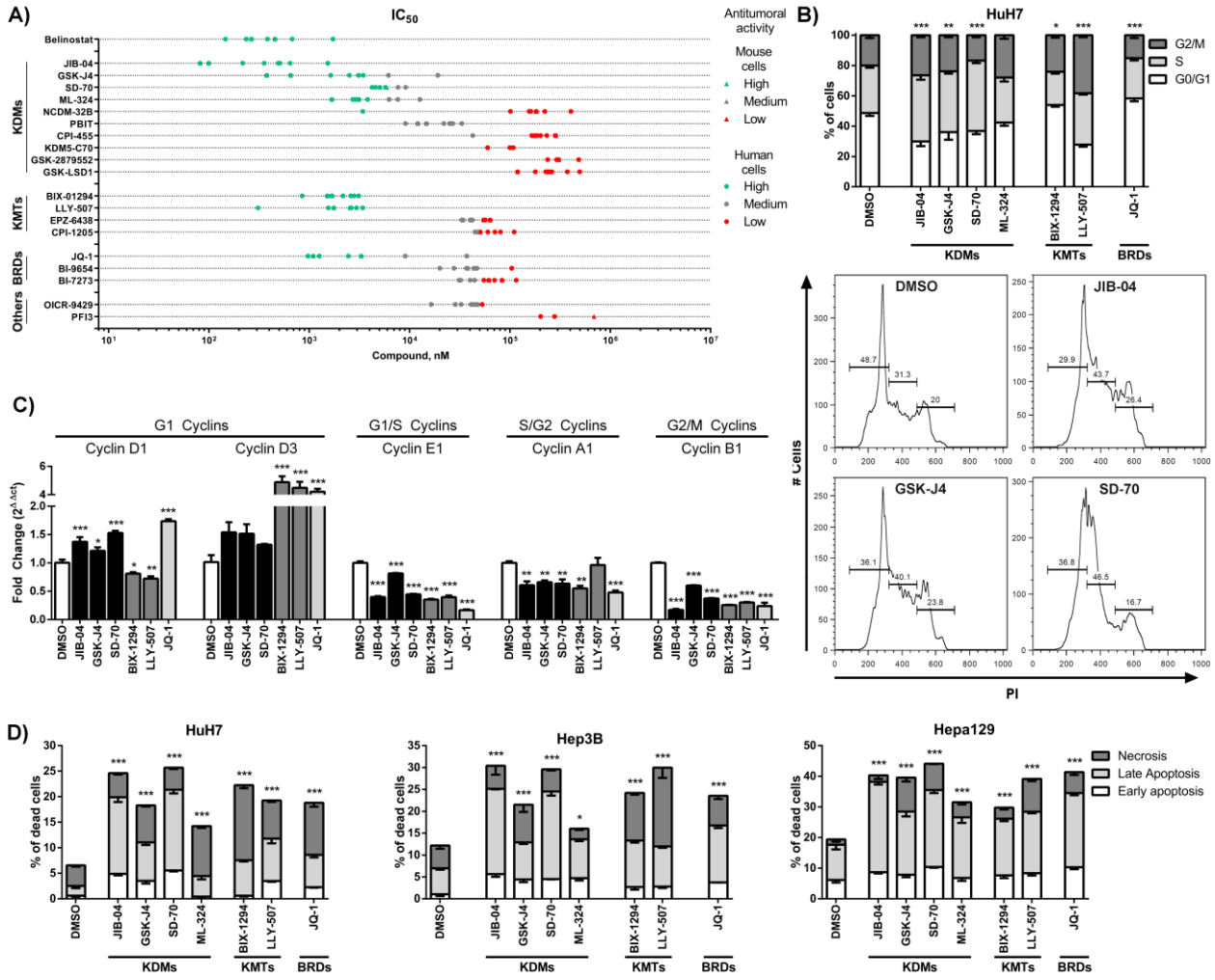
replicates analyzed by RNA-seq. ***p<0.001 vs DMSO, FDR by Benjamini and Hochberg's procedure. D) Pie charts showing number of genes depleted or enriched in HuH7 cells by JmjC inhibitor treatment regulating "cell proliferation", "cell death" and "WNT pathway signaling" that correlate with patient prognosis when highly expressed in HCC patients. 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).

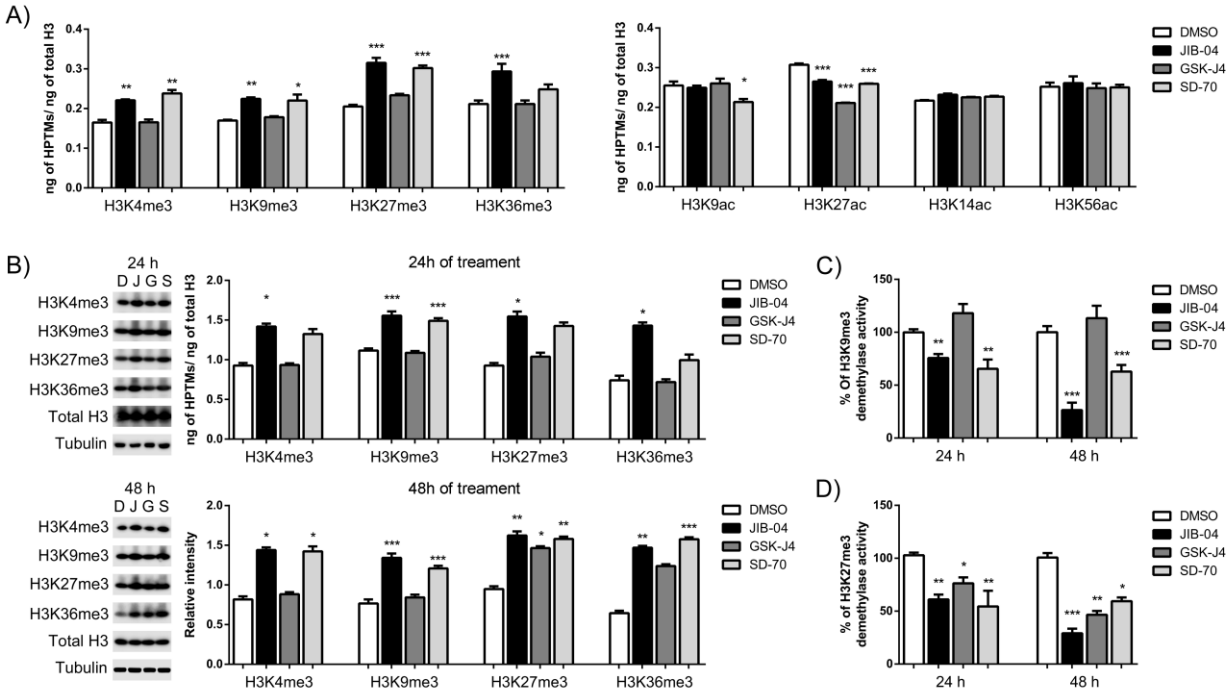
Figure 5. The gene signature formed by CENPA, KIF20A, NCAPG and PLK1 defines a group of patients with poor survival that could benefit from JmjC inhibitor therapy. A) Venn diagram showing overlap of genes related with "cell proliferation", "cell death" and "WNT pathway" that are depleted by JmjC inhibitors in HCC cells, are up-regulated 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 down-regulated in tumor vs normal tissues and correlates 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 $\pm$ 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 HCC patients 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 HCC patients (n = 221) segregated the patients into 2 major

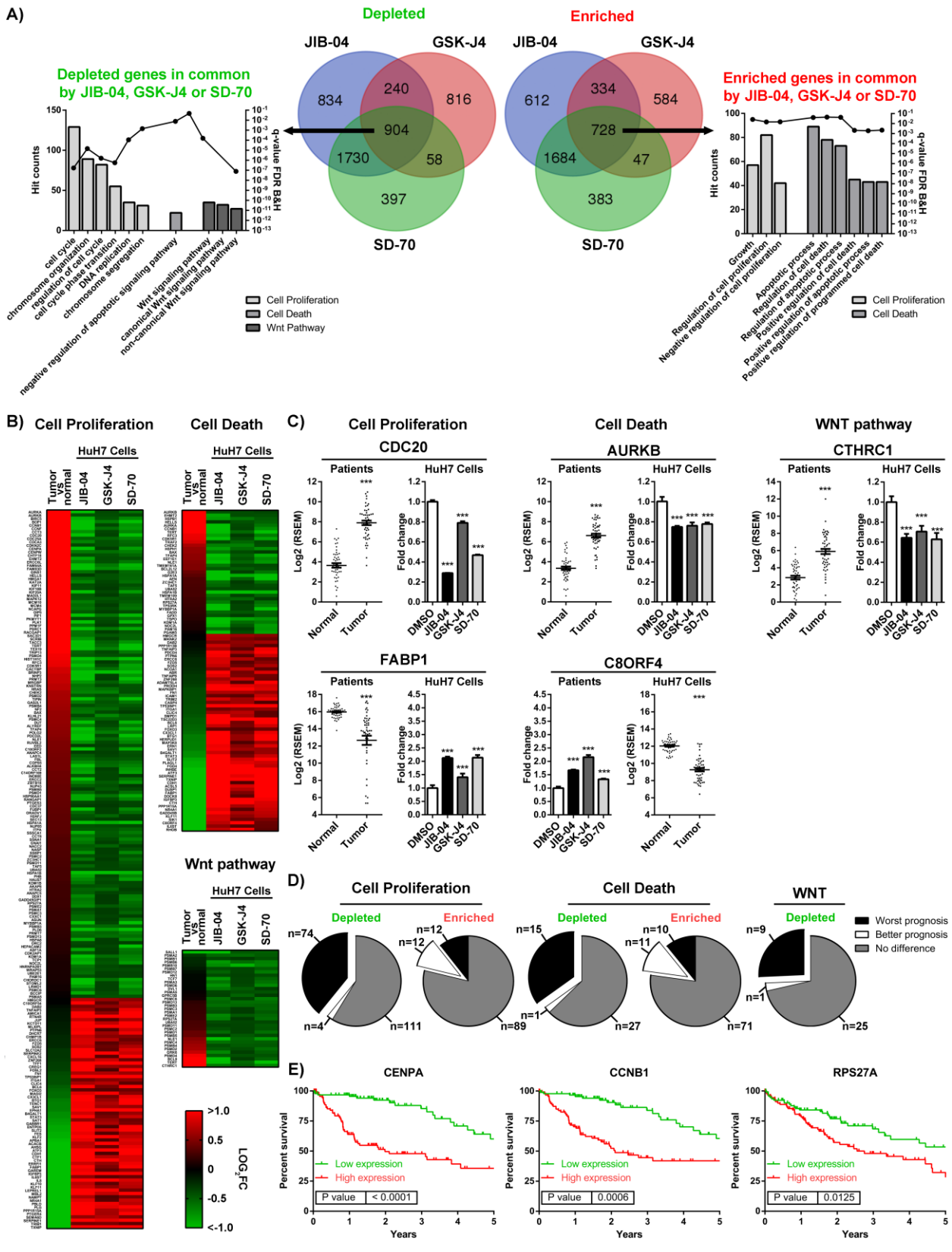
groups with increasing expression of CENPA, KIF20A, NCAPG and PLK1. Kaplan-Meier survival analysis revealed significant differences in overall survival across groups ($p < 0.001$, left panel). Right panel graph bar shows average expression levels ± SEM of CENPA, KIF20A, NCAPG and PLK1 genes in the 2 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 three independent biological replicates analyzed by RNA-seq. ***p<0.001 vs DMSO, FDR by Benjamini and Hochberg's procedure.

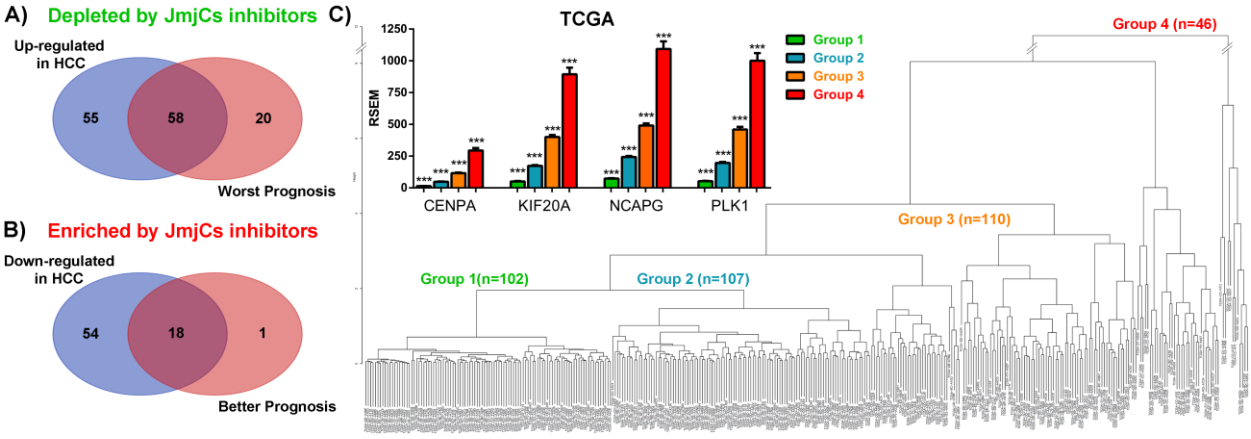
Figure 6. The JmjC inhibitor JIB-04 exerts a potent antitumor effect on HCC *in vivo*. A) Experimental protocol. Fibrosis was induced by TAA I.P. injections 3 times at week for 6 weeks. At week 4 orthotopic tumors were generated by intra-hepatic injection of Hepa129 cell by laparotomy; one week later 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 I.P. administration respectively. D) Representative photographs of livers of mice bearing Hepa129 orthotopic tumors treated with I.P. injection of vehicle or JIB-04 at the indicated doses.

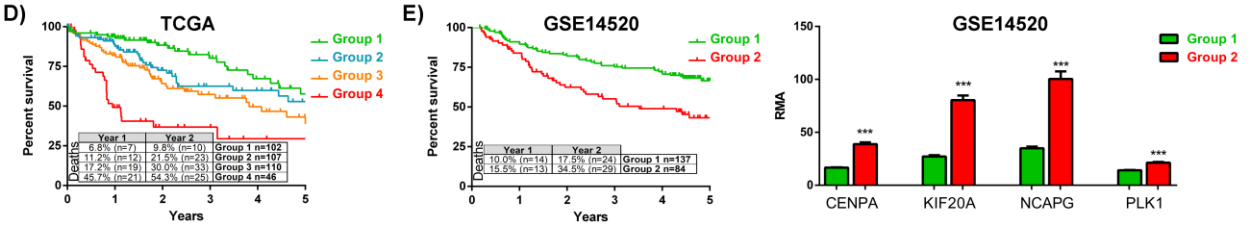



A)
IC50
Antitumoral activity
Mouse cells
High
Medium
Low
Human cells
High
Medium
Low
Belinostat
JIB-04
GSK-J4
SD-70
ML-324
NCDM-32B
PBIT
CPI-455
KDM5-C70
GSK-2879552
GSK-LSD1
BIX-01294
LLY-507
EPZ-6438
CPI-1205
JQ-1
BI-9654
BI-7273
OICR-9429
PFI3
KDMs
KMTs
BRDs
Others
Compound, nM
B)
HuH7
% of cells
G2/M
S
G0/G1
DMSO
JIB-04
GSK-J4
SD-70
ML-324
BIX-1294
LLY-507
JQ-1
KDMs
KMTs
BRDs
DMSO
JIB-04
GSK-J4
SD-70
Cells
PI
C)
G1 Cyclins
Cyclin D1
Cyclin D3
G1/S Cyclins
Cyclin E1
S/G2 Cyclins
Cyclin A1
G2/M Cyclins
Cyclin B1
Fold Change (2^-ΔΔCt)
DMSO
JIB-04
GSK-J4
SD-70
BIX-1294
LLY-507
JQ-1
D)
HuH7
Hep3B
Hepa129
% of dead cells
Necrosis
Late Apoptosis
Early apoptosis
KDMs
KMTs
BRDs




A)
Depleted
Enriched
JIB-04
GSK-J4
SD-70
834
240
816
904
1730
58
397
612
334
584
728
1684
47
383
Depleted genes in common by JIB-04, GSK-J4 or SD-70
Enriched genes in common by JIB-04, GSK-J4 or SD-70
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
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
Cell Proliferation
Cell Death
Wnt Pathway
B)
Cell Proliferation
Cell Death
Wnt pathway
HuH7 Cells
Tumor vs normal
JIB-04
GSK-J4
SD-70
LOG2FC
>1.0
0.5
0
-0.5
<-1.0
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
Worst prognosis
Better prognosis
No difference
n=74
n=4
n=111
n=12
n=12
n=89
n=15
n=1
n=27
n=11
n=10
n=71
n=9
n=1
n=25
E)
CENPA
CCNB1
RPS27A
Percent survival
Low expression
High expression
P value
< 0.0001
0.0006
0.0125
Years



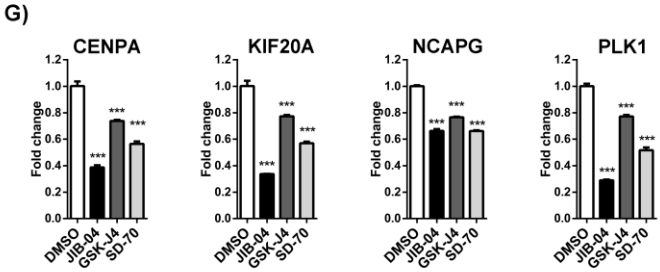


F)

$$\text{Predicted class } (Group_i) = \arg\min_{k=1..4}(Distance(sample_i, Group_k))$$

$$(Distance(sample_i, Group_k)) = \sum_{t=1..4} \frac{(RSEM(sample_i, gene_t) - \mu(Group_k, gene_t))^2}{\sigma(gene_t)}$$

μ		Group $_{k=1..4}$				σ
		Group 1	Group 2	Group 3	Group 4	
Gene $_{t=1..4}$	CENPA	12.2308	49.5708	112.403	251.913	1676.5
	KIF20A	45.1047	176.935	367.336	823.177	16256.3
	NCAPG	70.6232	247.396	473.431	1058.64	29456.9
	PLK1	50.0886	193.397	442.129	890.456	21139.6



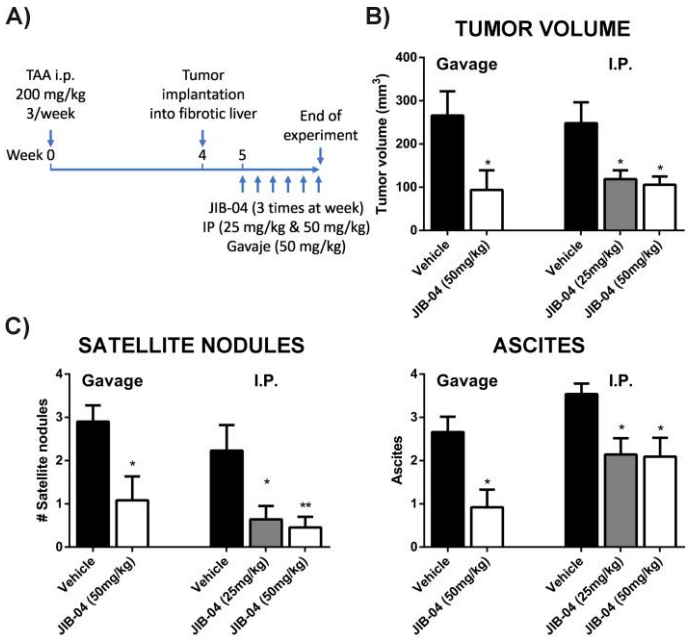


Table 1: Uni- and multi-variate analysis of overall survival TCGA and GSE14520 datasets

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 ****				.642
TNM Stage								
Stage I	RV			<0.001 ****				.159
Stage II	1.42	.87	2.31	0.16				.834
Stage III	2.67	1.75	4.07	<0.0001 ****				.084
Vascular invasion								
Negative				0.077	RV			0,007 **
Microscopic				0.633	1.348	.612	2.969	.458
Macroscopic				0.03 *	5.306	1.889	14.905	0,002 **
Surgical margin resection				0.327	.848			.357
Systemic adjuvant treatment	0.669	0.450	0.994	0.047 *	.076			.783
Signature								
Group 1	RV			<0.0001 ****	RV			0.006 **
Group 2	1.361	0.803	2.306	0.252	1.641	.605	4.450	.331
Group 3	2.001	1.225	3.266	0.006 **	2.231	.882	5.640	.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			<0.001 ****	RV			<0.001 ****
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	<0.001 ****	4.701	2.664	8.297	<0.001 ****
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								
HVB (yes/no)	0.0750	0.1154								
HVC (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 *

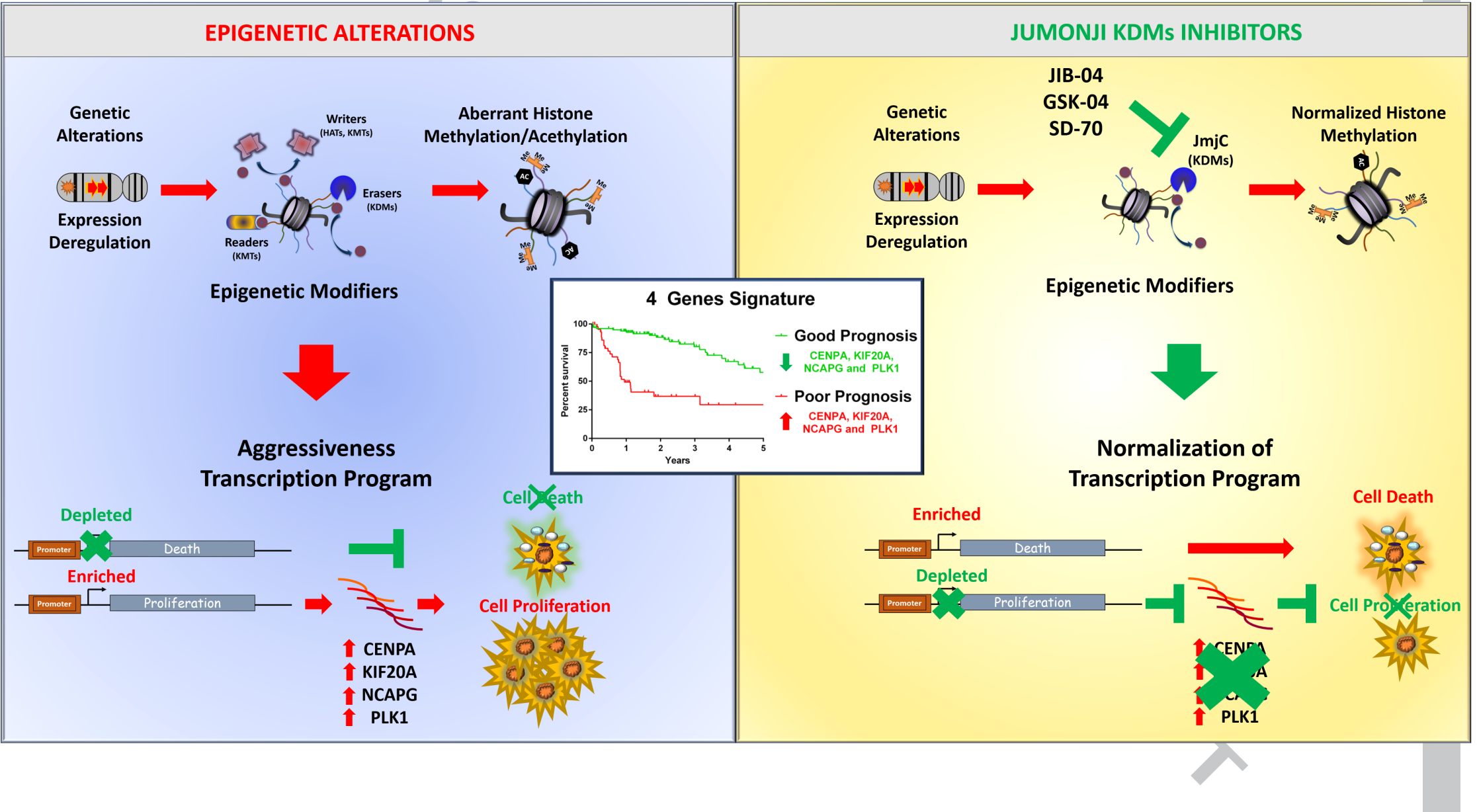
WT: wild type; Mut: mutant; NA: Not applicable

Mutations and expression alterations of epigenetic modifiers are frequent in HCC.

Jumonji lysine demethylases normalize aggressive transcriptions programs in HCC.

CENPA, KIF20A, NCAPG and PLK1 gene expression signature defines prognosis in HCC.

Epigenetic inhibitors are a new potential therapeutic tools for HCC.


EPIGENETIC ALTERATIONS
Genetic Alterations
Expression Deregulation
Writers (HATs, KMTs)
Erasers (KDMs)
Readers (KMTs)
Aberrant Histone Methylation/Acethylation
Epigenetic Modifiers
4 Genes Signature
Percent survival
Years
Good Prognosis
CENPA, KIF20A, NCAPG and PLK1
Poor Prognosis
CENPA, KIF20A, NCAPG and PLK1
Aggressiveness Transcription Program
Depleted
Promoter
Death
Enriched
Promoter
Proliferation
CENPA
KIF20A
NCAPG
PLK1
Cell Death
Cell Proliferation
JUMONJI KDMs INHIBITORS
JIB-04
GSK-04
SD-70
JmjC (KDMs)
Genetic Alterations
Expression Deregulation
Normalized Histone Methylation
Epigenetic Modifiers
Normalization of Transcription Program
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Promoter
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Cell Proliferation