Research Article
Hepatic and Biliary Cancer

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Identification and experimental validation of druggable epigenetic targets in hepatoblastoma

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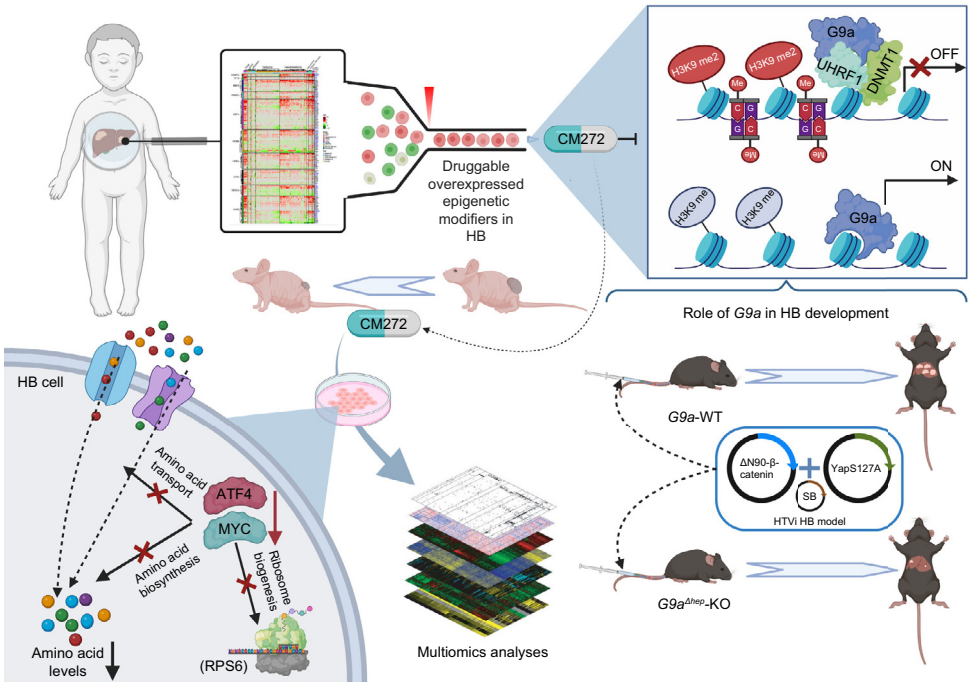
Graphical abstract



Highlights

- Hepatoblastomas display marked changes in the expression of epigenetic genes.
- Epigenetic transcriptome dysregulation correlates with poor clinical outcomes.
- The histone-methyltransferase G9a is a key gene in hepatoblastoma development.
- Targeting G9a activity abrogates metabolic reprogramming in hepatoblastoma cells.
- Epigenetic drugs may hold promise for the treatment of patients with hepatoblastoma.

Impact and implications

In spite of recent advances in the management of hepatoblastoma (HB), treatment resistance and drug toxicity are still major concerns. This systematic study reveals the remarkable dysregulation in the expression of epigenetic genes in HB tissues. Through pharmacological and genetic experimental approaches, we demonstrate that the histone-lysine-methyltransferase G9a is an excellent drug target in HB, which can also be harnessed to enhance the efficacy of chemotherapy. Furthermore, our study highlights the profound pro-tumorigenic metabolic rewiring of HB cells orchestrated by G9a in coordination with the c-MYC oncogene. From a broader perspective, our findings suggest that anti-G9a therapies may also be effective in other c-MYC-dependent tumors.

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Identification and experimental validation of druggable epigenetic targets in hepatoblastoma

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Background & Aims: Hepatoblastoma (HB) is the most frequent childhood liver cancer. Patients with aggressive tumors have limited therapeutic options; therefore, a better understanding of HB pathogenesis is needed to improve treatment. HBs have a very low mutational burden; however, epigenetic alterations are increasingly recognized. We aimed to identify epigenetic regulators consistently dysregulated in HB and to evaluate the therapeutic efficacy of their targeting in clinically relevant models.
Methods: We performed a comprehensive transcriptomic analysis of 180 epigenetic genes. Data from fetal, pediatric, adult, peritumoral (n = 72) and tumoral (n = 91) tissues were integrated. Selected epigenetic drugs were tested in HB cells. The most relevant epigenetic target identified was validated in primary HB cells, HB organoids, a patient-derived xenograft model, and a genetic mouse model. Transcriptomic, proteomic and metabolomic mechanistic analyses were performed.
Results: Altered expression of genes regulating DNA methylation and histone modifications was consistently observed in association with molecular and clinical features of poor prognosis. The histone methyltransferase G9a was markedly upregulated in tumors with epigenetic and transcriptomic traits of increased malignancy. Pharmacological targeting of G9a significantly inhibited growth of HB cells, organoids and patient-derived xenografts. Development of HB induced by oncogenic forms of β-catenin and YAP1 was ablated in mice with hepatocyte-specific deletion of *G9a*. We observed that HBs undergo significant transcriptional rewiring in genes involved in amino acid metabolism and ribosomal biogenesis. G9a inhibition counteracted these pro-tumorigenic adaptations. Mechanistically, G9a targeting potently repressed the expression of c-MYC and ATF4, master regulators of HB metabolic reprogramming.
Conclusions: HBs display a profound dysregulation of the epigenetic machinery. Pharmacological targeting of key epigenetic effectors exposes metabolic vulnerabilities that can be leveraged to improve the treatment of these patients.

Introduction

Hepatoblastoma (HB) is the most common pediatric liver malignancy and its incidence has tripled over the past 30 years.[1] Significant advances in the clinical management of patients with HB have recently been achieved thanks to the efforts of different international consortia.[2] An efficient combination of surgery and chemotherapy at early disease stages may result in a 5-year survival rate of over 80% for these patients.[1] However, those diagnosed with advanced unresectable tumors, lesions that remain inoperable after chemotherapy, or recurrent disease face a much worse prognosis.[1,2] On the other hand, currently implemented chemotherapy may result in lifelong severe toxic effects. Therefore, a better understanding of the pathobiology of HB is still needed to provide more effective treatments, particularly for the most aggressive forms.[3]

From a genetic perspective, HBs display the lowest rate of somatic mutations across childhood cancers.[4] Indeed, HBs only harbor an average of 2.9 mutations per tumor, and most of these alterations consist in activating mutations or deletions of the *CTNNB1* gene encoding β-catenin, which are present in over 70% of cases in association with higher malignancy.[5] The second most frequently mutated gene, ~10% of cases, is *NFE2L2*.[6,7] Transcriptomic studies identified distinct HB subclasses corresponding to tumors representing different stages of liver development or differentiation and aggressiveness.[8–11] The paucity of genetic alterations in HB, together with the

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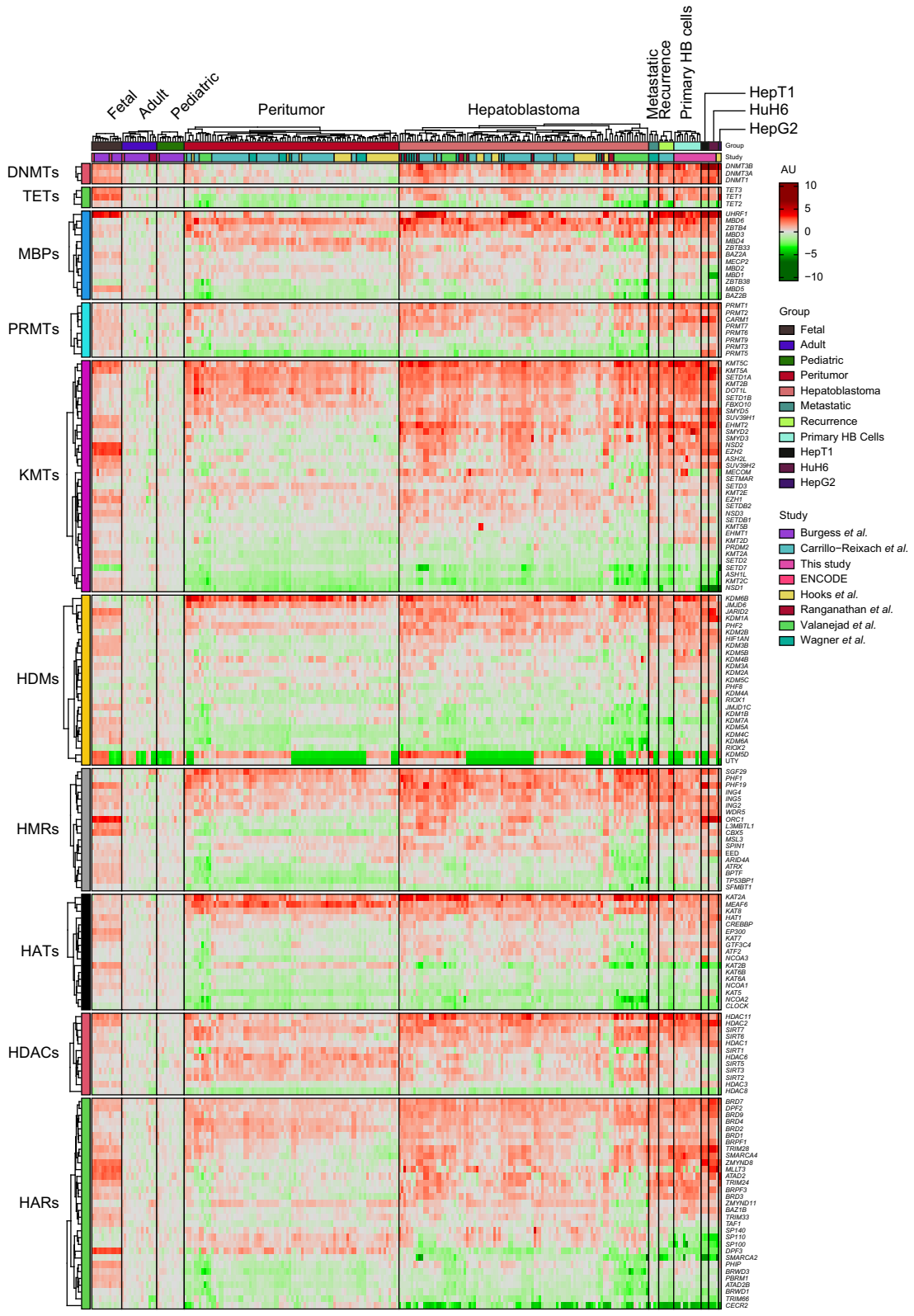

Fig. 1. Heatmap showing the expression of epigenetic modifiers in healthy liver tissues, peritumoral liver tissues, HB tissues and HB cells. Expression levels (RNA-sequencing data) are relative to those in healthy pediatric livers. Epigenetic modifiers are functionally grouped as DNMTs, TETs, MBPs, PRMTs, KMTs, HDMs, HMRs, HATs, HDACs and HARs. Transcriptomic data are integrated from the indicated studies. DNMTs, DNA methyltransferases; HARs, histone acetyl-readers; HATs, histone acetyltransferases; HB, hepatoblastoma; HDACs, histone deacetylases; HDMs, histone-lysine demethylases; HMRs, histone methyl-readers; KMTs, histone-lysine methyltransferases; MBPs, DNA-methyl-binding proteins; PRMTs, protein-arginine methyltransferases; TETs, DNA demethylases.

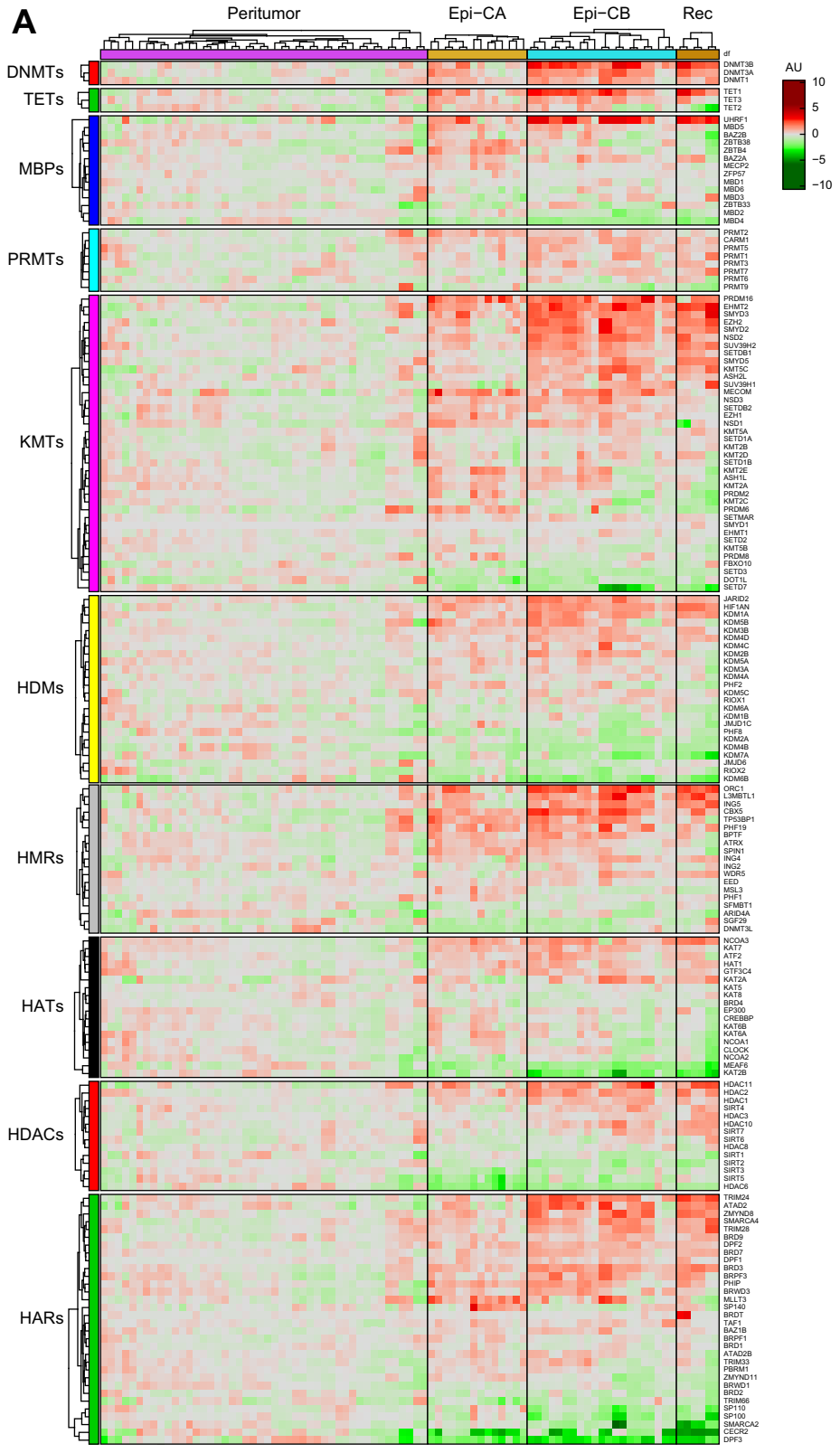

Fig. 2. Heatmap showing the expression of epigenetic modifiers in peritumoral liver tissues and HB tissues classified according to: (A) Genome-wide DNA methylation status in the epigenetic clusters in Epi-CA and Epi-CB.[16] (B) Transcriptomic molecular subgroups C1, C2A and C2B defined in Ref. [10]. Epi-CA/B, epigenetic cluster A/B; HB, hepatoblastoma.

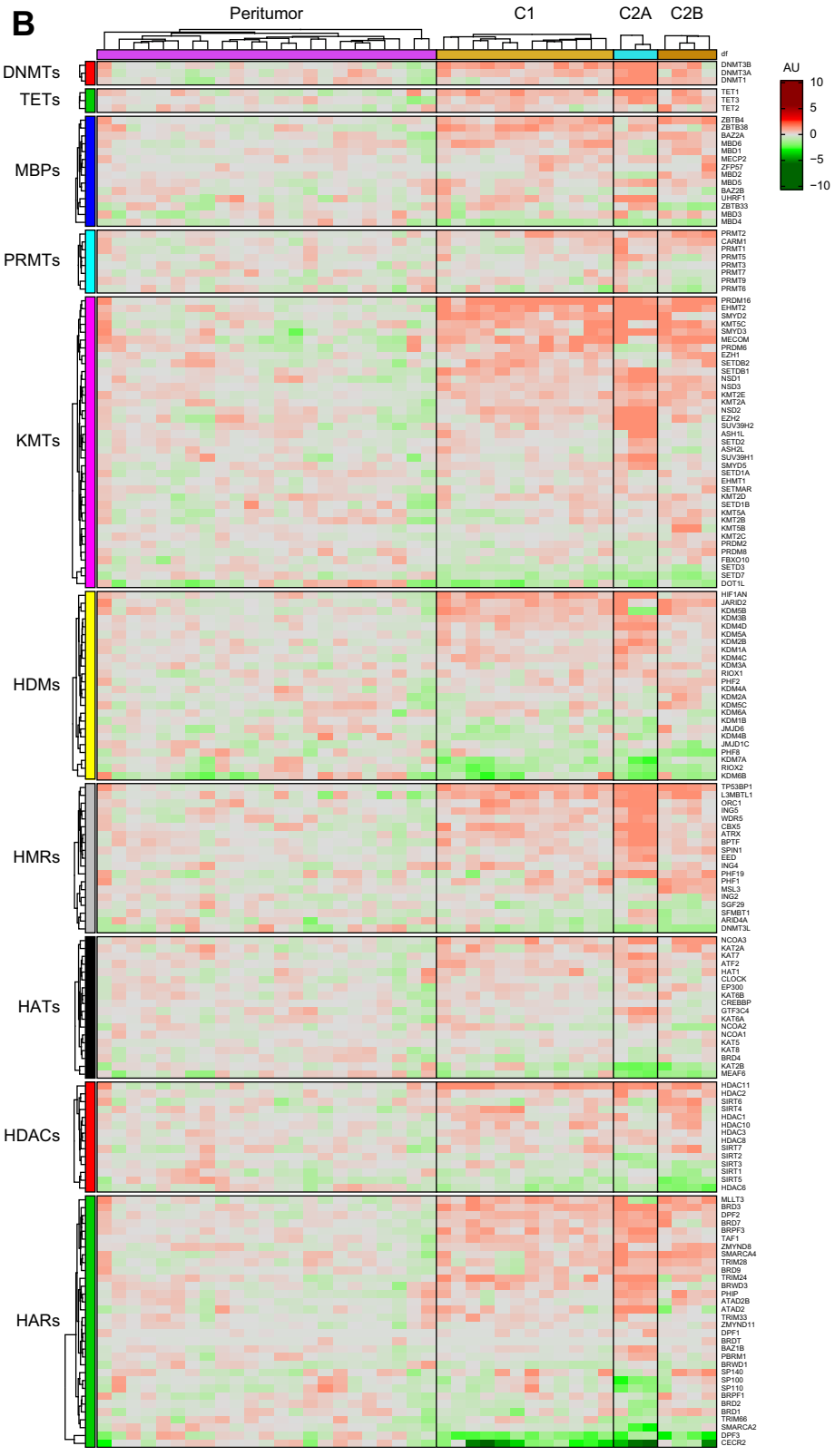

Fig. 2. Continued.

identification of robust transcriptomic subclasses suggest that mechanisms other than structural genetic variations likely play important roles in HB development. Dysregulation of epigenetic mechanisms is currently recognized to participate in carcinogenesis in many organs, including the liver.[12] Earlier studies in HB already identified alterations in genomic DNA methylation, finding low levels of global DNA methylation and hypermethylation at the promoters of putative tumor suppressor genes.[13–15] Importantly, more recent works including a higher number of patients with HB have provided comprehensive DNA-methylome analyses, enabling the establishment of epigenetic clusters with histological and clinical correlations,[16]

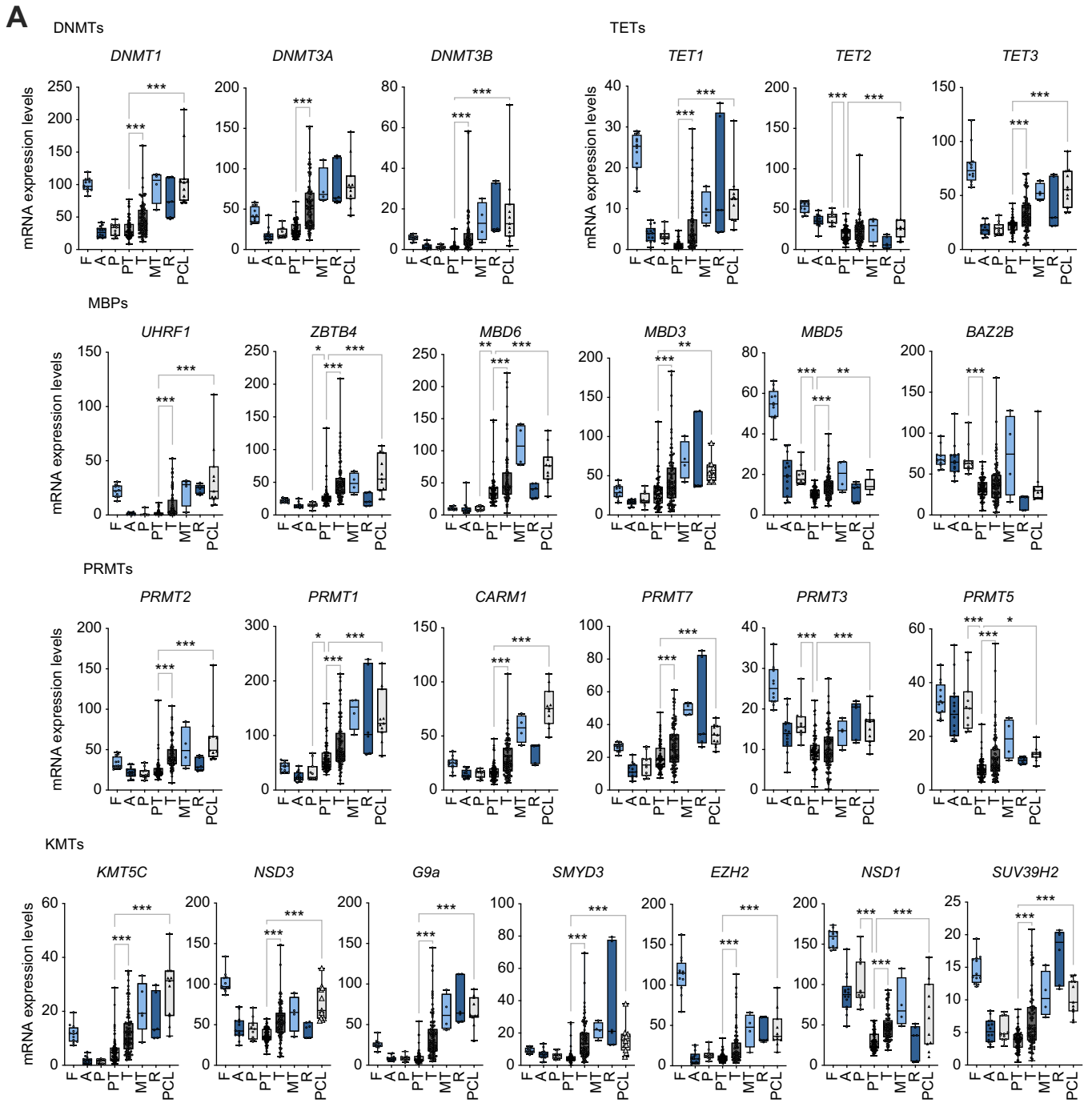

Fig. 3. Expression of selected epigenetic modifiers in healthy liver tissues, peritumoral liver tissues, HB tissues and cultured HB cells. Expression levels in fetal (F), adult (A), peritumor (PT), tumor (T), metastatic tissues (MT), recurrent tumors (R), and primary HB cell lines (PCL) relative to those found in pediatric livers (P). Data are individual values and means ± SD. Non-parametric Kruskal-Wallis ANOVA test was used. *$p<0.05$, **$p<0.01$, ***$p<0.001$. DNMTs, DNA methyltransferases; HARs, histone acetyl-readers; HATs, histone acetyltransferases; HB, hepatoblastoma; HDACs, histone deacetylases; HDMs, histone-lysine demethylases; HMRs, histone methyl-readers; KMTs, histone-lysine methyltransferases; MBPs, DNA-methyl-binding proteins; PRMTs, protein-arginine methyltransferases; TETs, DNA demethylases.

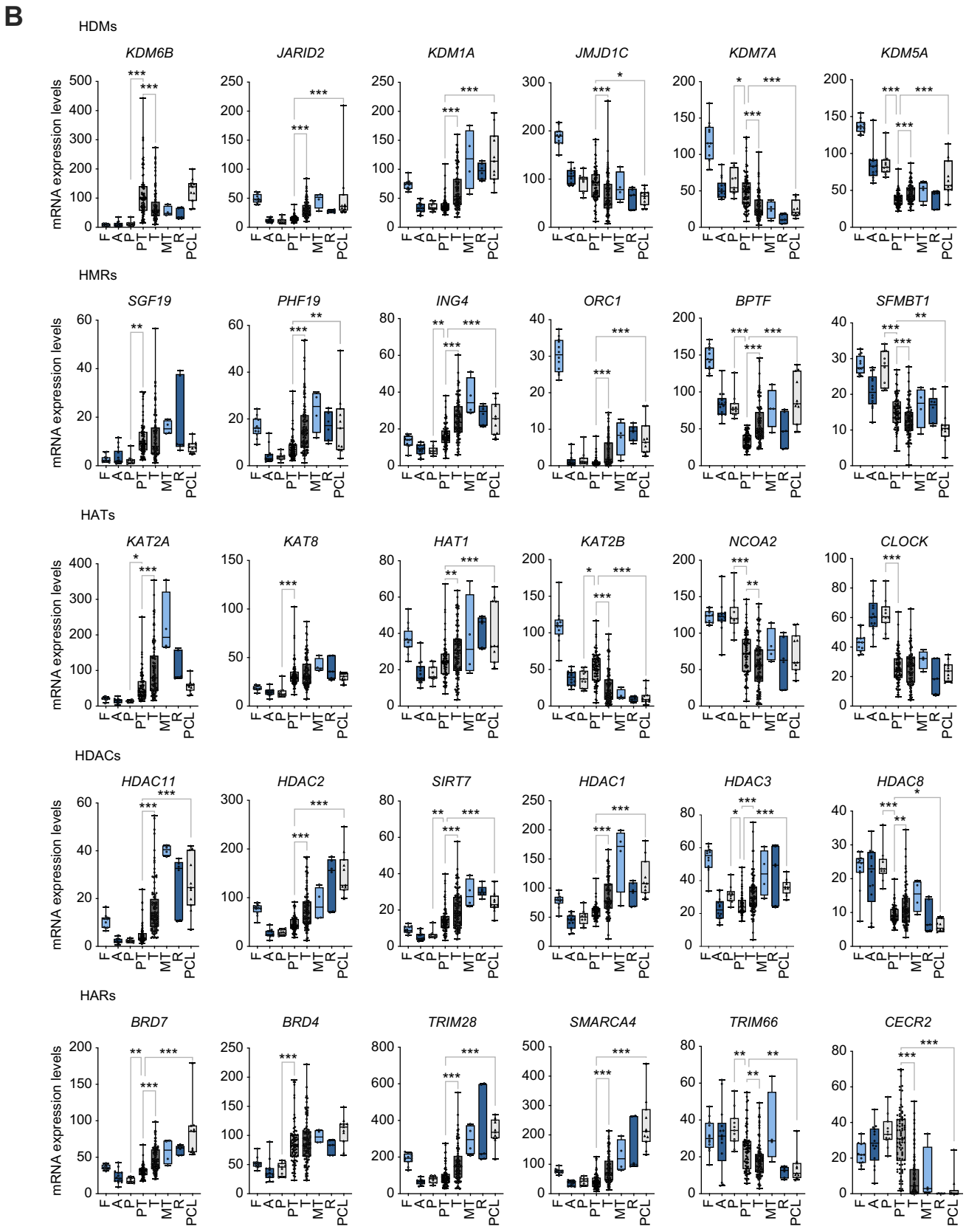

Fig. 3. Continued.

Table 1. GI_{50} of epigenetic inhibitors in HB cell lines.

	HB cell line inhibitor	HuH6 (μM)	HepT1 (μM)	HepG2 (μM)
DNMT	**DNMTi** (5-Azacytidine)	8.31	11.6	15.3
PRMT	**PRMT1i** (EPZ019997)	>50	>50	>50
KMT	**NSD3i** (BI-9321)	>100	>100	75
	G9ai (BIX01246)	2.67	5.71	1.7
	G9ai (UNC0642)	4.1	4.5	4.4
	G9ai + DNMT1i (CM272)	0.130	0.290	0.495
	SMYD3i (EPZ031686)	>100	>100	>100
	EZH2i (UNC1999)	5.8	9.2	7.7
	SUV39H2i (OTS186935)	3.8	3.6	2.9
HDM	**KDM1Ai** (Ladademstat)	20.3	16.2	16.5
HDAC	**HDAC1i** (Entinostat)	9.1	6.5	5.0
HAR	**BRD4i** (JQ1)	17.8	20.68	17.8

DNMT, DNA methyltransferase; HB, hepatoblastoma; HAR, histone acetyl-reader; HDAC, histone deacetylase; HDM, histone-lysine demethylases; KMT, histone-lysine methyltransferase; PRMT, protein-arginine methyltransferases.

that have been further validated.[11,17,18] Notably, these epigenomic traits were markedly associated with the previously defined transcriptomic subclasses, supporting the significance of epigenetic dysregulation in HB pathogenesis.

The ultimate causes leading to DNA methylation abnormalities in HB are not completely understood. Recent studies described the upregulation of DNA methyltransferases 1 and 3A (DNMT1, DNMT3A) and the DNMT adaptor protein UHRF1, along with the DNA demethylases TET1 and TET2 in HB.[16,19,20] Besides DNA methylation, epigenetic mechanisms regulating gene expression encompass other molecular processes including chromatin remodelers, non-coding RNAs and the covalent modification of histones.[12,20,21] Histone modifications comprise a growing list of reversible post-translational modifications (PTMs), such as acetylation, methylation, phosphorylation and sumoylation, among others.[22] Histone PTMs often work in concert with other epigenetic mechanisms like DNA methylation to control the recruitment of remodeling complexes and transcription factors.[22] As occurs for DNA methylation, histone PTMs are introduced, removed and recognized by a complement of epigenetic modifiers known as epigenetic writers, erasers and readers.[12] Unraveling the intricate processes of epigenetic regulation is important not only to understand carcinogenic mechanisms, but also to elucidate novel therapeutic strategies. Indeed, a variety of "epidrugs" targeting epigenetic effectors are actively being developed with promising preclinical antitumoral results, including sensitization to chemotherapy.[21] Regarding HB, some studies highlighted the inhibitory potential of epigenetic drugs targeting histone deacetylases or the acetylated histone reader BRD4.[20,23] In this work we performed an integrative transcriptional analysis of 180 epigenetic modifiers in a broad set of tumor tissues and evaluated the antitumoral effects of the inhibition of selected targets in relevant HB models. Our findings underscore the profound dysregulation of epigenetic mechanisms in HB and their involvement in key aspects of tumor biology. We have also identified new vulnerabilities in the metabolic reprogramming of HB cells that can be tackled with epigenetic inhibitors.

Materials and methods

Transcriptomic data

Transcriptomic data were obtained from the following sources: GSE133039:[16] 34 tumoral and 32 peritumoral tissues; GSE104766:[10] 19 tumoral and 23 peritumoral tissues; GSE81928:[24] 21 tumoral and 6 peritumoral tissues; GSE75271:[9] 50 tumoral and 5 peritumoral tissues; GSE111845:[25] 10 fetal, 10 pediatric and 10 adult healthy liver tissues; GSE89775:[26] 10 tumoral and 3 healthy pediatric liver tissues. GSE151347:[27] 7 tumoral, 11 peritumoral and 4 metastasized primary tumors. ENCODE: 2 fetal, 1 pediatric and 1 adult healthy liver tissues.

Human tissue samples

Written informed consent was obtained from each patient. The study was approved by the Human Ethics Committee of the Hospital Universitari Germans Trias i Pujol, Badalona, Spain (protocol PI15-057), according to the 1975 Declaration of Helsinki guidelines.

Additional information is provided in the supplementary materials.

Results

Landscape of epigenetic gene expression in healthy liver, peritumoral liver and HB tissues

We performed a combined analysis of three previous RNA-sequencing studies comprising a total of 72 peritumoral and 91 tumoral HB tissues, four samples of metastasized tumors, plus six samples of recurrent tumors.[10,16,24] For comparisons, transcriptomes from normal human fetal, pediatric and adult liver tissues, as well as 11 primary HB cell lines and three established HB cell lines (HepG2, HuH6 and HepT1), were integrated. Gene expression in healthy pediatric liver samples was used as reference. We analyzed 180 genes from three different categories: i) epigenetic writers: DNMTs, histone-

Table 2. GI_{50} of CM272 in HB primary cell lines.

PDX cell line	CM272 (nM)
HB-279	1,700
HB-282	420
HB-284-M	760
HB-243	796
HB-295	400
HB-303	530
HB-214	540
HB-229	1,600
HB-233	280
HB-305	445
HB-310	375

HB, hepatoblastoma; PDX, patient-derived xenograft.

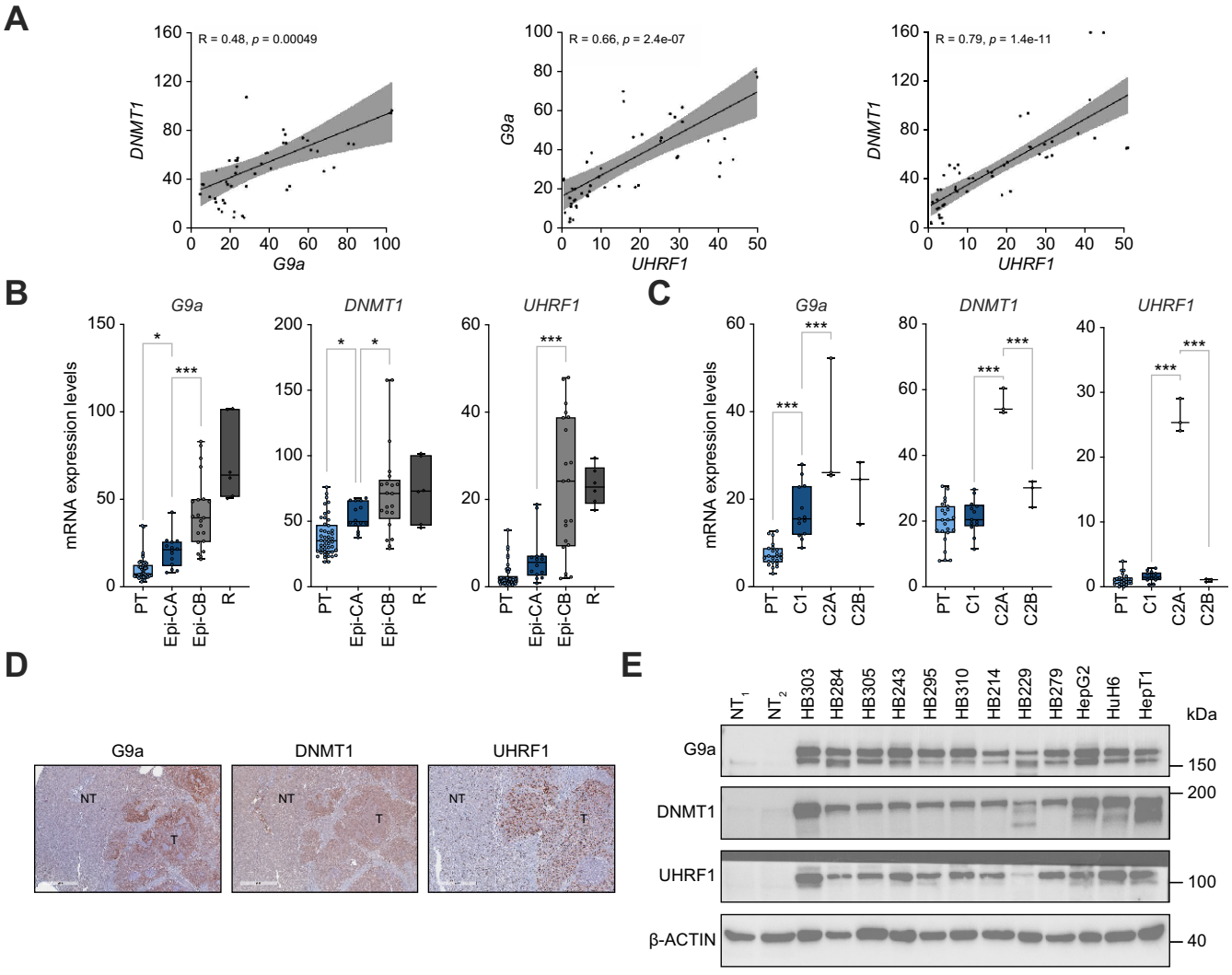

Fig. 4. Analysis of *G9a*, *DNMT1*, and *UHRF1* expression in HB. (A) Spearman correlation analyses of *G9a*, *DNMT1*, and *UHRF1* mRNA levels in HB tissues. The regression coefficient (R) and *p* value of each correlation are indicated. (B) *G9a*, *DNMT1* and *UHRF1* mRNA levels in peritumoral tissues (PT), HB tissues classified into the Epi-CA and Epi-CB epigenetic groups and in recurrent tumors (R). (C) *G9a*, *DNMT1* and *UHRF1* mRNA levels in peritumoral tissues (PT), HB tissues classified into the C1, C2A and C2B transcriptomic groups. **p* <0.05, ****p* <0.001. (D) Representative immunohistochemistries of G9a, DNMT1, and UHRF1 in HB tissues. Tumoral (T) and non-tumoral (NT) areas. (E) Western blot analysis of G9a, DNMT1 and UHRF1 proteins in nine primary human HB cell lines and three established HB cell lines. Samples of pediatric non-tumoral liver tissue (NT_1 and NT_2) are included. Data are individual values and means ± SD. Non-parametric Kruskal-Wallis ANOVA test was used. Epi-CA/B, epigenetic cluster A/B; HB, hepatoblastoma. (This figure appears in color on the web.)

lysine methyltransferases (KMTs), protein-arginine methyltransferases (PRMTs), histone acetyl-transferases (HATs); ii) epigenetic erasers: DNA demethylases (TETs), histone-lysine demethylases (HDMs), histone deacetylases (HDACs); and iii) epigenetic readers: DNA-methyl-binding proteins (MBPs), histone methyl-readers (HMRs) and histone acetyl-readers (HARs) (Table S1).[12] As observed in Fig. 1, and in an additional cohort of patients[9] (Fig. S1), marked changes in the expression of numerous epigenetic genes, mostly upregulation, were observed between HB samples and peritumoral tissues. Interestingly, many genes upregulated in HB were also highly expressed in fetal liver compared to pediatric liver. Overall, these differences were preserved in primary HB cell lines. A recent study identified two distinct epigenetic clusters in HB (Epi-CA and Epi-CB) according to genome-wide DNA methylation status.[16] As compared to Epi-CA tumors, Epi-CB tumors have a more profound dysregulation of DNA methylation, a high-risk transcriptomic signature and are associated with a worse prognosis.[16] The expression of epigenetic genes in tumors classified as Epi-CA and Epi-CB was more significantly altered in the latter group (Fig. 2A, Fig. S2A). Consistently, overall expression of epigenetic genes was more dysregulated in the transcriptomic subgroup C2A defined by Hooks *et al.*,[10] which identifies highly proliferative and high-risk tumors, compared to C1 tumors with good prognosis and C2B tumors of intermediate risk (Fig. 2B, Fig. S2B).

Expression of genes selected according to their alteration in HB tissues is shown in Fig. 3A,B. Although transcriptional downregulation in tumor tissues was observed in some cases, increased expression was prevalent. In all categories we identified genes whose expression was high in the fetal liver, became reduced in healthy pediatric and adult organs, and was

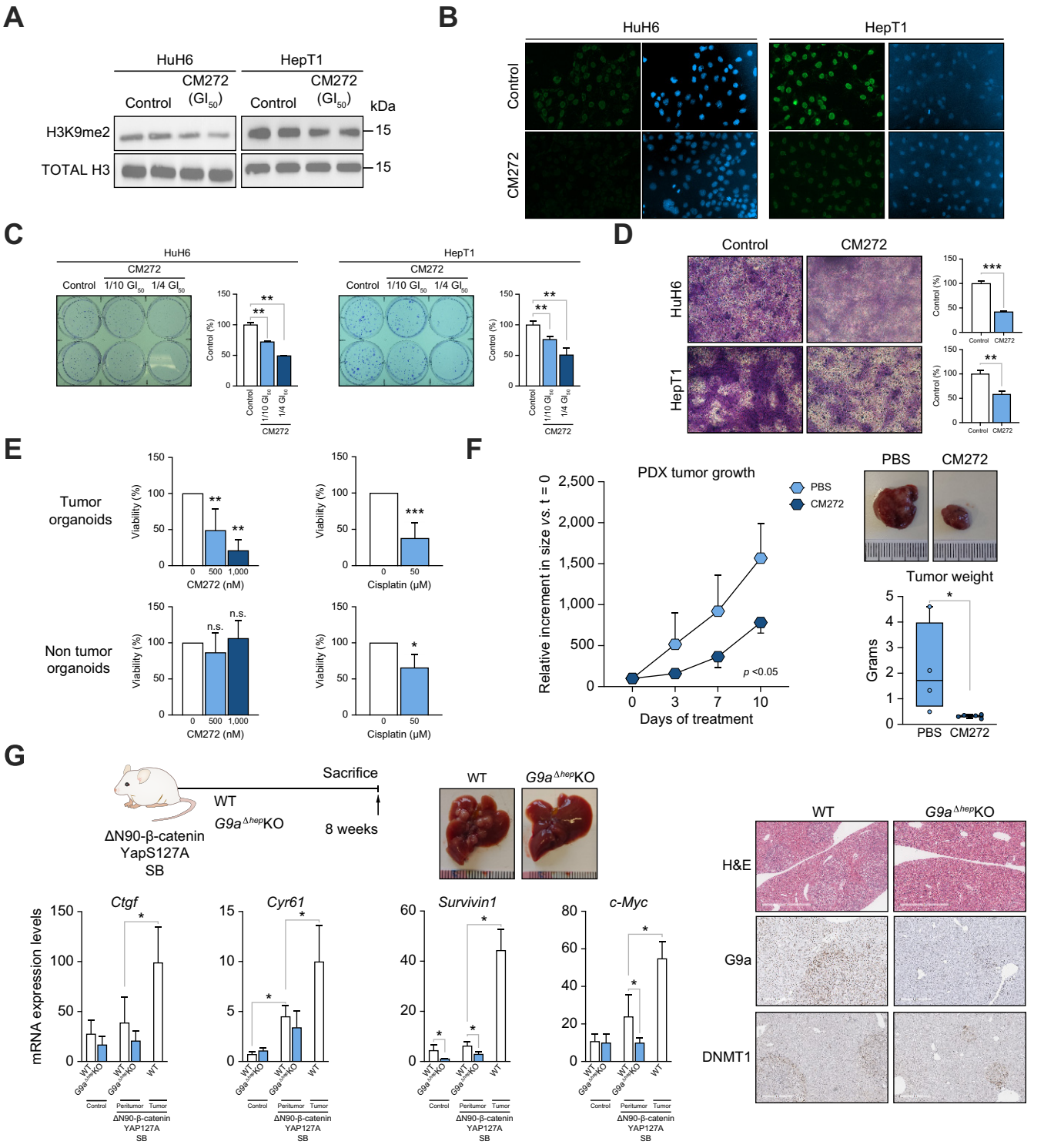

Fig. 5. Antitumoral effects of CM272 and role of G9a in genetic mouse HB model. (A) Effect of CM272 treatment (48 h) on the levels of H3K9me2 in HuH6 and HepT1 cells. (B) Effect of CM272 treatment (48 h) on the global levels of DNA CpG methylation in HuH6 and HepT1 cells. (C) Colony formation assays in HuH6 and HepT1 cells treated with CM272 at their respective GI_{50}. (D) Effect of CM272 on HuH6 and HepT1 cells migration. (E) Effect of CM272 on the viability of HB tumoroids and healthy pediatric hepatocyte organoids. (F) Effect of CM272 on the growth of HB-303 primary HB cells subcutaneously implanted into nude mice. Representative images of tumors and tumor weight at the end of treatments are shown. (G) Effect of constitutively active YAP1 (S127A-YAP1) and β-catenin (ΔN90-β-catenin) coexpression on HB development in WT and G9a$^{\Delta hep}$KO mice. Macroscopic images of livers from WT and G9a$^{\Delta hep}$KO mice are shown. Representative H&E, and immunohistochemical stainings for G9a and DNMT1 are shown. Hepatic expression of HB relevant genes in WT and G9a$^{\Delta hep}$KO mice (uninjected), plasmid injected non-tumor tissues (G9a$^{\Delta hep}$KO) and tumor tissues (WT) are shown. Data are means ± SD. Paired two-tailed Student's *t* tests were used (C–F). Non-parametric Kruskal-Wallis ANOVA test was used (G). **p* <0.05, ***p* <0.01, ****p* <0.001. HB, hepatoblastoma; KO, knockout; WT, wild-type. (This figure appears in color on the web.)

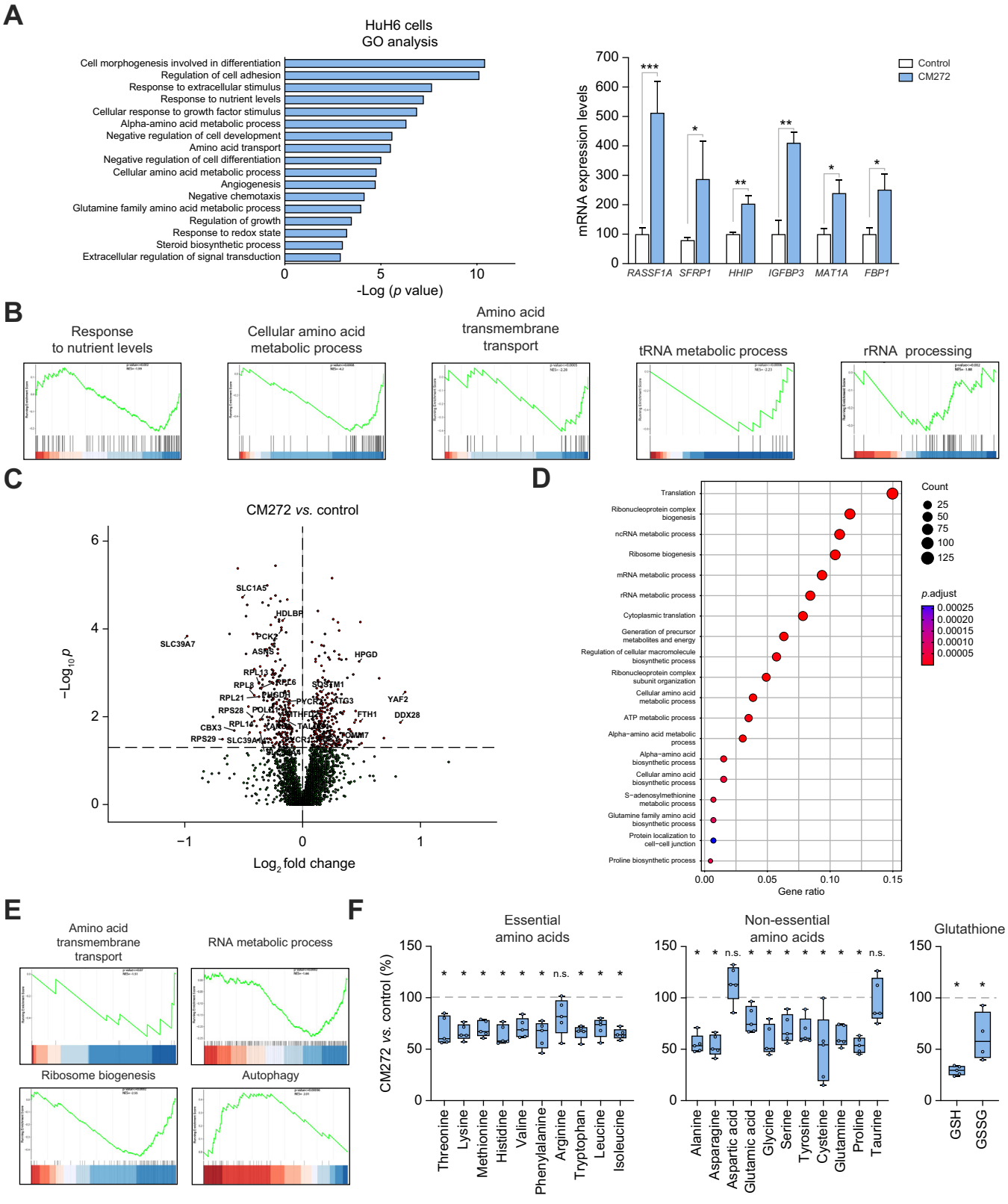

Fig. 6. Mechanisms involved in the antitumoral effects of CM272 in HB. (A) Most relevant GO functional categories of genes undergoing changes in expression identified by RNA-sequencing in HuH6 cells treated with CM272 (GI_{50}, 48 h). Right panel shows the effect of CM272 (GI_{50}, 48 h) on the expression of tumor suppressor genes epigenetically downregulated in HB. **p* <0.05, ***p* <0.01, ****p* <0.001. (B) GSEA revealed that CM272 inhibited the expression of genes involved in nutrient, amino acid, tRNA, and rRNA metabolism in HuH6 cells. (C) Volcano plot of the proteins differentially expressed in HuH6 cells treated with CM272 (GI_{50}, 48 h). Selected functionally relevant proteins are indicated. (D) Most relevant GO categories of proteins undergoing changes in expression in HuH6 cells treated with CM272. (E) GSEA confirming inhibition of the expression of proteins involved in amino acid and RNA metabolism, and ribosome biogenesis, and enrichment of the expression of

induced in tumors, including metastatic and recurrent tissues. We confirmed the recently described upregulation of *DNMT1*, *DNMT3A*, *DNMT3B*, *TET1*, *TET2*, *TET3*, *UHRF1*, *EZH2*, *HDAC1* and *HDAC2* in HB tissues.[16,19,23,28,29] We also observed the marked induction of other epigenetic genes involved in tumorigenic processes, including *MBD3*, *PRMT1*, *KMT5C*, *G9a*, *NSD3*, *SMYD3*, *SUV39H2*, *JARID2*, *KDM1A*, *KAT2A*, *HDAC11*, *SIRT7*, *BRD4* and *SMARCA4*[30–36] among others. Interestingly, many genes overexpressed in HB samples were already upregulated in peritumoral tissues compared to healthy pediatric livers (*e.g. ZBTB4*, *MBD6*, *PRMT1*, *KMTC5C*, *KDM6B*, *SGF19*, *ING4*, *KAT8*, *KAT2B*, *SIRT7*, *BRD7*, *BRD4*).

Pharmacological targeting of epigenetic regulators in HB cells

We next evaluated the antiproliferative potential of selected available epigenetic drugs in HB cell lines. We tested molecules targeting different classes of epigenetic modifiers significantly upregulated in tumor tissues, such as: DNMTs, KMTs (SUV39H2, SMYD3, G9a, NSD3, EZH2), KDMs (KDM1A), HATs (KAT2A), HDACs (HDAC1/3), PRMTs (Type-I PRMTs) and HARs (BRD4). Albeit some inhibitors had limited efficacy (GI_{50} 20-100 μM), others showed GI_{50} values in the low micromolar range (Table 1). Most effective compounds were among KMTs inhibitors, and those targeting the KMT G9a stood out. The best response was obtained with CM272, a potent substrate-competitive inhibitor of G9a that also targets DNMT1.[37] In view of this, we tested CM272 in 11 well-characterized patient-derived HB cell lines,[38] validating a robust growth-inhibitory effect (Table 2).

Characterization of G9a and DNMT1 as epigenetic targets in HB

Epigenetic effectors undergo extensive functional crosstalk among themselves and with other transcriptional regulators.[12,39] This has been well described for G9a, DNMT1, and the epigenetic scaffold UHRF1 in different tumor types.[19,39–41] We found a significant positive correlation between the expression of *G9a*, *DNMT1* and *UHRF1* mRNA levels in HB tissues (Fig. 4A). Moreover, the expression of these three genes was higher in tumors within the epigenetic cluster Epi-CB and the transcriptomic group C2A, which include patients with poorer prognosis[10,16](Fig. 4B,C). Increased levels of G9a, DNMT1 and UHRF1 proteins, and the G9a-mediated H3K9me2 histone mark,[41] were also validated by immunohistochemistry in representative HB tissues *vs.* peritumoral parenchyma (Fig. 4D, Fig. S2C), and in HB cell lines (Fig. 4E).

These findings, together with the observed antiproliferative efficacy of G9a inhibitors, particularly CM272, on HB cells supported an oncogenic role for G9a and DNMT1. Thus, we further evaluated the effects of CM272 on HB cells and relevant *in vitro* and *in vivo* models. Consistent with its pharmacological targets, CM272 treatment decreased total levels of H3K9me2 and DNA methylation (Fig. 5A,B, Fig. S3A). CM272 markedly inhibited HB cells' clonogenic and migratory capacities (Fig. 5C,D), without inducing apoptosis (not shown). Cisplatin-based therapy is regularly implemented in patients with HB; however, resistance frequently occurs or develops.[1–3] Epigenetic drugs may increase the therapeutic response to chemotherapy.[21,22] Thus, we tested the effect of CM272 on cisplatin efficacy in HuH6 and HepT1 cells. We found that cisplatin GI_{50} was reduced by 59% (from 2.7 to 1.1 μg/ml) and 62% (from 3.2 to 1.2 μg/ml) in HuH6 and HepT1 cells, respectively. Moreover, CM272 had a synergistic growth-inhibitory effect when combined with cisplatin or the PARP inhibitor olaparib (Fig. S3B,C). We also evaluated the antitumoral efficacy of this molecule in human HB organoids. CM272 inhibited organoids' growth at significantly lower concentrations than cisplatin (Fig. 5E). Importantly, as opposed to cisplatin, it had no effect on healthy organoids (Fig. 5E).

The antitumoral properties of CM272 were also validated in a xenograft mouse model with a primary HB cell line from a well-characterized tumor[38] (Fig. 5F). Tumor growth inhibition was accompanied by a tendency to reduced mitotic figures (14–54 *vs.* 13–45 mitoses/20x field) and increased areas of necrosis in treated mice, while no differences were found in apoptotic cells (not shown). No signs of toxicity such as weight loss or serum parameters of liver and kidney injury were observed in treated mice (not shown). The significant antiproliferative efficacy of G9a inhibitors in HB cells suggests that this KMT plays an important role in HB. Therefore we evaluated tumor development in mice expressing constitutively active forms of β-catenin (ΔN90-β-catenin) and YAP1 (YapS127A) upon tail vein injection of hydrodynamic plasmids, a recognized model for HB,[42] using wild-type (WT) mice, and mice with hepatocyte-specific deletion of *G9a* (*G9a*$^{\Delta hep}$KO) (Fig. 5G). We observed that while WT mice had tumor-laden livers, *G9a*$^{\Delta hep}$KO animals showed very few and smaller nodules (Fig. 5G, Fig. S4A, B). Lesions in WT mice had more prominent cellular crowding and more frequent mitoses (Fig. S4C). G9a and DNMT1 proteins were readily detected in tumor nodules (Fig. 5G). Expression of genes involved in HB development and known to be induced in this mouse model, such as *Ctgf*, *Cyr61*, *Survivin1* and *c-Myc*,[42] was markedly induced in WT mice tumors, but not in *G9a*$^{\Delta hep}$KO liver tissues (Fig. 5G). Consistently, RNA-sequencing analyses in this genetic model indicated that G9a was a key determinant in the transcriptomic rewiring observed in HB, and other pediatric tumors, including hepatocellular dedifferentiation and the establishment of a c-MYC signature characteristic of most aggressive HBs (Fig. S5A,B).

Mechanisms involved in the antitumoral effects of CM272 in HB

In view of the marked inhibition of tumorigenesis triggered by ΔN90-β-catenin and YapS127A expression in *G9a*$^{\Delta hep}$KO mouse livers, we first evaluated if CM272 had a direct effect on the activity of these pathways, or if it affected the expression of β-catenin and YAP inhibitors in HB cells. However, CM272 treatment did not affect β-catenin nor YAP nuclear

autophagy-related proteins in CM272-treated HuH6 cells. (F) Metabolomic analysis of HuH6 cells treated with CM272 (GI_{50}, 48 h). Levels of amino acids, GSH and GSSG relative to untreated controls are shown. *p <0.05. Data are means ± SD. Paired two-tailed Student's t tests were used (A). NES and significance are shown (B, E). Data are individual values with means ± SD. Paired two-tailed Student's t tests were used (F). GO, gene ontology; GSH, reduced glutathione; GSSG, oxidized glutathione; HB, hepatoblastoma; NES, normalized enrichment score. (This figure appears in color on the web.)

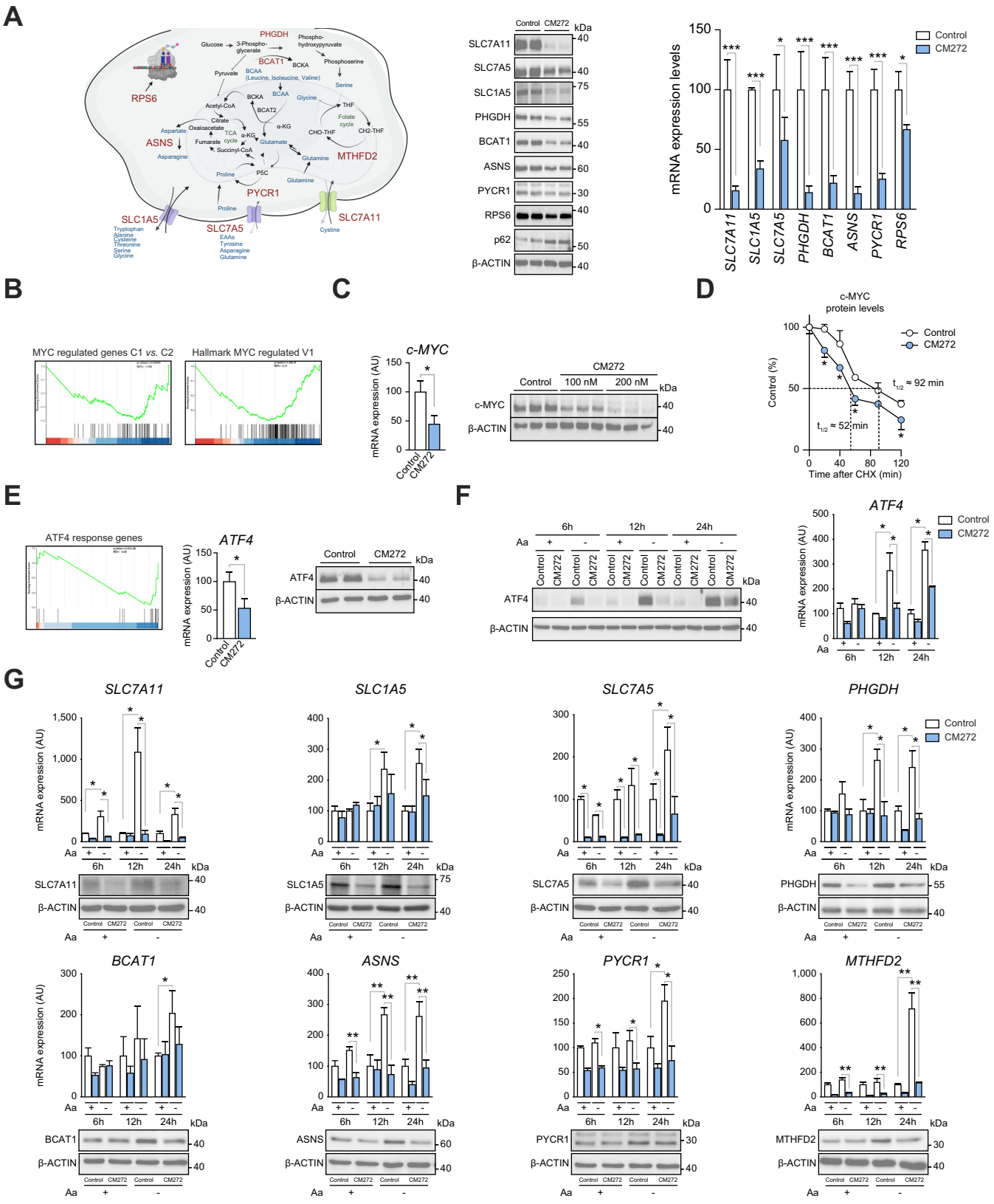

Fig. 7. CM272 inactivates the metabolic rewiring of HB cells that supports tumor growth. (A) Diagram representing key cellular pathways and mechanisms activated in cancer counteracted by CM272. Right panels show the effects of CM272 (GI_{50}, 48 h) in HuH6 cells on the expression of relevant metabolic genes commonly upregulated in tumors, and p62. (B) GSEA revealed that CM272 inhibits c-MYC transcriptional activity in HuH6 cells. (C) Effects of CM272 (GI_{50}, 48 h) on c-MYC mRNA and protein levels in HuH6 cells. (D) Effect of CM272 on c-MYC protein stability in HuH6 cells. (E) GSEA of the effect of CM272 (GI_{50}, 48 h) on ATF4-driven gene expression, and ATF4 mRNA and protein levels in HuH6 cells. (F) CM272 pretreatment (GI_{50}, 24 h) blunts the upregulation of *ATF4* expression in response to

translocation, and with the exception of *SFRP1* (see below) it did not increase the expression of negative modulators of these pathways (data not shown). To explore the antitumor mechanisms of CM272, we examined the transcriptional responses of HuH6 and HepT1 cells. In agreement with the observed antiproliferative and anticlonogenic activities, gene ontology (GO)-based functional classification of differentially expressed genes identified general categories related to cell growth, differentiation and interaction with the cellular microenvironment (Fig. 6A). According to the pharmacological activity of CM272, the expression of *RASSF1A*, *HHIP*, *SFRP1*, *IGFBP3*, *MAT1A* and *FBP1*, tumor suppressors and key metabolic genes epigenetically repressed in HB[19,20] were induced (Fig. 6A, Fig. S6A), and this response was accompanied by a decrease in the levels of the G9a-mediated H3K9me2 repressive mark in their proximal promoters (Fig. S6B). Perhaps more interestingly, and aligned with categories identified by GO, gene set-enrichment analysis (GSEA) revealed significant changes in the expression of genes involved in the response to nutrient levels, amino acid transport and metabolism, mRNA translation and rRNA processing (Fig. 6B). These transcriptional effects were also captured by proteomic studies in HuH6 cells. We identified 504 differentially expressed proteins, of which 264 were upregulated and 240 downregulated. Downregulated proteins included membrane transporters (SLC1A5, SLC39A7, SLC39A14), enzymes involved in amino acid metabolism (PHGDH, PYCR1, PYCR2, ASNS), and other metabolic pathways (TALDO1, HDLBP, PCK2, MTHFD2), ribosomal proteins (RPL13, RPL21, RPL14, RPL8) and additional proteins commonly upregulated in cancer (POLD1, CBX3, YARS1) (Fig. 6C). Among the upregulated proteins, we found enzymes (AHCY, HPGD) and negative regulators of tumor growth (FTH1, DDX28, YAF2) frequently repressed in cancer. Interestingly, we also detected increased levels of key autophagy proteins (TOMM7, SQSTM1, ATG3) (Fig. 6C). GO functional analysis of differentially expressed proteins overlapped to a great extent with transcriptomic data (Fig. 6D). Similarly, GSEA revealed categories related to amino acid transmembrane transport, RNA metabolism, ribosome biogenesis and autophagy (Fig. 6E). To evaluate if the effects of CM272 on the expression of genes involved in amino acid transport and metabolism were functionally translated we performed targeted metabolomic analyses. We observed significant reductions in the intracellular levels of several essential and non-essential amino acids, as well as in total glutathione upon CM272 treatment (Fig. 6F, Fig. S7).

CM272 impairs the adaptive response of HB cells to high metabolic demand

To sustain rapid proliferation and survival, tumor cells reprogram their metabolism to meet exacerbated bioenergetic and biosynthetic demands. According to our multiomic analyses, interference with this biosynthetic rewiring may underlie CM272 antitumoral activity. Consistently, we found that CM272 downregulated genes frequently induced in tumors such as the amino acid transporters SLC7A11, SLC7A5, and SLC1A5; the proline biosynthetic enzyme PYCR1;[43] ASNS (asparagine synthetase); the enzymes involved in serine and one carbon/folate metabolism PHGDH and MTHFD2; and BCAT1 (branched-chain aminotransferase 1)[43–45] (Fig. 7A, Fig. S8A). *SLC7A11*, involved in cysteine uptake and commonly induced in tumors contributing to malignancy,[45] was among the most potently downregulated genes. To test the functional significance of SLC7A11 inhibition on the antitumoral effects of CM272 we measured its efficacy in the presence of N-acetylcysteine, a cell membrane-permeable cysteine precursor. We observed that by replenishing intracellular cysteine levels the GI_{50} of CM272 increased by threefold (Fig. S8B). Notably, we also observed that CM272 reduced the expression of RPS6 (ribosomal protein S6), upregulated in cancer with protumorigenic consequences.[46] Importantly, the expression of most of these genes was significantly induced in HB tissues and in correlation with that of *G9a* (Fig S9A,B). Interestingly, we also validated the accumulation of the autophagy adaptor protein p62 (sequestosome/SQSTM1) (Fig. 7A), indicative of impaired autophagic flux.[47]

Cancer metabolic rewiring is frequently orchestrated by oncogenes, among which c-MYC, with a central role in HB,[5,8] stands out.[44,45,48] Cairo *et al.* distinguished two transcriptomic subclasses of HB, C1 and C2, with C2 tumors being less differentiated and more aggressive.[8] C2 tumors showed a strong enrichment in the expression of *bona fide* MYC target genes. We performed GSEA using this gene set, and the "Hallmark MYC-targets V1" gene set, on the transcriptomic data from HuH6 cells treated with CM272. CM272 induced a significant negative enrichment in the expression of c-MYC target genes (Fig. 7B). Consistently, *c-MYC* expression was downregulated by CM272 in HuH6 and HepT1 cells (Fig. 7C, Fig. S10A). Interestingly, CM272 not only downregulated *c-MYC* mRNA levels, but it also reduced c-MYC protein half-life (Fig. 7D, Fig. S10B). The effects of CM272 on c-MYC expression were reproduced with the G9a inhibitor UNC0642 (Fig. S10C). The involvement of c-MYC inhibition in the antitumoral effects of CM272 was tested using HB cells overexpressing the oncogene. We observed that the growth-inhibitory activity of CM272, as well as its effects on the expression of key metabolic genes, were attenuated upon c-MYC overexpression (Fig. S10D,E).

Activating transcription factor 4 (ATF4) is also a critical mediator of metabolic changes supporting cancer cell survival, often in cooperation with c-MYC.[45,49,50] We found that *ATF4* expression is upregulated in HB in correlation with that of *G9a* (Fig. S11A, B). Moreover, the expression of the lysine demethylase *KDM4C*, recently reported to contribute to *ATF4* transcriptional induction in cooperation with G9a,[51] was also upregulated in HB tissues (Fig. S11C). Notably, GSEA of a well-characterized ATF4 target gene set[52] indicated that CM272 reduces ATF4 transcriptional activity (Fig. 7E). Consistently, CM272 lowered ATF4 mRNA and protein levels in HB cells (Fig. 7E, Fig. S10F). ATF4 is essential in the adaptive cellular

amino acid deprivation in HepT1 cells. (G) Effect of CM272 pretreatment (GI_{50}, 24 h) on the response of the indicated genes to amino acid deprivation in HepT1 cells. *p <0.05, **p <0.01, ***p <0.001. Data are means ± SD. Paired two-tailed Student's t tests were used (B–E). NES and significance are shown (B, E). Non-parametric Kruskal-Wallis ANOVA test was used (F, G). GSEA, gene set-enrichment analysis; HB, hepatoblastoma; NES, normalized enrichment score. (This figure appears in color on the web.)

response to reduced amino acids levels, triggering the expression of genes involved in amino acid synthesis and uptake.[45] We observed that CM272, and UNC0642, markedly blunted ATF4 upregulation induced by amino acid restriction in HB cells (Fig. 7F, Fig. S10G). Consistently, the expression of ATF4 target genes, including membrane amino acid transporters and metabolic enzymes was markedly abated (Fig. 7G). In agreement with these observations, we also found that the expression of *Atf4*, and that of the ATF4 and c-MYC target genes involved in amino acid transport, metabolism and ribosome biogenesis discussed above, was significantly reduced in $G9a^{\Delta hep}$KO *vs.* WT mice in our HB model (Fig. S12A). Moreover, overexpression of G9a in HB cells enhanced the expression of c-MYC and ATF4, and regulated the levels of downstream metabolic genes like *SLC1A5* and *FBP1* (Fig. S12B).

Taken together, all these findings suggested that CM272 can reprogram the transcriptome of HB towards a less malignant and more differentiated phenotype. To further confirm this tenet, we evaluated the effects of CM272 on the expression of a gene set that defines the transcriptome of adult healthy hepatocytes.[53] To this end we selected HepT1 cells, which according to the expression of this gene set are significantly less differentiated than HuH6 cells (Fig. S12C). Interestingly, CM272 treatment induced a significant positive enrichment in the expression of this complement of genes in HepT1 cells (Fig. S12C). These transcriptomic effects had a functional translation, as CM272 treatment promoted a shift in the relative production of ATP from glycolysis to mitochondrial respiration, thus counteracting the aerobic glycolysis characteristic of tumoral cells (Fig. S12D).

Discussion

Accumulating evidence indicates that epigenetic alterations can play a role in HB development and response to therapy. Dysregulation of DNA methylation has been recognized for almost 20 years, and is currently considered a hallmark of HB, enabling the identification of epigenetic subtypes with differential biological characteristics and prognosis.[16,18,29] Yet, epigenetic mechanisms are very complex and intertwined, extending well beyond the control of DNA methylation. Mutational burden is low in HB, even in its most aggressive forms,[6,54] and to our knowledge mutations in epigenetic modifiers have not been reported. Conversely, the altered expression/activity of epigenetic modifiers has increasingly been recognized as contributing to malignancy in multiple cancer types.[12,22] In this study, we performed what we believe is the most comprehensive analysis of the expression of epigenetic modifiers in HB tissues. We observed significant and consistent alterations, mostly upregulation, in the expression of genes belonging to every category of epigenetic gene. Interestingly, the overall pattern of epigenetic gene expression in HB was reminiscent of that in the fetal liver, and is aligned with the global DNA hypomethylation reported in fetal as well as HB tissues.[16] This finding, which is consistent with the global transcriptomic reprogramming of HB towards prenatal phases,[5] further underscores the involvement of epigenetic mechanisms in HB development. In fact, we observed that the upregulation of epigenetic genes was more pronounced in the Epi-CB and C2A molecular subgroups, corresponding to aggressive tumors resembling early developmental stages.[10,16] Notably, the expression of a significant number of epigenetic modifiers in peritumoral tissues was already elevated compared to healthy pediatric livers. This might indicate that epigenetic alterations predisposing liver tissue to neoplastic conversion may already occur before tumor development. This "field cancerization" effect is well recognized in hepatocellular carcinoma development,[55] as these tumors normally arise on a background of chronic injury and inflammation, while in HB, underlying liver disease is generally absent at diagnosis. However, recent observations indicate that field cancerization may also be involved in HB,[56] and our findings suggest that dysregulated expression of epigenetic modifiers may be part of it.

The reversible nature of epigenetic PTMs makes targeting these mechanisms an attractive strategy for cancer therapy.[21] Our transcriptomic screening for relevant epigenetic genes in HB identified the consistent upregulation of the KMT G9a, particularly in tumors with more aggressive clinical and molecular profiles. Pharmacological interference with G9a methyltransferase activity markedly inhibited the *in vitro* and *in vivo* growth of primary HB cells, as well as that of HB tumoroids. The involvement of G9a in HB development suggested by our molecular tool CM272 was further substantiated *in vivo*. Tumor development upon hepatic delivery of oncogenic forms of β-catenin and YAP1, a genetic model of HB,[42,48] was blunted in mice lacking *G9a* expression in hepatocytes. As shown by ourselves and others, elevated G9a expression contributes to malignant traits in solid tumors.[32,41,57] Notably, the protumorigenic activities of G9a, including the repression of tumor suppressor genes (TSGs), are frequently mediated in association with the DNMT1/UHRF1 complex.[32,57] We found a strong correlation between *G9a*, *DNMT1* and *UHRF1* expression in HB tissues and, most interestingly, the dual G9a/DNMT1 inhibitor CM272 reactivated the expression of key TSGs known to be repressed by the DNMT1/UHRF1 complex in HB cells in association with increased H3K9 methylation, the outcome of G9a activity.[19]

To elucidate the antitumor mechanisms of CM272 in HB in an unbiassed manner and beyond the reactivation of TSGs, we performed a series of multiomic analyses. Cancer cells reprogram their metabolism to boost biosynthesis of cellular building blocks and fuel bioenergetic demands to sustain uncontrolled proliferation and growth.[44,45] These critical responses encompass activation of the uptake and synthesis of amino acids, including non-essential amino acids,[43,45] ribosome biogenesis, protein synthesis, and salvage autophagy, among other reactions supporting macromolecule synthesis.[44,49,52] We observed that CM272 markedly reduced the expression of amino acid transporters (SLC1A5, SLC7A5, SLC7A11), metabolic enzymes (BCAT1, PYCR1, ASNS), as well as genes involved in ribosome biogenesis, RNA processing and translation, such as RPS6, which are often upregulated in cancer cells.[43,44,46] These genes, which we also found overexpressed in HB tissues, are known to be important for malignant cell growth.[44–46] Moreover, the downregulation of SLC7A11 expression and the concomitant reduction in intracellular reduced glutathione levels may underlie the increased efficacy of cisplatin in CM272-treated HB cells.[58]

Taken together, our findings indicate that c-MYC antagonism could be a major determinant of the antitumor effects of CM272. Indeed, G9a inhibition markedly antagonized the c-MYC-driven transcriptome in HB cells. This oncogene, a well-

known target of the Wnt/β-catenin pathway, is frequently overexpressed in HB.[5,8,56] Furthermore, *c-MYC* is essential for the development of experimental HB triggered by oncogenic β-catenin and YAP1 expression in mouse livers.[42,48] Notably, when this same HB model was implemented in *G9a*$^{\Delta hep}$KO mice, *c-MYC* expression was blunted and tumor development was drastically reduced, phenocopying the response of *c-MYC*-KO animals.[48] These observations may have broader significance, as they support an important role for G9a in c-MYC-driven tumorigenesis *in vivo*.[59] c-MYC is a master regulator of the transcriptional metabolic reprogramming occurring in cancer, and this reprogramming is central to c-MYC-induced tumor growth.[44,50] Direct targets of c-MYC include the aforementioned amino acid transporters, enzymes, and ribosomal proteins downregulated by CM272 in HB cells. We found that G9a inhibition reduced *c-MYC* mRNA levels, which is consistent with recent findings in multiple myeloma cells.[60] Additionally, we also observed that G9a targeting significantly decreased c-MYC protein half-life. Although the underlying mechanisms merit further investigation, our current observations, together with the notion that c-MYC can in turn trigger *G9a* expression in breast cancer cells,[59] emphasize the strong interaction between these two genes in cancer.

Interestingly, the transcription factor ATF4 cooperates with c-MYC in regulating multiple metabolic genes, with ATF4 being critical for c-MYC-dependent tumorigenesis in certain cell types.[45,50] *ATF4* expression is mainly regulated at the translational level; however, increased *ATF4* transcription has been found in certain tumors.[49,52] We showed that *ATF4* mRNA levels are elevated in HB tissues in correlation with those of *G9a*. Activation of ATF4 is essential for the adaptation of cancer cells to nutrient limitation, prominently to the lack of amino acids, as occurs inside a growing tumor mass.[49] Concomitant with the reduction in amino acid levels detected in CM272-treated HB cells, we observed a significant downregulation in *ATF4* gene expression. Furthermore, the potent induction of *ATF4* expression elicited by amino acid restriction was abated upon G9a inhibition. It has been shown that *ATF4* transcription is activated by the histone demethylase KDM4C, which reduces the repressive H3K9me2 and H3K9me3 marks on the *ATF4* promoter.[51] As we observed increased *KDM4C* expression in HB tissues, it is tempting to speculate that the concomitant upregulation of *KDM4C* and *G9a* could result in increased levels of the activating H3K9me1 mark on the *ATF4* promoter. Nevertheless, the interplay between these two epigenetic effectors in *ATF4* regulation in HB requires further elucidation.

Altogether, we have shown a remarkable alteration in the landscape of epigenetic effector expression in HB tissues. Among them, we identified the KMT G9a as a strong candidate involved in HB growth. Our mechanistic studies also led us to expose the extensive metabolic reprogramming taking place in HB. As observed in other solid tumors, c-MYC, in cooperation with ATF4, may play a central role in this metabolic rewiring. However, pharmacological inhibition of c-MYC has not been achieved yet, and thus alternative pathways to antagonize c-MYC function need to be defined. We demonstrated that G9a inhibition impaired c-MYC expression, inducing a state of metabolic anergy and leading to growth suppression in HB cells. These effects were accompanied by the induction of genes characteristic of the adult differentiated hepatocyte and the consistent reprogramming of ATP metabolism. This study suggests that epigenetic drugs may hold promise for the treatment of patients with HB, particularly those with more aggressive forms of the disease.

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Abbreviations

ATF4, activating transcription factor 4; DNMTs, DNA methyltransferases; Epi-CA/B, epigenetic cluster A/B; GO, gene ontology; GSEA, gene set-enrichment analyses; HARs, histone acetyl-readers; HATs, histone acetyltransferases; HB, hepatoblastoma; HDACs, histone deacetylases; HDMs, histone-lysine demethylases; HMRs, histone methyl-readers; KMTs, histone-lysine methyltransferases; KO, knockout; MBPs, DNA-methyl-binding proteins; PDX, patient-derived xenograft; PRMTs, protein-arginine methyltransferases; PTMs, post-translational modifications; TETs, DNA demethylases; TSG, tumor suppressor gene; WT, wild-type.

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Conflict of interest

C. Alonso is employed by OWL Metabolomics; the other authors declare no conflict of interest.

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

Authors' contributions

Experiments and procedures: ACC, JMH, MUL, MA, IU, APL, FP, PF, RA, PB, CA, BS, JJGM, MLMC, SC, FJC, JZR, SC, MDS, LZ, PSB, CA, CB; concept and design: ACC, JMH, MGFB, MAA; supervision: MGFB, MAA; writing of article: MAA.

Data availability statement

RNA-sequencing data reported in this study have been deposited in the Sequence Read Archive (SRA) of NCBI, ref. PRJNA900056.

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Supplementary data

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Author names in bold designate shared co-first authorship.

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