Up-Regulation of Histone Methyltransferase SETDB1 by Multiple Mechanisms in Hepatocellular Carcinoma Promotes Cancer Metastasis

Chun-Ming Wong,* Lai Wei, Cheuk-Ting Law, Daniel Wai-Hung Ho, Felice Ho-Ching Tsang, Sandy Leung-Kuen Au, Karen Man-Fong Sze, Joyce Man-Fong Lee, Carmen Chak-Lui Wong, and Irene Oi-Lin Ng

Epigenetic deregulation plays an important role in liver carcinogenesis. Using transcriptionome sequencing, we examined the expression of 591 epigenetic regulators in hepatitis B-associated human hepatocellular carcinoma (HCC). We found that aberrant expression of epigenetic regulators was a common event in HCC. We further identified SETDB1 (SET domain, bifurcated 1), an H3K9-specific histone methyltransferase, as the most significantly up-regulated epigenetic regulator in human HCCs. Up-regulation of SETDB1 was significantly associated with HCC disease progression, cancer aggressiveness, and poorer prognosis of HCC patients. Functionally, we showed that knockdown of SETDB1 reduced HCC cell proliferation in vitro and suppressed orthotopic tumorigenicity in vivo. Inactivation of SETDB1 also impeded HCC cell migration and abolished lung metastasis in nude mice. Interestingly, SETDB1 protein was consistently up-regulated in all metastatic foci found in different organs, suggesting that SETDB1 was essential for HCC metastatic progression. Mechanistically, we showed that the frequent up-regulation of SETDB1 in human HCC was attributed to the recurrent SETDB1 gene copy gain at chromosome 1q21. In addition, hyperactivation of specificity protein 1 transcription factor in HCC enhanced SETDB1 expression at the transcriptional level. Furthermore, we identified miR-29 as a negative regulator of SETDB1. Down-regulation of miR-29 expression in human HCC contributed to SETDB1 up-regulation by relieving its post-transcriptional regulation. Conclusion: SETDB1 is an oncogene that is frequently up-regulated in human HCC; the multiplicity of SETDB1 activating mechanisms at the chromosomal, transcriptional, and posttranscriptional levels together facilitates SETDB1 up-regulation in human HCC. (Hepatology 2016;63:474-487)

Hepatocellular carcinoma (HCC) is a major type of primary liver cancer and one of the most prevalent human malignancies globally.1 Due to late symptom presentation and frequent metastasis, most HCC patients are diagnosed at a late stage of disease progression and, therefore, are not eligible for curative surgical resection or liver transplantation. Currently, the treatment options for inoperable HCC patients are very limited. Sorafenib is the only Food and Drug Administration-approved drug for advanced HCC. However, the clinical benefit of sorafenib treatment remains unsatisfactory.2 In this regard, new biomarkers for early diagnosis and new targets for therapy are eagerly awaited to improve the survival rate of HCC patients.
HCC is etiologically associated with chronic hepatitis B viral (HBV) and hepatitis C viral infections, and progress in a multistep fashion typically involves sequential development of cirrhotic liver, premalignant dysplastic nodule, early HCC, and eventually metastatic HCC. It is commonly believed that liver carcinogenesis is fueled by accumulation of various genetic and epigenetic alterations. However, detailed molecular mechanisms underlying the disease progression remain to be elucidated. Mounting evidence has recently linked aberrant promoter DNA methylation to the transcriptional silencing of crucial tumor suppressor genes and highlighted the roles of epigenetic alterations in human carcinogenesis. Nonetheless, the pathological implications of histone modification, another major epigenetic event, have not been fully investigated. Histone proteins are the structural components of chromatin. Recent evidence has suggested that posttranslational modifications (methylation, acetylation, sumoylation, etc.) on the N-terminal tail of core histone proteins form a unique signature (histone code) that governs the chromatin structure and transcriptional competency. Different histone modifications are regulated by specific enzymes; for example, H3K27 methylation is mediated by EZH2 (enhancer of zeste homolog 2) and its associated polycomb repressive complex, whereas H3K9 methylation is specifically catalyzed by SUV39H1/2 (suppressor of variegation 3-9 homolog 1), SETDB1 (SET domain, bifurcated 1), and G9a. Interestingly, various histone methyltransferases, such as EZH2, SYMD3, and SUV39H1, are frequently up-regulated in human cancers, suggesting that deregulation of histone modifications may be a common mechanism underlying human carcinogenesis.

In this study, we investigated the expression of 591 known epigenetic regulators that covered the full spectrum of epigenetic events in human HCC by whole-transcriptome sequencing (RNA-Seq). This analysis led to the identification of SETDB1 as the most significantly up-regulated epigenetic regulator in human HCC. SETDB1 (also known as ESET and KMT1E) was first identified as a SET domain-containing protein through an \textit{in silico} search. The SETDB1 gene is located at chromosome 1q21 and encodes a 143-kDa protein with multiple functional domains. The Tudor domain in the N-terminal region is involved in protein-protein interactions, the MBD domain in the middle is thought to mediate methyl-CpG binding, and the SET domain in the C-terminal region is responsible for H3K9-specific lysine methylation. Different from another H3K9 methyltransferase, SUV39H1, which is mainly responsible for structural pericentromeric heterochromatin formation, SETDB1 is linked to transcriptional repression of euchromatin. SETDB1 activity is important for the maintenance of the embryonic stem cell state by repressing lineage-specific gene expression. SETDB1 also plays a central role in mediating permanent endogenous retrovirus silencing in embryonic stem cells during early embryonic development, which is an important process to protect genome integrity. However, little is known about the role of SETDB1 in liver carcinogenesis. Therefore, a comprehensive investigation into the functional and pathological roles of SETDB1 in HCC is warranted.

Materials and Methods

**Clinical Specimens and Cell Lines.** Primary HCC and corresponding adjacent nontumorous (NT) liver specimens were collected at HCC tumor resection at Queen Mary Hospital, Hong Kong. The use of clinical samples was approved by the institutional review board of the University of Hong Kong and the Hong Kong Hospital Authority. HCC cell lines used in this study, Hep3B and MHCC97L, were obtained from ATCC and Fudan University, Shanghai (Dr. Z.Y. Tang), respectively.

**Transcriptome Sequencing.** Transcriptome sequencing (RNA-Seq) was performed in 16 pairs of primary HCC and corresponding NT liver samples. A polyA+ messenger RNA (mRNA) library was prepared with the TruSeq standard mRNA sample Prep kit (Illumina). A 100-bp paired-end sequencing was performed in Illumina HiSeq2000 by Axeq Technologies (Seoul, South Korea). RNA-Seq data were analyzed in TopHatCufflinks pipeline and are presented as fragments per kilobase transcript sequence per million mapped reads (FPKM). The expression level of 591 epigenetic regulators encompassed in the ChromoHub database were

Address reprint requests to: Chun-Ming Wong, Ph.D., or Irene Oi-Lin Ng, M.B.B.S., M.D., Ph.D., State Key Laboratory for Liver Research and Department of Pathology, University Pathology Building, The University of Hong Kong, Queen Mary Hospital, Pokfulam, Hong Kong. E-mail: jackwong@pathology.hku.hk or iolng@hku.hk.

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extracted from the global RNA-Seq data for further analysis. Unsupervised clustering and a heat-map diagram was done with the Cluster and TreeView algorithms.\textsuperscript{18} Transcriptome analysis of Hep3B and MHCC97L short hairpin nontarget control and shSETDB1 was performed by RNA-Seq, as described above. Gene signature and pathway analysis was performed by DAVID and gene set enrichment analysis (GSEA) algorithms.

**Complementary DNA Preparation and Quantitative Reverse-Transcription Polymerase Chain Reaction.** Total RNA was extracted from snap-frozen clinical specimens and HCC cell lines by TRIZOL reagent (Life Technologies) according to the manufacturer’s protocol. Complementary DNA (cDNA) was prepared from 1 µg of total RNA using the GeneAmp RNA PCR kit (Life Technologies). The expression level of SETDB1 mRNA was determined by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) using TaqMan gene expression assay and normalized with hypoxanthine-guanine phosphoribosyltransferase (HPRT) endogenous control. For microRNA expression analysis, cDNA was synthesized from 10 ng total RNA using the TaqMan MicroRNA Reverse Transcription kit with miR-29a-specific and RNU44-specific stem-loop primers. MicroRNA expression level was determined by qRT-PCR using the microRNA-specific TaqMan probe (Life Technologies).

**Immunohistochemistry and Western Blotting.** The expression of SETDB1 protein was determined by immunohistochemistry (IHC) in formalin-fixed, paraffin-embedded section and tissue microarrays using rabbit monoclonal antibody specifically against SETDB1 (1:50; Cell Signaling Technology). Western blotting was performed with HCC cell protein lysate in radio immunoprecipitation assay (RIPA) buffer and SETDB1-specific rabbit monoclonal antibody (1:1000; Cell Signaling Technology). The housekeeping gene α-tubulin was used as the loading control. Histone was extracted from HCC cell lines using an acid-base histone extraction protocol as described.\textsuperscript{27} Global histone modification levels were determined by western blot with specific antibodies against histone 3 lysine 9 tri-methylation (H3K9me3; Abcam), histone 3 lysine 4 tri-methylation (H3K4me3) (Millipore), and histone 3 lysine 27 tri-methylation (H3K27me3) (Millipore). Histone 3 antibody (Millipore) was used as the loading control.

**Establishment of SETDB1 Stable Knockdown Cells.** Two SETDB1 targeting short hairpin RNA (shRNA) sequences (shSETDB1 #2 CCGGGCTCAGA TGATAACTTCTGTACGAGTACAGAAGTTATC ATCTGAGCTTTTTG and shSETDB1 #5 CCGGA GTTAGAGACATGGTATACCTCGAGGTATTAC CCATGTTCCTAAGTTTTTGT) were cloned into a lentiviral expression vector (pLKO.1) and stably infected into HCC cell lines MHCC97L and Hep3B. Nontarget control shRNA, obtained from Sigma-Aldrich, was included as a negative control. Infected cells were subjected to puromycin selection, and successful knockdown of SETDB1 in stably infected cells was confirmed by both qRT-PCR and western blotting.

**Cell Proliferation Assay and Cell Migration Assay.** For the cell proliferation assay, 5 × 10⁴ cells were seeded onto a 24-well cell culture plate in triplicate. Cells were counted using a Z1 Coulter Counter Cell and Particle Counter (Beckman Coulter) for 6 consecutive days to generate cell proliferation curves. For the cell migration assay, 5 × 10⁴ cells were resuspended in serum-free medium and seeded into the upper chamber of the Transwell. Serum-containing culture medium was added to the bottom chamber as a chemoattractant to drive directional cell migration. At 24 hours, migrated cells were fixed with methanol, stained with crystal violet, and counted under a microscope.

**In Vivo Orthotopic Tumor Implantation Model.** Orthotopic tumor implantation was performed as described.\textsuperscript{9} In brief, 2 × 10⁶ luciferase labeled, MHCC97L stably expressing short hairpin nontarget control or shSETDB1 #2 were resuspended in 25 µL Dulbecco’s modified Eagle’s medium, high glucose/ Matrigel (1:1) mixture and injected into the left liver lobe of male BALB/C nude mice at 6-8 weeks old. After inoculation, tumor formation in the liver of nude mice was assessed by bioluminescent imaging using the IVIS 100 Imaging System (Xenogen). Mice were sacrificed at 5 weeks, and livers and lungs were collected. Formation of extrahepatic metastasis was detected by ex vivo bioluminescent imaging.

**Chromatin Immunoprecipitation Assay.** A chromatin immunoprecipitation (ChIP) assay was performed as described.\textsuperscript{19} Briefly, HCC cells were crosslinked with formaldehyde and lysed with sodium dodecyl sulfate lysis buffer. Chromatin fragmentation was performed with a Bioruptor Pico (Diagenode). ChIP grade H3K9me3-specific antibody (Abcam) and immunoglobulin G (Santa Cruz) were used to immunoprecipitate the fragmented chromatin. Protein A agarose beads were used to pull down the antibody/chromatin complex. After de-cross-linking in 0.2 M NaCl, DNA was extracted by phenol chloroform. Enrichment of H3K9me3 modified histone in specific loci was determined by qPCR. The mRNA expression of the corresponding genes was also assessed by qRT-PCR.
**Gene Copy Number Assay.** Genome DNA was extracted from snap-frozen clinical specimens using the standard phenol-chloroform extraction protocol. The SETDB1 gene copy number was quantified by TaqMan gene copy number assay (Life Technologies). The SETDB1 gene copy number was further normalized against the RPPH1 gene on chromosome 14q11.2, a standard reference gene for copy number assay as recommended by the manufacturer (Life Technologies).

**Luciferase Reporter Assay.** For SETDB1 promoter activity, a putative SETDB1 promoter region was amplified from the normal liver cell line MIHA and cloned into pGL3-Basic luciferase reporter vector (Promega). Luciferase reporter constructs were transiently transfected into Hep3B cells by FuGENE 6 transfection reagent (Roche). Luciferase activity of SETDB1 promoter was determined by dual luciferase assay reagent II (Promega) and normalized based on cotransfected pGK Renilla luciferase control. Specificity protein 1 (SP1) expression construct and 3× SP1 binding site luciferase reporter were prepared as described. Control and SP1-specific small interfering RNA were obtained from Santa-Cruz. The SP1 inhibitor mithramycin A was purchased from Sigma-Aldrich.

For SETDB1 post-transcriptional regulation, wild-type and mutated miR-29 binding sequences in the SETDB1 3′-untranslated region (3′UTR) were cloned into pmirGLO dual-luciferase microRNA target expression vector (Promega). The miR-29a complement sequence was also cloned in pmirGLO vector for positive control. The miR-29a precursor and microRNA negative control were obtained from Life Technologies. The miR-29a precursor and luciferase reporter construct were sequentially transfected as described. The activities of Firefly luciferase and Renilla luciferase were quantified 48 hours after transfection.

**The Cancer Genome Atlas Data Set.** Level 3 mRNA and mature microRNA RNA-Seq data of 50 paired HCC samples were retrieved from The Cancer Genome Atlas (TCGA) through the Broad Institute (http://gdac.broadinstitute.org/). The copy number alterations of 204 TCGA HCC samples were calculated by the copy number analysis algorithm GISTIC and accessed through cBioPortal (http://www.cbioportal.org/).

**Statistical Analyses.** Statistical analyses were performed in IBM SPSS Statistics 20 or PRISM 5 software. A paired t test was used to compare the expression changes of epigenetic regulators in RNA-Seq studies. P values of multiple comparisons were further subjected to false discovery rate (FDR) correction. The nonparametric Mann-Whitney test and Wilcoxon test were used to compare SETDB1 expression in qRT-PCR analyses of independent and paired samples, respectively. Significance of SETDB1 expression changes in multistep liver carcinogenesis were determined by Kruskal-Wallis test. Clinicopathological correlations were performed with Fisher’s exact test. Survival test was done by Kaplan-Meier curve and log-rank test. Association between SETDB1 copy number and mRNA expression was determined by one-way analysis of variance. Coexpression analyses were assessed by linear regression test. The Student t test was used to compare data obtained from in vitro and in vivo experiments. P < 0.05 was considered statistically significant.

**Results**

**Deregulation of Epigenetic Regulatory Genes in Human HCC.** Epigenetic modifications are regulated by a highly complicated mechanism and involve the orchestrated functions of multiple epigenetic modifying complexes. We hypothesized that deregulation of epigenetic regulators may be the underpinning mechanism of various epigenetic alterations detected in human cancers. We therefore compared the expression levels of a total of 591 known epigenetic regulatory proteins (as listed in the ChromoHub database) in 16 pairs of HBV-associated primary HCC and their corresponding NT liver samples by RNA-Seq. In this analysis, expression of 473 epigenetic regulatory genes was readily detected by RNA-Seq, while 118 genes that had low or undetectable expression (median expression <1 FPKM in both primary HCC and NT liver samples) were excluded from this study. The expression profiles of these 473 epigenetic regulators can unambiguously distinguish primary HCC and their NT livers under an unsupervised clustering analysis (Fig. 1A). Intriguingly, in contrast to other protein-coding genes, we observed a global up-regulation of the epigenetic regulatory genes in primary HCC (Fig. 1B). Significant deregulation ($FDR < 0.05$) was detected in 351 epigenetic regulators. Of these, 341 genes were up-regulated in primary HCC and only 10 were down-regulated (Fig. 1C; Supporting Table S1). These findings indicate that up-regulation of epigenetic regulators was a common event in human HCC, implying an increased demand of epigenetic modifications for sustaining HCC cell growth.

**Frequent Up-Regulation of SETDB1 in Human HCCs.** In the above-mentioned RNA-Seq analysis, we identified SETDB1 as the most significantly deregulated epigenetic regulator ($P = 1.2 \times 10^{-6}$, $FDR = 5.7 \times 10^{-9}$; Fig. 1C,D). SETDB1 transcript variant 1 was the predominant form of SETDB1 expressed in HCCs and NT livers (Supporting Fig. S1). Consistent with our
findings, SETDB1 was also found to be significantly up-regulated in 50 paired HCC samples available in the TCGA RNA-Seq data set (Fig. 1E), thus reinforcing our initial observation and prompting us to further investigate the role of SETDB1 in liver carcinogenesis. We first validated the expression changes of SETDB1 in an expanded sample set consisting of five normal livers and 92 pairs of primary HCC/NT liver samples by qRT-PCR. Consistently, we found that SETDB1 was significantly up-regulated in primary HCCs when compared to their corresponding NT livers as well as normal liver samples (\( P < 0.001 \); Fig. 2A). In this sample set, up-regulation of SETDB1 was detected in 45% (41/92) of primary HCC cases, including 27 cases with moderate up-regulation (greater than two-fold) and 14 cases with high up-regulation (greater than four-fold). On the other hand, down-regulation of SETDB1 was detected in 12% (11/92) of the cases, but none of them reached the four-fold cutoff threshold (Fig. 2A). Next, we tested whether up-regulation of SETDB1 was also detected at the protein level. IHC was performed in tissue sections with HCC and NT liver boundary as well as tissue microarrays consisting of 89 pairs of primary HCC/NT liver samples. The results showed that SETDB1 expression was mainly confined to the nucleus and apparently up-regulated in HCC cells compared to the adjacent hepatocytes (Fig. 2B). Up-regulation of SETDB1 protein was observed in 39% of (35/89) cases in the tissue microarray (Fig. 2C). With the above findings, we concluded that SETDB1 was frequently up-regulated in human HCC at both the mRNA and protein levels.
Clinicopathological Significance of SETDB1 Up-Regulation in Human HCC. Liver carcinogenesis is a multiple-step process. We found that there was a step-wise increase of SETDB1 expression in HCC progression. The median SETDB1 expression level increased progressively from normal liver and chronic hepatitis (median 2.71 and 2.70) to cirrhotic livers (4.52) and early HCCs (5.13) and was further elevated in late-stage HCCs (6.50) ($P < 0.001$; Fig. 2D). This finding suggested a possible engagement of SETDB1 deregulation in promoting HCC development. To investigate the potential clinical implications of SETDB1 up-regulation in human HCC, (A) Expression of SETDB1 mRNA assessed by qRT-PCR in an expanded HCC cohort consisting of five normal livers and 92 paired HCC and NT liver samples. Left: Scatter plot shows the significant up-regulation of SETDB1 in human HCCs ($P < 0.001$, Wilcoxon test). Right: Up-regulation of SETDB1 (greater than two-fold) was detected in 45% (41/92) of primary HCCs, including 14 cases that showed a high up-regulation (greater than four-fold). (B) Expression of SETDB1 protein determined by IHC. SETDB1 protein expression was predominantly detected at the nucleus. A representative case shows the up-regulation of SETDB1 protein in HCC when compared to its adjacent NT liver. (C) IHC on tissue microarray consisting of 89 pairs of primary HCC and NT samples revealed that up-regulation of SETDB1 protein was detected in 39% of HCC cases. (D) SETDB1 mRNA expression was elevated in a step-wise manner along disease progression ($P < 0.001$, Kruskal-Wallis test). (E,F) Up-regulation of SETDB1 mRNA was significantly correlated with the formation of metastatic tumor microsatellites ($P = 0.044$, Fisher’s exact test) and poorer 5-year actual survival of HCC patients ($P = 0.047$, log-rank test).

Abbreviations: HPRT, hypoxanthine-guanine phosphoribosyltransferase; NL, normal liver; pTNM, pathological tumor-node-metastasis.
regulation in human HCC, the expression change of SETDB1 mRNA was correlated with various clinicopathological features and survival rates of the patients. We found that high up-regulation of SETDB1 (greater than four-fold) was significantly associated with tumor microsatellite formation in the adjacent NT liver ($P = 0.044$, odds ratio = 3.96; Fig. 2E), which indicates a prometastatic role of SETDB1 in human HCC. In addition, we found that the median survival of SETDB1 highly up-regulated patients and the others were 23.2 and 62.8 months, respectively. Up-regulation of SETDB1 was significantly associated with a poorer 5-year survival rate of the patients ($P = 0.047$, hazard ratio = 2.60; Fig. 2F). These findings together suggested that up-regulation of SETDB1 had significant pathological implications in HCC development.

Knockdown of SETDB1 Inhibited HCC Growth.
Similar to primary HCC samples, up-regulation of SETDB1 was detected in multiple HCC cell lines when compared to the immortalized normal liver cell line MIHA (Fig. 3A). To investigate the functional roles of SETDB1 in HCC, we established the SETDB1 stable knockdown model in Hep3B and MHCC97L cell lines with two independent shRNA sequences (shSETDB1 #2 and shSETDB1 #5). Successful knockdown of SETDB1 was confirmed with qRT-PCR and western blotting (Fig. 3B,C). We found that stable knockdown of SETDB1 significantly suppressed proliferation of both Hep3B and MHCC97L cells as demonstrated by growth curve analysis (Fig. 3D). Similar findings were also obtained from a MTT assay and a colony formation assay (Supporting Fig. S2). However, knockdown of SETDB1 exhibited no growth inhibitory effect in MIHA cells (Supporting Fig. S3A). On the other hand, overexpression of SETDB1 significantly enhanced cell proliferation and colony formation abilities of MIHA cells (Supporting Fig. S3B,C). To further verify the oncogenic function of SETDB1, an orthotopic liver implantation experiment was performed to test the effect of SETDB1 knockdown in HCC tumorigenicity. In this in vivo model, we found that stable knockdown of SETDB1 in MHCC97L cells significantly reduced the size of HCC tumors formed in the orthotopic liver microenvironment compared to the nontarget shRNA control ($P = 0.048$; Fig. 3E). This observation echoed the in vitro experiments, and together they suggested that SETDB1 acts as an oncogene that is essential for HCC proliferation.

Up-Regulation of SETDB1 Promoted HCC Extrhepatic Metastasis.
As our clinicopathological correlation analysis suggested a prometastatic function of SETDB1, we further explored the implication of SETDB1 in HCC cell motility. Using a Transwell cell migration assay, we showed that knockdown of
SETDB1 remarkably suppressed the HCC cell migratory ability \textit{in vitro} (Fig. 4A). Importantly, we demonstrated that knockdown of SETDB1 also attenuated lung metastasis of HCC cells when orthotopically implanted in nude mice (Fig. 4B). The above findings provided experimental evidence to support the functional implication of SETDB1 in controlling HCC motility and metastasis.

To substantiate the notion that up-regulation of SETDB1 is important for HCC metastasis, we further analyzed the expression of SETDB1 protein in a tissue microarray consisting of 23 individual sets of NT livers, primary HCC, and HCC metastases at distant organs detected by IHC on tissue microarray. (C) Expression of SETDB1 protein in NT liver, primary HCC, and HCC metastases at distant organs detected by IHC on tissue microarray. Abbreviation: NTC, nontarget control.

Transcriptomic Changes of SETDB1 Knockdown Cells. To explore the function of SETDB1 in epigenetic regulation, we compared the change of histone modification levels in SETDB1 knockdown cells. We showed that depletion of SETDB1 significantly reduced global H3K9me3 level, while the levels of H3K27me3

Fig. 4. Knockdown of SETDB1 impeded HCC metastasis. (A) Stable knockdown of SETDB1 in Hep3B and MHCC97L cells suppressed HCC cell migration as revealed by transwell cell migration assay. (B) Knockdown of SETDB1 diminished lung metastasis in an orthotopic tumor implantation model in nude mice. Left: \textit{Ex vivo} bioluminescent images detecting the presence of luciferase labeled HCC cells in the lungs. Right: Hematoxylin and eosin staining confirming the formation of HCC tumor foci in the lungs. (C) Expression of SETDB1 protein in NT liver, primary HCC, and HCC metastases at distant organs detected by IHC on tissue microarray. Abbreviation: NTC, nontarget control.
and H3K4me3 were unaffected (Supporting Fig. S4). As SETDB1 functions as an H3K9-specific histone methyltransferase and is responsible for euchromatin epigenetic transcriptional repression, altered SETDB1 expression may have a profound impact on HCC gene expression globally. To this end, we performed RNA-Seq to investigate the transcriptomic changes of HCC cell lines upon stable knockdown of SETDB1. We identified 828 genes that were reactivated upon knockdown of SETDB1 by greater than two-fold in Hep3B and MHCC97L cells (Supporting Table S3). The effects of SETBD1 and H3K9me3-mediated epigenetic silencing have been further validated by ChIP assay and qRT-PCR in multiple target genes, such as IGFBP3, CXCL3, and NOS3 (Supporting Fig. S5). Consistently, the expression of these SETDB1 target genes was down-regulated in human HCC and negatively correlated with SETDB1 expression (Supporting Fig. S6). Pathway and gene ontology term analyses revealed that SETDB1 target genes were significantly enriched in multiple cancer-regulated pathways, including cadherin mediated cell adhesion and Wnt signaling pathways. In addition, SETDB1 target genes were also significantly associated with common diseases including prostate, liver, and colon cancers (Supporting Table S4). The above findings together suggest that SETDB1 may contribute to HCC development by aberrant epigenetic gene silencing.

SETDB1 Gene Copy Number Gain at Chromosome 1q21. As SETDB1 was frequently up-regulated in human HCC, we investigated the underlying mechanisms contributing to this phenomenon. The SETDB1 gene is located at chromosome 1q21, a chromosomal location which was frequently amplified in human HCC as reported by previous comparative genomic hybridization studies available in OncoDB.HCC (Supporting Fig. S7). To directly assess the gene copy number of SETDB1, a qPCR-based gene copy number assay was performed in 32 pairs of primary HCCs and their corresponding NT livers. The results revealed that all NT livers had two copies of the SETDB1 gene. In contrast, gain of the SETDB1 gene was detected in 75% of the primary HCCs (Fig. 5A). In line with our findings, analysis of TCGA gene copy number data also revealed a frequent gain (56%) or amplification (14%) of the SETDB1 gene in HCC samples (Fig. 5B). Notably, SETDB1 gene copy gain was found to be associated with increased SETDB1 mRNA expression in the TCGA sample set (P < 0.001; Fig. 6C). These findings collectively demonstrated that SETDB1 gene copy gain was common in human HCCs and significantly contributed to the aberrant SETDB1 expression.

Overexpression of SP1 Transcription Factor Promoted SETDB1 Up-Regulation in Human HCCs. Apart from gene copy number changes, hyperactivation of
upstream transcription factors is also a possible mechanism for the aberrant up-regulation of SETDB1. To this end, in silico analysis was performed to study the putative transcription factor binding sites 1 kb upstream from the transcription start site of the SETDB1 gene. As predicted by PROMO 3.0, this region contains multiple GC boxes (GGGCGG), the consensus binding site of SP1 and other SP family transcriptional factors. To test if SP1 was involved in regulating SETDB1 expression, a luciferase reporter assay was conducted. We showed that ectopic overexpression of SP1 augmented the promoter activity of the SETDB1 gene (Fig. 6A). Consistently, SETDB1 promoter activity was significantly suppressed upon inactivation of SP1 by mithramycin A treatment or small interfering RNA knockdown (Fig. 6B). In addition, inactivation of SP1 reduced the endogenous SETDB1 mRNA and protein expression in a dosage-dependent manner in HCC cell lines (Fig. 6C). We found that SP1 was significantly up-regulated in human HCCs at both the mRNA and
protein levels (Fig. 6D). The expression levels of SETDB1 and SP1 were positively correlated with each other as revealed by RNA-Seq analysis of the TCGA sample set (Fig. 6E). Based on the above findings, we speculated that overexpression of SP1 might facilitate SETDB1 up-regulation in human HCC.

**Loss of miR-29 Contributed to SETDB1 Up-Regulation in Human HCC.** The emerging role of microRNAs as post-transcriptional regulators prompted us to investigate whether deregulation of microRNA contributes to SETDB1 up-regulation in human HCC. To this end, we interrogated the potential microRNA binding sites in the 3′UTR of SETDB1 transcript using TargetScan, miRANDA, and PicTar microRNA target prediction algorithms. Results from the in silico analyses revealed that the SETDB1 3′UTR harbors a putative binding sequence of nine different microRNAs, of which the miR-29 family targeted sequence was commonly identified by all three algorithms (Fig. 7A). The miR-29 family binding site was evolutionarily conserved among different species and is predicted to form a stable duplex with miR-29 family members (Fig. 7B). We previously reported that miR-29 was frequently down-regulated in human HCC, raising the possibility that loss of miR-29 expression might contribute to the up-regulation of SETDB1. To experimentally validate the negative regulatory function of miR-29 on SETDB1 expression, wild-type and mutated miR-29 binding sequences of the SETDB1 3′UTR were fused with a luciferase reporter. We showed that overexpression of miR-29a significantly repressed the luciferase activity of wild-type SETDB1 3′UTR but not the miR-29 binding site mutant. Overexpression of miR-29a significantly inhibited endogenous SETDB1 mRNA and protein expression levels in Hep3B and MHCC97L cells. Expression of miR-29a was negatively correlated with SETDB1 expression in the TCGA HCC sample set (R² = 0.088, P = 0.003, linear regression). Abbreviations: mi, micro; RLU, relative light unit; RSEM, RNA-Seq by expectation maximization.

**Discussion**

In this study, by examining the expression change of 473 genes involved in epigenetic machinery, we revealed that deregulation of epigenetic regulatory genes was indeed an extremely common event in human HCC. Intriguingly, 72% (341/473) of the epigenetic regulators...
were significantly up-regulated in human HCC when compared to NT livers. These up-regulated genes encompassed epigenetic writers, readers, and erasers with different functional consequences. Some are associated with active gene transcription, while others are responsible for epigenetic repression. The biological reasons behind this global up-regulation of epigenetic regulators in human HCC remain to be elucidated. One possible explanation is that cancer cells are actively dividing and undergoing more dynamic epigenetic reprogramming during cell cycles and, therefore, may require increasing amounts of epigenetic regulators to sustain their continuous growth and clonal evolution.

In this study, we identified SETDB1 as the most up-regulated epigenetic regulator. This finding was validated in an expanded cohort and consistently observed in the TCGA samples. Although up-regulation of SETDB1 is common in human cancers, its functional roles in carcinogenesis are still largely unknown. It has been reported that ectopic overexpression of SETDB1 in melanocytes was able to abrogate BRAF mutation-induced senescence and accelerate melanoma onset in a zebrafish model with a BRAF (V600E) mutation and p53 null background. More recently, studies showed that depletion of SETDB1 in lung cancer cell lines suppressed cell growth. Consistent with the previous findings, we demonstrated that overexpression of SETDB1 increased cell colony formation of immortalized hepatocytes, whereas stable knockdown of SETDB1 drastically inhibited HCC cell proliferation and alleviated tumor formation in the orthotopic liver microenvironment. These findings together suggested that SETDB1 is a bona fide oncoprotein in human cancers. Nevertheless, the implication of SETDB1 in cancer metastasis remains unclear. In a melanoma zebrafish model, overexpression of SETDB1 increased local invasiveness of the tumor. However, in another study, inactivation of SETDB1 promoted metastasis of lung cancer cells in zebrafish and mouse models. Interestingly, both prometastatic and antimetastatic functions of SETDB1 have been observed in lung cancer with different cell line models used. In the present study, we showed that knockdown of SETDB1 remarkably suppressed HCC cell migration in vitro and diminished lung metastasis in nude mice. In line with these findings, we observed that up-regulation of SETDB1 in human HCC was associated with a more aggressive clinicopathological phenotype and poorer prognosis of HCC patients. Importantly, in HCC patients, SETDB1 expression was consistently up-regulated in all metastatic foci found in different organs, implying that SETDB1 is essential for HCC metastatic progression. Thus, our findings strongly suggested that SETDB1 up-regulation facilitates cancer metastasis in human HCC.

In the past decade, several SETDB1 binding partners have been identified by yeast two-hybrid screening and co-immunoprecipitation experiments. These binding partners include various transcriptional repressors and epigenetic regulators, including, KAP-1, HP1, MBD1, HDAC1/2, Sin3A/B, DNMT3A/B, and SUV39H1. Recent evidence also indicated that SETDB1 and EZH2 work collaboratively in RNA-induced transcriptional silencing of the androgen receptor gene. It has been proposed that SETDB1 is present in a mega-epigenetic complex. Interestingly, gene set enrichment analysis revealed that the gene expression signature of SETDB1-knockdown cells correlated with the H3K27me3-enriched and DNA methylation-silenced gene signatures of embryonic stem cells and cancer cells. These findings raised the possibility that SETDB1 may work in concert with other components of the complex to mediate proper epigenetic modifications during DNA replication and cell differentiation. Further investigations are required to test this notion.

In this study, we focused on investigating the molecular mechanisms that contributed to the frequent SETDB1 up-regulation in human HCC at the chromosomal, transcriptional, and post-transcriptional levels. Similar to our findings in human HCC, overexpression of SETDB1 has also been reported in melanoma and lung cancers. In these cancers, overexpression of SETDB1 was attributed to the recurrent amplification of SETDB1 gene at chromosome 1q21. In the present study, gain of SETDB1 gene copy number was detected in more than 70% of human HCCs in our Hong Kong cohort that is mainly associated with chronic HBV infection as well as the TCGA cohort with mixed etiologies including chronic hepatitis C virus, HBV, and alcoholism. In fact, analysis of TCGA data revealed that SETDB1 gene copy gain or amplification was frequently detected in most of the solid tumors and profoundly associated with SETDB1 overexpression. The available data consistently underscore the importance of recurrent gene amplification in SETDB1 up-regulation in different human cancers. Besides gene amplification, we noted that the SETDB1 promoter contains multiple SP1 binding sites. SP1 has been implicated in regulating SETDB1 expression in Huntington’s disease. We demonstrated that SP1 was frequently up-regulated in human HCC. Inactivation of SP1 by RNA interference knockdown or mithramycin A treatment successfully blocked SETDB1 expression in HCC cell lines. However, SP1 is considered a general
transcriptional factor and is involved in the regulation of a wide array of housekeeping genes. The pathological contribution of SP1 up-regulation in HCC development remains to be confirmed. Previous studies showed that deregulation of microRNAs profoundly influences the expression of histone methyltransferases. For example, loss of miR-101 is associated with EZH2 up-regulation in different cancers. We also have reported that underexpression of miR-125b facilitated SUV39H1 overexpression in human HCC. Inspired by these studies, we therefore considered the possible engagement of microRNA in regulating SETDB1 expression at the post-transcriptional level. In this study, we identified the miR-29 family as a negative regulator of SETDB1. The miR-29 family interacted with SETDB1 3’UTR in a seed sequence-dependent manner and repressed SETDB1 expression. MiR-29 is a frequently down-regulated microRNA in human HCCs and is negatively associated with SETDB1 expression in human HCCs, implying that loss of miR-29 stabilizes SETDB1 and thereby contributes to its up-regulation.

In the work presented here, we demonstrated that SETDB1 is a bona fide oncogene that is functionally important to HCC tumor growth and metastasis. The multiplicity of SETDB1 activating mechanisms at the chromosomal, transcriptional, and post-transcriptional levels together results in the frequent up-regulation of SETDB1 in human HCC.

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Author names in bold denote shared co-first authorship.

Supporting Information

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