Supplementary materials and methods

Cell culture

Hepatoma cell lines used in this research were obtained from the Cell Bank of Type Culture Collection (Chinese Academy of Sciences, Shanghai, China). The identification and viability of cell lines were determined by third-party biology services (GeneCreate Biological Engineering Co., Ltd). All cells were cultured in minimum essential medium (Gibco, Carlsbad, CA, USA) with the recommended media supplemented with 10% fetal bovine serum (Gibco) at 37 ℃ in an incubator with 5% CO_2.

Plasmid construction and cell transfections

Full-length LINC00662 was cloned into the pEZ-Lv201 (GeneCopoeia) expression vector for overexpression. For MAT1A and AHCY overexpression, pEZ-Lv201 was also applied to add the respective homologous sequences. These vectors and a control eGFP plasmid were generated by the cotransfection of hepatoma cells with each expression plasmid together with the GeneCopoeia Lenti-Pac Expression Packaging Kit, according to the manufacturer's instructions (Lifesciences, Source Bioscience). Moreover, shRNAs were designed and inserted into the psi-LVRH1GP vector by GeneCopoeia (GeneCopoeia, Inc.) for experiments *in vivo*.

siRNA transfection

To knock down LINC00662, 2 different small interfering RNAs (siRNAs) (Supplementary Table S1) targeting LINC00662 were generated and tested by GeneCopoeia (GeneCopoeia, Inc.). The cells were transfected with 20 nmol/L of siRNA using the Lipofectamine RNAiMAX reagent (Invitrogen) following the manufacturer's protocol.

RNA and DNA extraction and preparation

Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The total RNA extracted was quantified using a NanoDrop spectrophotometer (Thermo Scientific 183 Inc.) at 260 and 280 nm. RNA was used for reverse transcription if A260/A280 ≥ 2.0. Total RNA was used to synthesize first

strand cDNA using random primers and SuperScript II reverse transcriptase (Invitrogen, Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. Reverse transcription (RT) reactions comprising a total volume of 20 μl were performed using a PrimeScript RT reagent kit (Takara Bio Inc.). The DNAzol Reagent kit (Thermo Fisher Scientific) was used to isolate DNA from tissues and cells according to the manufacturer's instructions. The isolated DNA was air-dried, dissolved in 20 ml DNase-free water, and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific) at 260 and 280 nm. All cDNA and DNA samples were stored at -20 ℃ until use.

Quantitative polymerase chain reaction (q-PCR)

Using the abovementioned cDNA samples, a 20 μl systematic amplification reaction with 10 μl of the 2 × SYBR Master mix (Toyobo Co., Ltd.) was conducted. Amplification was performed with an iQ5 quantitative PCR system (Bio-Rad Laboratories Inc.). q-PCR was conducted in triplicate and included the nontemplate controls. GAPDH was used for expression normalization, with the $2^{-\Delta\Delta CT}$ values being normalized to GAPDH levels.

Cell proliferation assay

Cell proliferation assays were conducted using a Cell Counting Kit-8 (Dojindo Molecular Technologies Inc., Kumamoto, Japan) according to the manufacturer's protocol. Hepatoma cells were plated in 24-well plates in triplicate at a density of 2-5 × 10^4 cells per well and cultured in growth medium. Cells were treated with the siRNAs or plasmids, and the numbers of cells per well were measured by the (450 nm) at the indicated time points. Additionally, for the colony formation assay, 500 cells were seeded in 6-well plates for 10 days, and colonies were fixed and stained with crystal violet solution. Each cell line was evaluated in three parallel replicates.

5-ethynyl-2'-deoxyuridine (EdU) assay

5-ethynyl-2'-deoxyuridine (EdU) is readily incorporated into cellular DNA during DNA replication. We evaluated cell proliferation by using an EdU Cell Proliferation Kit with Alexa Fluor 488 (BeyoClick™, Shanghai, China) as described by the manufacturer. Cells were incubated with 50 μM EdU for 2 h at 37 °C and fixed

with 4% formaldehyde. Images were acquired by fluorescence microscopy and overlapped using Image-Pro Plus software (Version 6.0; Media Cybernetics, Inc.).

Flow cytometric analysis

Hepatoma cells (2- 5 × 10^5) were then treated with the LINC00662 vector or siRNAs were plated in 6-well plates. After 12 h of transfection, cells were treated with 0.1 mmol/L H_2O_2 dissolved in complete medium for 3 h. Then, the recruited cells were treated with only complete medium until 48 h after transfection before being harvested by trypsinization. The cultures were double stained with Annexin V and propidium iodide (China Sinopharm International, Co., Ltd.) for 30 minutes in the dark. Cultures were collected and analyzed for cell apoptosis using a flow cytometer (FACScan; BD Biosciences Franklin Lakes) equipped with CellQuest 3.3 software. Cells were categorized as early apoptotic cells, late apoptotic cells, dead cells, or viable cells. The ratio of early apoptotic cells was compared with that in the controls from each experiment. Flow cytometry was performed with three parallel replicates of each cell line.

Wound healing assay

Wounds were made using a 100-μl plastic pipette tip in incubation wells (1 × 10^6). After a 24 h incubation, wound sizes were accessed and imaged. The cell migration area was measured between dashed regions using ImageJ software (NIH, Bethesda) and normalized to control cells.

Transwell assay

Cell invasion was assessed using Transwell experiments. Briefly, quantitative cell migration assays were performed using 24-well plates containing chambers with 8-μm polycarbonate filters. Hepatoma cells (1 × 10^5) were seeded in serum-free medium and allowed to translocate toward complete media supplemented with 10% fetal bovine serum. The cells that invaded through the membrane into the lower surface after 24 h were fixed, stained and counted at 37 ℃.

Western blot analysis

Total cellular protein was evaluated by western blot analysis. Samples were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF)

membranes (Millipore). Sealed protein samples were incubated with primary antibodies (Supplementary Table S2) overnight at 4 ℃ After the membranes were incubated with secondary antibodies, they were subjected to immunoblot analysis using an ECL immunoblotting kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Each band was normalized with respect to its corresponding GAPDH band. Band Scan (Glyko, Inc., USA, version 5.0) was used to quantitatively analyze each band for statistical comparison.

Hematoxylin-eosin (HE), immunochemical and immunofluorescence staining

For the HCC and adjacent tissue samples, tissue sections were deparaffinized in xylene and rehydrated with ethanol before paraffin embedding. All the tissue samples were sectioned to produce 4-mm thick slices. To perform HE staining, slices were stained with hematoxylin and eosin for 3 minutes and 5 seconds after dewaxing. For immunohistochemical staining, slices were incubated in boiling 0.01 mol/L citric acid buffer for 15 minutes for antigen repair before incubating with primary antibodies (Supplementary Table S2). After incubation with secondary antibody, DAB solution (Dako Denmark A/S) and hematoxylin were used for staining before sealing. Immunocytochemical staining was performed under similar conditions after the cells were seeded onto slides. For immunofluorescence staining, cells were fixed with 4% paraformaldehyde/PBS before incubating with the anti-AHCY antibody. Incubation with the Alexa-488 secondary antibody (Invitrogen) lasted for 1 h before fluorescence observations.

Fluorescence in situ hybridization (FISH)

RNA FISH was used to observe the relative subcellular localization and molecular abundance. FISH was performed according to the Ribo Fluorescence In Situ Hybridization Immobilized Kit (RN: 10910; RiboBio Co., Ltd.) protocol. After prehybridization buffer treatment, the probe mixture was diluted in hybridization buffer after removing prehybridization buffer and was incubated overnight at 37 ℃. The DNA was dyed with DAPI for 10 minutes before sealing. The subcellular localization and molecular abundance were observed under the same optical conditions with a Double Disc Laser Confocal Imaging System (UltraVIEW VOX &

1 × 81; Perkin Elmer & Olympus).

Transfection and luciferase reporter assays

To verify the activity of mRNA and its 3'UTR, a luciferase reporter was used for indirect quantitative analysis. All transfections were performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Luciferase activity was measured using a Dual Luciferase Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The wild-type 3'UTR cDNA of MAT1A (containing a putative binding site) was inserted into a pGL3-basic vector (Promega, USA) downstream of the luciferase reporter gene, generating the RLuc-MAT1A-3'UTR-WT plasmid. All plasmids were verified by DNA sequencing, and fluorescence intensity was measured using a luminometer (Promega, USA).

Ultra-performance liquid chromatography (UPLC)

Ultra-performance liquid chromatography was utilized for the quantitative analysis of S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) levels. The UPLC-LC/MS measurements were performed using an ACQUITY Ultra Performance LC system (Waters) that was coupled to an API4000 tandem mass spectrometer (AB Sciex). The samples were separated on a Waters ACQUITY UPLC1 BEH Amide (2.1 mm × 100 mm, 1.7-mm particle size) column. The column temperature was 40 ℃ with 5-ml injections. The retention time was set according to the peak value of SAM and SAH standards with ± 0.1 minutes.

Enzyme linked immunosorbent assay (ELISA)

5-Methylcytosine (5mC), which represents proximate global methylation [1], was detected according to the MethylFlash Global DNA Methylation ELISA Easy Kit (Epigentek Group Inc. USA) protocol. Briefly, 14 wells were included in the reactions for the construction of the standard curve (ranging from 0.1% to 5%). DNA samples were diluted in 100 ml binding solution in each well. The absorbance values were calculated according to the standard curve to obtain the final results.

Methylation microarray profiling

Aberrant methylation profile patterns were detected by methylation microarray

profiling, as illustrated previously [2- 3]. Bisulfite conversion was performed using the EZDNA Methylation kit (Zymo Research) for DNA samples. Scanning (iScan) of the Human Methylation 850 K BeadChip arrays was performed according to the manufacturers' instructions. The data from all the samples were imported in the idat file format into Genomic Suite 6.6 (Partek). Different gene segments, including CpG islands and promoter regions, were labeled. Raw methylation values were compared between the groups with at least a 1.5-fold change, and differences were determined significant if $P < 0.05$. All the original methylated values were normalized to a respective average value. The difference for each locus is presented as homogenized data ranging from -0.15 to 0.15. Moreover, multiple hypothesis tests using the FDR values were conducted to identify HCC-promoting genes with significant methylation changes.

Pharmacological treatment

Hepatoma cells were treated with actinomycin D (ActD) (Sigma-Aldrich) to verify RNA stability. The cells were harvested from 6-well plates at 0, 1, and 2 h for q-PCR after adding 15 μg ActD. The relative expression levels of each mRNA at 0 h were normalized to 1. Additionally, the cell lines were incubated in the presence or absence of the protein-synthesis inhibitor cycloheximide (CHX, 100 μg/μL) for different lengths of time (0 h, 4 h, 8 h). The protein levels in the whole cell extracts of the experimental and control groups were determined by western blotting. Moreover, hepatoma cells were treated with SAM (10 μmol/L) or SAH (250 μmo/L; Sigma-Aldrich) dissolved in PBS every 12 h for 48 h and then harvested for subsequent tests. An isodose PBS solution was used as the control in the same batch of cells.

RNA pulldown

LINC00662 was synthesized *in vitro* by Ribo Biotech, and Biotin labeling was performed with the Pierce RNA 3’ End Desthiobiotinylation Kit (Thermo Scientific, USA). RNA pulldown was performed with a Pierce Magnetic RNA-Protein Pull-Down Kit (Thermo Scientific, USA). In brief, biotin-labeled LINC00662 and negative control RNAs were incubated with hepatoma cell lysates and streptavidin

magnetic beads. The beads were washed, and the proteins were eluted and resolved by PAGE. Then, silver staining was conducted. Gel fragments were excised and analyzed by western blotting.

RNA immunoprecipitation (RIP) analysis

RNA immunoprecipitation was performed using the RNA-Protein Immunoprecipitation Kit (Magna RIP™) according to the manufacturer's protocol. Briefly, HCCLM9 and Huh7 cells were scraped and lysed at 80-90% confluency in 15-cm culture dishes and incubated on ice for 5 minutes. After washing and magnetic suspension, AHCY antibody (Proteintech) was used for incubation. RNA quality assessment and RT were performed using a Nanodrop™ spectrophotometer (Thermo Scientific) and an EZ-Magna RIP kit (Magna). Finally, q-PCR was performed to examine AHCY-bound RNA, which was normalized to the input and compared to IgG-bound RNA. Agarose gel electrophoresis was conducted to visualize the cDNA fragments after q-PCR.

Ubiquitination assay

Hepatoma cells transfected with LINC00662 or empty vector were incubated in the presence or absence of 30 μM MG132 (Cell Signaling Technology) for 24 h. Total proteins were extracted using RIPA buffer supplemented with proteinase inhibitor. Then, immunoprecipitation was performed using anti-AHCY or anti-IgG. The immunoprecipitated proteins were subjected to western blotting using anti-ubiquitin (Proteintech) to evaluate the ubiquitination level. The inputs were subjected to western blot analysis with an anti-AHCY antibody to quantify the levels of AHCY, which were normalized to GAPDH.

Methylated DNA Immunoprecipitation (MeDIP)-PCR assay

MeDIP-PCR was used to evaluate the methylation status of specific promoters. This method is based on quantitative analysis of methylated-specific compounds and relative q-PCR, which has been described previously [4- 6]. Briefly, genomic DNA was extracted and randomly sheared to an average length of 0.3-1.0 kb by sonication (Diagenode). Antibodies (Eurogentec) against 5-methylcytosine were utilized for methylated precipitation, and purified DNA was analyzed by q-PCR on a ViiA 7

Real-time PCR system (Applied Biosystems). Changes in methylation were determined by measuring the amount of immunoprecipitated DNA after normalization and comparing that value with the amount of input DNA (MeDIP/Input%).

RNA-binding protein purification and identification (RaPID) analysis

An RNA-binding protein purification and identification (RaPID) methodology was designed to examine the interactions among miRNAs, lncRNAs, and mRNAs [7]. To this end, the RNA of interest is tagged with the MS2 vector and coexpressed with a fusion protein of an MS2-binding protein and a streptavidin-binding protein (SBP). The RNA immunoprecipitation complex of SBP and binding RNAs was tested by q-PCR to illustrate the specific binding RNAs. In this study, the MAT1A 3'UTR was loaded onto the MS2 vector to evaluate its interactions with LINC00662. The RNA fraction isolated by RIP was quantified with a NanoDrop spectrophotometer (Thermo Fisher Scientific), and the RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent).

***In vivo* experiments**

The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Wuhan University in Wuhan, China. Male athymic 4-week-old BALB/c nude mice were obtained from the Animal Center of the Chinese Academy of Medical Sciences (Beijing, China) and were maintained in a specific pathogen-free facility. HCCLM9 cells stably transfected with LINC00662-shRNA or scrambled control were harvested from 6-well plates. Next, we respectively conducted tumor growth and metastatic models based on subcutaneous and liver in situ injection. Tumor growth assays were performed in 10 nude mice (5 in each group) for 36 days after subcutaneously injecting 5×10^6 cells/ml HCCLM9 suspensions into the armpits of the mice, and subcutaneous tumor size was assessed every 3 days. For the observation of extra-organ metastases, 1×10^7 cells/ml suspensions were in situ injected into liver tissue for 7 weeks (5 in each group). At the end of the seventh week, the mice were sacrificed, and their lungs were removed to observe lung metastases. Relative lung tissue anatomy and slices were used for further statistical analysis.

Supplementary References

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[5] Zhao MT, Whyte JJ, Hopkins GM, Kirk MD, Prather RS. Methylated DNA immunoprecipitation and high-throughput sequencing (MeDIP-seq) using low amounts of genomic DNA. Cell Reprogram. 2014;16:175-84.

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Supplementary Tables

Supplementary Table S1. Primer sequences used in this research

Gene	Sequence (5'-3')
GAPDH-F	GGTATCGTGGAAGGACTCAT
GAPDH-R	CCTTGCCCACAGCCTTG
LINC00662-F	TTTGCCTTGTTCCTGAGCTT
LINC00662-R	CACCTCATGGATGCAGAGAA
MAT1A-F	AGTCCTCGCCTGTTCTCACG
MAT1A-R	CATCCACCGGTCCATTCATC
AHCY-F	ATTCCGGTGTATGCCTGGAAG
AHCY-R	GAGATGCCTCGGATGCCTG
MYC-F	CAAGAGGCGAACACACAACGTCT
MYC-R	AACTGTTCTCGTCGTTTCCGCAA
HRAS-F	TGAGGAGCGATGACGGAATA
HRAS-R	GTATCCAGGATGTCCAACAG
CTNNB1-F	CATCTACACAGTTTGATGCTGCT
CTNNB1-R	GCAGTTTTGTCAGTTCAGGGA
LINC00662-siRNA-1	GCUGCUGCCACUGUAAUAATT UUAUUACAGUGGCAGCAGCTT
LINC00662-siRNA-2	GCAGGCGUACAACUAACAATT UUGUUAGUUGUACGCCUGCTT
LINC00662-siRNA-control (scramble)	UUCUCCGAACGUGUCACGUTT ACGUGACACGUUCGGAGAATT
MedIP-MYC-F	CCCATATTCTCCCGTCTAGCAC
MedIP-MYC-R	CCAATTTCTCAGCCAGGTTTCA
MedIP-HRAS-F	GTTTGCCTGACTGTAAAACGC
MedIP-HRAS-R	GCAGGAGAATCGCTGGAAC
MedIP-CTNNB1-F	GATTCAGGTCGAAATTCAAGC
MedIP-CTNNB1-R	CAGCCCAGTCTCACAGCAC

Supplementary Tab. S2. Antibodies used in this study.

Antibody	Company	Specificity	Cat No.	Western Blot	IHC/ICC
GAPDH	Sungene Biotechnology	Mouse monoclonal	KM9002	1:2000	
MAT1A	Proteintech	Rabbit polyclonal	12395-1-AP	1:2000	
AHCY	Proteintech	Rabbit Polyclonal	10757-2-AP	1:1000	1:200
Ki-67	Santa Cruz Biotechnology	Rabbit polyclonal	sc-15402		1:200
MYC	Cell Signaling Technology	Rabbit monoclonal	#13987S	1:2000	
HRAS	Sigma-Aldrich	Mouse polyclonal	SAB1405964	1:2000	
CTNNB1	Cell Signaling Technology	Rabbit monoclonal	#8480S	1:1000	

Supplementary figure legends

Figure S1. Expression of LINC00662 in hepatoma cells.

(A) q-PCR detection of LINC00662 expression in different hepatoma cells. Error bars are SD (n = 3). (B) q-PCR analysis of LINC00662 after LINC00662 siRNA transfection in HCCLM9 cells. Error bars are SD (n = 3). (C) q-PCR analysis of LINC00662 after vector-LINC00662 transfection in Huh7 cells. Error bars are SD (n = 3).

Figure S2. Detailed aberrant genomic methylation patterns in HCCLM9 cells after LINC00662 knockdown.

(A) The beta distribution diagram of loci with methylation alterations after LINC00662 knockdown in HCCLM9 cells. (B and C) Summaries of loci with hypermethylation and hypomethylation in respective gene segments. (D) Heatmap of aberrantly methylated loci in the TSS 1500+200 regions. (E) Heatmap of aberrantly methylated loci in CpG islands.

Figure S3. Details aberrant genomic methylation patterns in Huh7 cells after LINC00662 overexpression.

(A) The beta distribution diagram of loci with methylation alterations after LINC00662 overexpression in HepG2 cells. (B and C) Summaries of loci with hypermethylation and hypomethylation in respective gene segments. (D) Heatmap of aberrantly methylated loci in TSS 1500+200 regions. (E) Heatmap of aberrantly methylated loci in CpG islands.

Figure S4. Methylation alternation was detected in the promoter regions of multiple HCC-promoting genes by multiple hypothesis testing.

(A) Top 33 genes with the greatest methylation differences after LINC00662 knockdown in HCCLM9. (B) Top 33 genes with the greatest methylation differences after LINC00662 overexpression in Huh7.

Figure S5. Efficiency of MAT1A and AHCY overexpression in hepatoma cells.

(A) q-PCR and (B) western blot analysis of MAT1A after vector-MAT1A transfection in HCCLM9 and Huh7 cells. Error bars are SD (n = 3). (C) q-PCR and (D) western blot analysis of AHCY after vector-AHCY transfection in HCCLM9 and Huh7 cells. Error bars are SD (n = 3).

Figure S6. Suppression of MAT1A on oncogenic behaviors.

(A) CCK-8 assay revealed that MAT1A inhibited the proliferation of HCCLM9 and Huh7 cells. Error bars are SD (n = 3). (B) Flow cytometric analysis determined that MAT1A could significantly enhance the apoptosis rate in HCCLM9 and Huh7 cells. Error bars are SD (n = 3). (C) Colony formation was reduced after MAT1A overexpression in HCCLM9 and Huh7 cells. Error bars are SD (n = 3). (D) A 5-ethynyl-2'-deoxyuridine assay was used to analyze DNA replication in HCCLM9 and Huh7 cells after MAT1A overexpression. (bar= 50 μm). Error bars are SD (n = 3). (E) Representative images of wound healing assays performed after MAT1A overexpression in HCCLM9 and Huh7 cells. Error bars are SD (n = 3). (F) Transwell assays performed using HCCLM9 and Huh7 cells after MAT1A overexpression (bar= 60 μm). Error bars are SD (n = 3). *P <0.05, **P < 0.01, ***P < 0.001.

Figure S7. AHCY exhibited anti-tumorigenicity in hepatoma cells.

(A) A CCK-8 assay was conducted to detect the proliferation of HCCLM9 and Huh7 cells after AHCY overexpression. Error bars are SD (n = 3). (B) Flow cytometric analysis of apoptosis in hepatoma cells after AHCY overexpression. Error bars are SD (n = 3). (C) Cell growth was investigated by colony formation in HCCLM9 and Huh7 cells after AHCY overexpression. Error bars are SD (n = 3). (D) The 5-ethynyl-2'-deoxyuridine assay was used to analyze DNA replication in HCCLM9 and Huh7 cells after AHCY overexpression. (bar= 50 μm). Error bars are SD (n = 3). (E) Representative images of wound healing assays performed after AHCY overexpression in HCCLM9 and Huh7 cells. Error bars are SD (n = 3). (F) Transwell

assays conducted in HCCLM9 and Huh7 cells after AHCY overexpression (bar= 60 μm). Error bars are SD (n = 3). *P <0.05, **P < 0.01, ***P < 0.001.