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Supplemental Information

Long Noncoding RNA HCAL Facilitates the Growth and Metastasis of Hepatocellular Carcinoma by Acting as a ceRNA of LAPTM4B

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Supplementary materials and methods

Microarray analysis

Fresh HCC and matched non-tumor tissue samples from six patients with HCC who initially underwent hepatectomy without any preoperative treatment at the Zhongshan Hospital of Xiamen University from 2011 to 2013 were randomly selected from the obtained samples for microarray analysis. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA). Next, cDNA was synthesized, labeled, purified, and hybridized with Human lncRNA Array v2.0 (Arraystar, Rockville, USA). After washing, slides were scanned using a DNA Microarray Scanner (Agilent). Data were extracted using Feature Extraction software (Agilent) and were analyzed using GeneSpring GX v11.5.1 software (Agilent). The experiments and data analyses were performed at Kang Chen Biology (Shanghai, China). Upregulated or downregulated lncRNAs and mRNAs in HCC and matched non-tumor samples were screened using a fold change of ≥ 2 and a p value of < 0.05 indicated significance.

RNA sequencing

Total RNA was extracted, and eukaryotic mRNA was enriched using oligo(dT) beads. Next, the enriched mRNA was fragmented in a fragmentation buffer and was reverse transcribed into cDNA using random primers. Second-strand cDNA was synthesized using DNA polymerase I, RNase H, dNTPs, and buffer. The cDNA fragments obtained were purified using a QIAquick PCR extraction kit and were end repaired. Next, poly(A) tails were added to the cDNA fragments, and the fragments were ligated with Illumina sequencing adapters. Ligation products were selected according

to size by performing agarose gel electrophoresis, amplified by PCR, and sequenced using Illumina HiSeq™2500 at Gene Denovo Biotechnology (Guangzhou, China). Upregulated or downregulated mRNAs were screened based on a fold change of ≥ 2 and a *p* value of <0.05.

Plasmid construction

Full-length HCAL was cloned into the pcDNA3.1 vector (Invitrogen), named pcDNA-HCAL. pSL-MS2-12X (Addgene) was double digested with EcoR I and Xho I, and the 12×MS2 fragment was cloned into pcDNA3.1 or pcDNA-HCAL, named pcDNA-MS2 or pcDNA-HCAL-MS2, respectively. The HCAL or LAPTM4B 3'UTR was amplified using PCR and cloned into the pmirGLO vector (Promega) for luciferase assay.

Transient transfection

Transfections were performed using the TurboFect Transfection Reagent (Thermo) according to the manufacturer's instructions. The empty vector pEnter or pEnter-LAPTM4B (purchased from Vigenebio Company, Shandong, China) was introduced into cells, respectively. The subsequent experiments were performed at 48 hour after transfection.

Western blot analysis

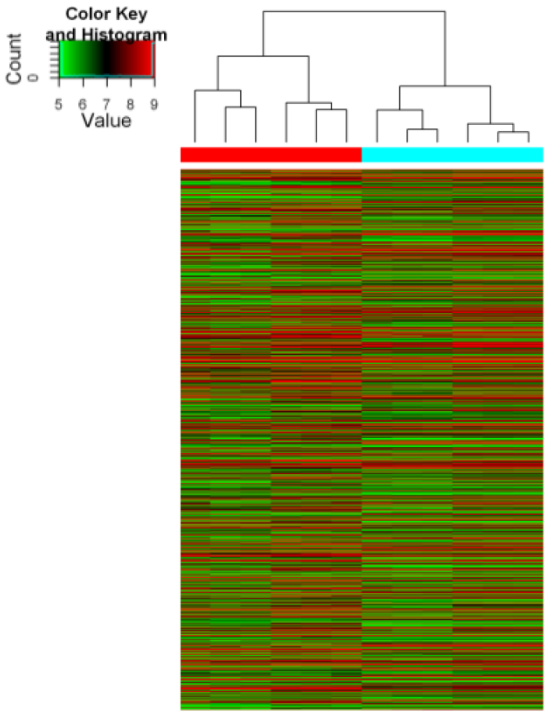
Cells and tissue samples were lysed by RIPA buffer (Beyotime Biotechnology, Beijing, China). Total proteins were separated by SDS-PAGE and then transferred to PVDF membranes (Millipore). Membranes were blocked in 5% non-fat milk and then incubated with anti-CDK4 (Proteintech), anti-CDK6 (Proteintech), anti-PARP (Cell

Signaling), anti-Caspase3 (Cell Signaling) or anti-LAPTM4B (Proteintech) antibodies at 4 ℃ overnight, and followed by incubation with secondary antibodies conjugated with horseradish peroxidase (HRP, Jackson). Immunoreactive proteins were visualized using the ECL detection system (Millipore).

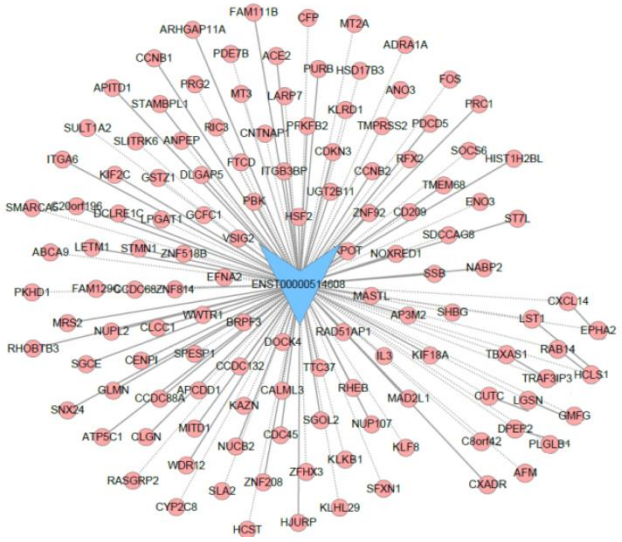
Cell cycle analysis

After trypsinization, cells were fixed by adding 70% ethanol at 4℃ overnight. The cells were collected by centrifugation and incubated in PBS containing 100 g/ml RNase A and 50 g/ml propidium iodide (PI) for 30 min at 37℃. Then samples were subjected to flow cytometry and the data were analyzed by Kaluza software.

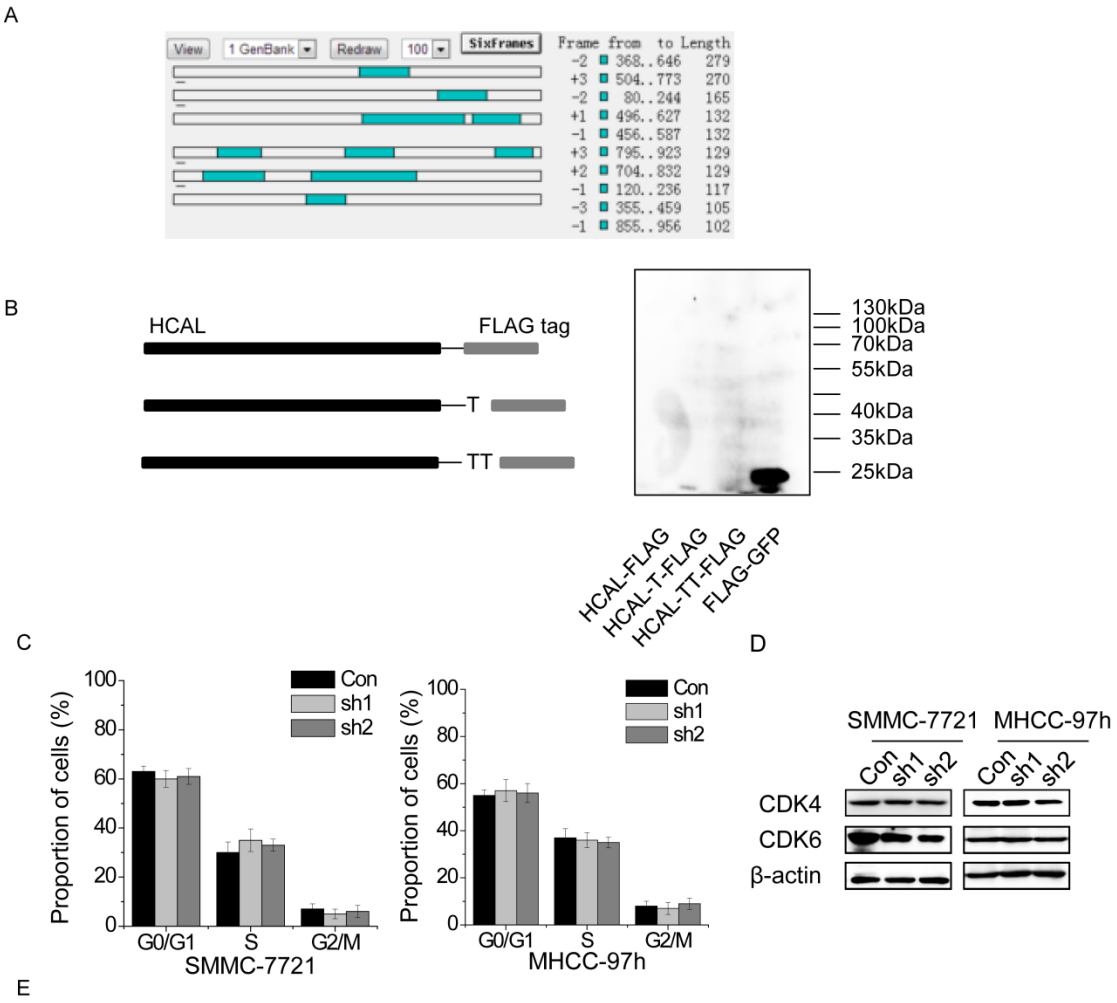
Supplemental figure and figure legends



Supplemental Figure 1. Hierarchical clustering analysis of the differential expressed mRNA (fold chage >2, $p < 0.05$) between HCC and paired peri-tumor samples.



Supplemental Figure 2. HCAL subnetwork in the HCC coexpression network. This subnetwork consists of HCAL (center) and its direct neighbors.



E

Group	Pulmonary metastasis	Intrahepatic metastasis
Con	5/7	6/7
sh1	1/7	3/7
sh2	1/7	3/7

Supplemental Figure 3. HCAL regulates cell proliferation, apoptosis, migration and invasion *in vitro* and *in vivo*.

A. Putative proteins possibly encoded by HCAL as predicted by the ORF Finder.

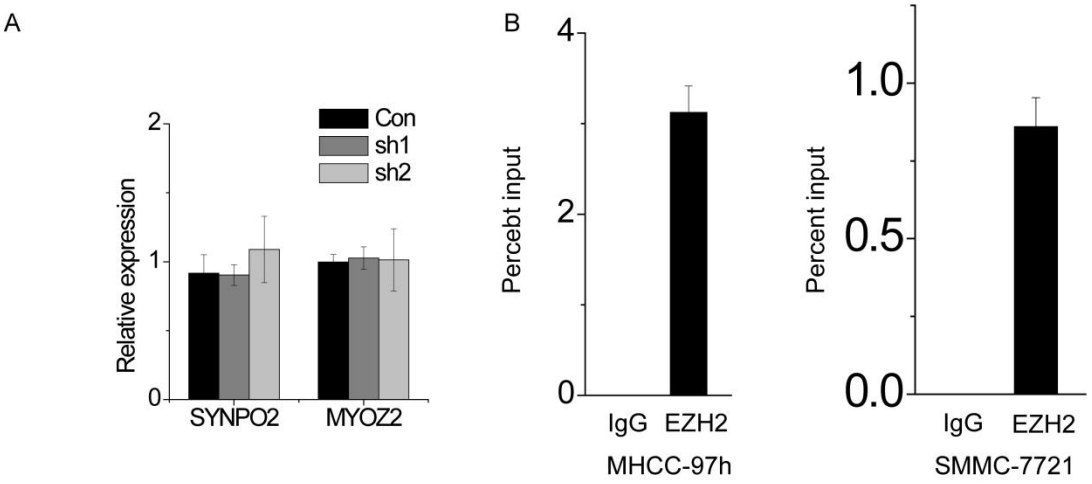
B. Full-length HCAL was cloned into the eukaryotic expression vector pcDNA3.1 C-terminal Flag tag in all three coding patterns and these plasmids subsequently transfected into HEK-293T cells respectively. After 48h, western blotting was used to detect the Flag-tagged protein. GFP with Flag tag severs as a positive

control.

C. The cell cycle distribution was determined in control and HCAL-silenced cells by flow cytometry.

D. The expression of checkpoint proteins, including CDK4 and CDK6, were determined in in cells expressing control and HCAL shRNAs by western blot.

E. The statistical results of pulmonary metastasis and intrahepatic metastasis in nude mice injected subcutaneously with the HCAL-silenced SMMC-7721 cells.



C

Score	Expect	Identities	Gaps
1561 bits(845)	0.0	937/981(96%)	8/981(0%)

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Query  61    CGGGCTTCACTAACCTTCCCTAGGCAGTGAAACATCCCCCAAATCTGATGGATCTAGAGT  120
Sbjct  1886  CGGGCTTCACTAACCTTCCCTAGGCATTGAAACTTCCCCCAAATCTGATGGA-C-----CT  1940
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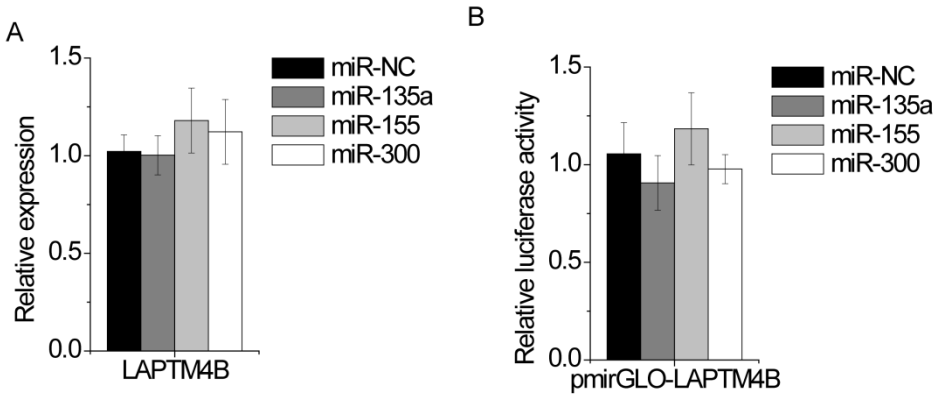
Supplemental Figure 4. *LAPTM4B* is a target gene of HCAL.

A. The relative mRNA expression of SYNPO2 and MYOZ2 were determined in control and HCAL-knockdown cells by qPCR.

B. Anti-EZH2 RIP was performed in SMMC-7721 and MHCC-97h cells, followed by qPCR.

C. The Blast comparison between HCAL and LAPTM4B 3'UTR.



Supplemental Figure 5. HCAL regulates LAPTM4B expression through competitively binding common miRNAs.

A. The mRNA level of LAPTM4B in SMMC-7721 cells transfected with miR-135a, miR-155 or miR-300.

B. The relative luciferase activity of LAPTM4B 3'UTR in SMMC-7721 cells transfected with miR-135a, miR-155 or miR-300.

Supplemental Tables

Supplemental Table 1. The primers for qPCR

Gene symbol	Sequences
ACTB	F：CACTCTTCCAGCCTTCCTTC
	R：GTACAGGTCTTTGCGGATGT
LAPTM4B	F：GGAACTGCTACCGATACATCAA
	R：TCACAGTGGCATCATCATACG
HCAL	F：TGATCATCTGCTGACTGTTCTC
	R：CAGTGCCAAACACATCCATTC
18s rRNA	F：ACACGGACAGGATTGACAGA
	R： GGACATCTAAGGGCATCACA
SYNPO2	F：ACCTCTCCTCCTTCCTTCTT
	R：GATGAGGCTGATTCTTGCTTTG
MYOZ2	F：CGTGGTGCCAGGCTATTTA
	R：TCCATCCACTTTCCCATTCTG
RP11-172E9.2	F：GAACAGCATGGAGCAAGGAA
	R：GCAGGATGGCACTACTGATAAC
LINC00598	F：TCCCAAAGTGCTGGGATTAC
	R：CTCTGGTGTGGGATGTTGATAG
LOC100128098	F：CCCAGAACCTATTGGAACTGAC
	R：CCATCTGCCCTTGCTTTCT
RP11-150O12.3	F：CGTCGTCTGACATCAGCTATT
	R：CCACGCATGCAGGAATAAAC
uc.197	F：CTGTCAGAGATCACGTAGAGTA
	R：GGGCCTTTACCACCATAAA

Supplemental Table 2. Predicted ceRNAs for lncRNA HCAL

Supplemental Table 3. Predicted miRNAs bind to lncRNA HCAL