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Supplemental Information

Novel Long Non-coding RNA lncAMPC Promotes Metastasis and Immunosuppression in Prostate Cancer by Stimulating LIF/LIFR Expression

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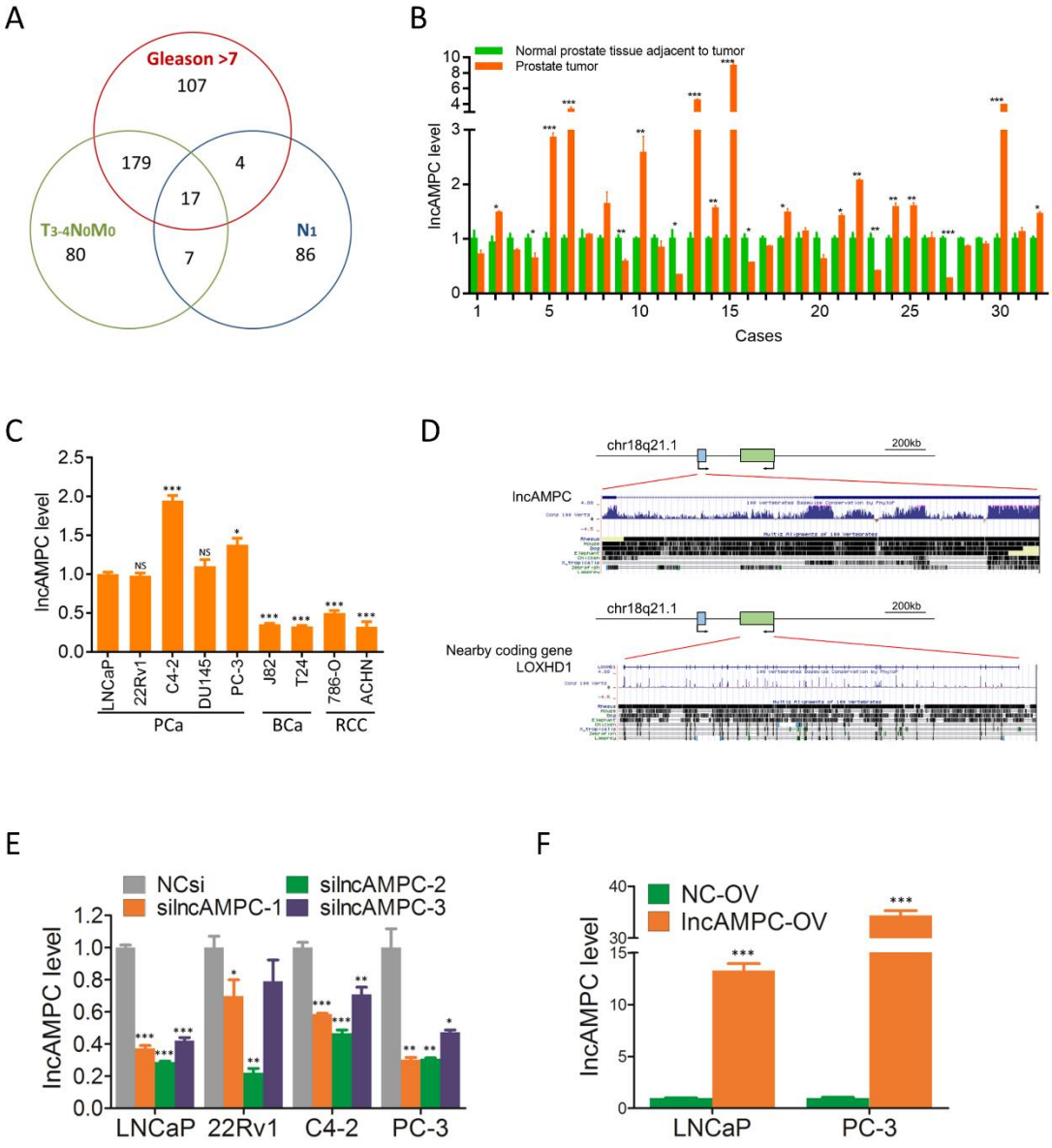


Figure S1. The targeted lncRNA selection and lncAMPC knockdown or overexpression efficiency. (A) The subgroup-analysis of identified lncRNAs according to the Gleason score and pathological TNM staging of patients. (B) qRT-PCR analysis of lncAMPC expression in an independent set of primary tumor tissue and its adjacent normal prostate tissue (n = 3). (C) Placental mammal conservation analysis of lncAMPC and its nearby coding gene LOXHD1 by PhyloP, UCSC. (D) qRT-PCR analysis of lncAMPC expression in a panel of PCa, bladder cancer, and renal cancer cell lines (n = 3). (E) qRT-PCR analysis of lncAMPC expression after siRNAs transfection (n = 3). (F) qRT-PCR analysis of lncAMPC expression after pcDNA3.1-lncAMPC transfection (n = 3). Results are presented as mean ± SD; *p < 0.05, **p < 0.01, ***p < 0.001.

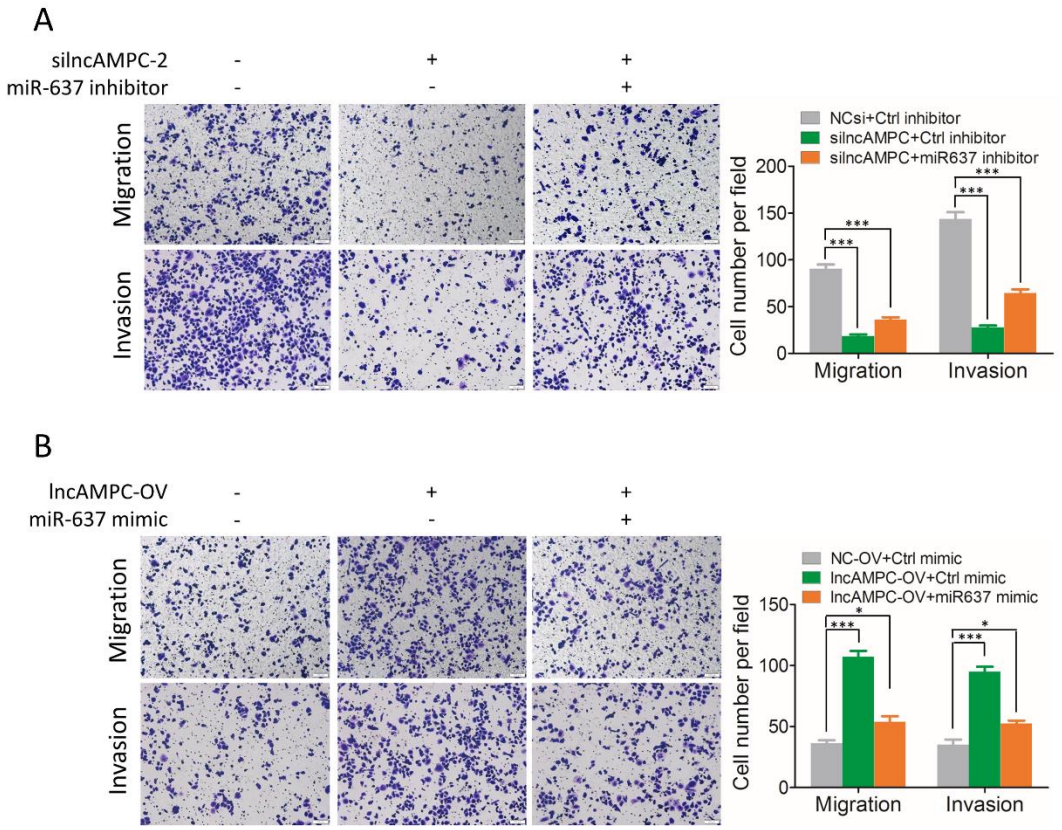
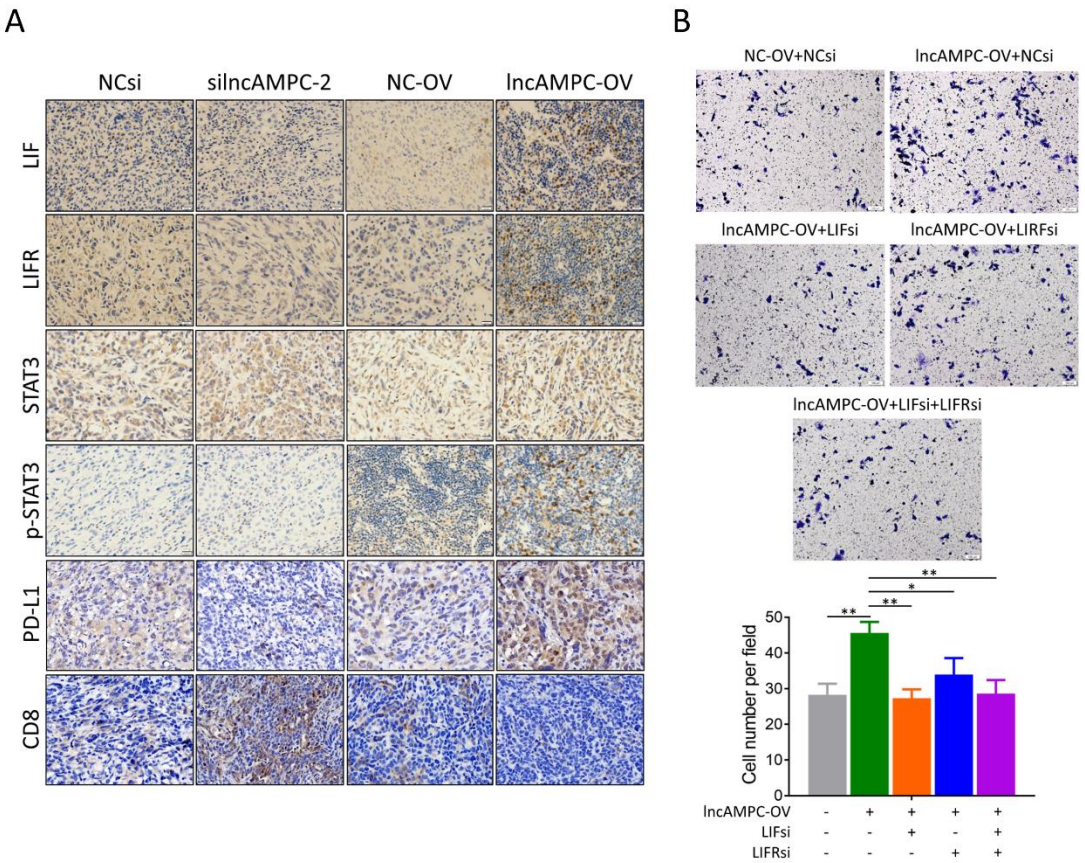


Figure S2. The rescue experiment evaluating the role of miR-637, LIF and LIFR on cell migration and invasion. (A) Transwell assay testing the cell migration and invasion of PC-3 transfected with silncAMPC-2 alone or combined with miR-637 inhibitor simultaneously (n = 3). (B) Transwell assay testing the cell migration and invasion of PC-3 transfected with lncAMPC-OV alone or combined with miR-637 mimics simultaneously (n = 3). Results are presented as mean ± SD; *p < 0.05, **p < 0.01, ***p < 0.001.



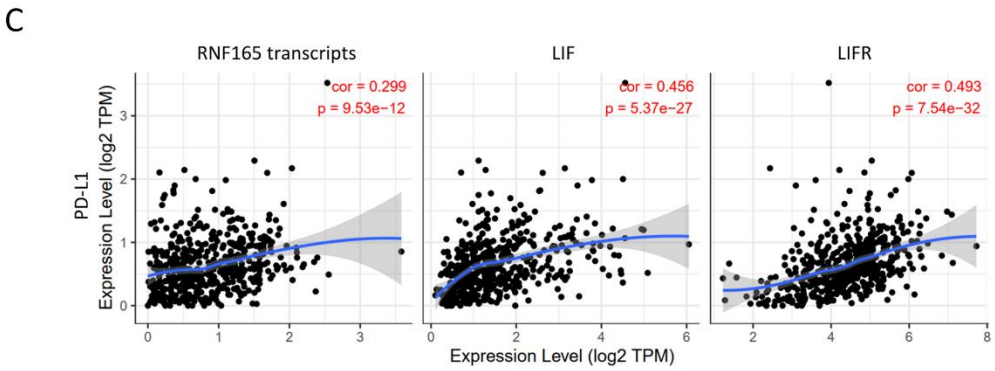


Figure S3. LIF/LIFR-activated Jak1-STAT3 signaling pathway is essential for lncAMPC oncogenic and immunosuppression activities in PCa. (A) Immunohistochemistry analysis evaluating the status of LIF/LIFR/Jak1/STAT3 signaling pathway and PD-L1, CD8 in the tumors from subcutaneous xenograft models (n = 3). (B) Transwell assay testing the cell invasion of PC-3 transfected with lncAMPC-OV alone or combined with LIFsi/LIFRsi simultaneously (n = 3). (C) The public human PCa dataset evaluating the correlation between RNF165 transcripts, LIF, LIFR and PD-L1 expression levels. Results are presented as mean ± SD; *p < 0.05, **p < 0.01, ***p < 0.001.

Table S1. The clinical baseline characteristics of 65 patients for RNA-seq analysis and 32 patients for qRT-PCR validation

Variable	No. cases (%)	
	RNA-seq analysis	qRT-PCR validation
PSA level (ng/μl)		
<10	14 (21.5%)	11 (34.4%)
10-20	36 (55.4%)	12 (37.5%)
>20	15 (23.1%)	9 (28.1%)
Gleason score		
<7	4 (6.2%)	8 (25.0%)
7	36 (55.4%)	11 (34.4%)
>7	25 (38.5%)	13 (40.6%)
TNM stage		
$T_2N_0M_0$ or lower	37 (56.9%)	21 (65.6%)
$T_{3-4}N_0M_0$	23 (35.4%)	1 (3.1%)
$T_xN_1M_x$ or $T_xN_xM_1$	5 (7.7%)	10 (31.3%)

Table S2. Prediction of miRNA target sites for lncAMPC

miRNA name	Predicted target site
hsa-miR-655-3p	TGTATTA
hsa-miR-637	CCCCCAG
hsa-miR-646	TGCTGCTA
hsa-miR-196a-5p	ACTACCT
hsa-miR-196b-5p	ACTACCT
hsa-miR-922	TCTGCTG

Table S3. siRNA sequence

siRNA name	Sequence	
silncAMPC-1	Sense（5’-3’）	GUAUUGUGACACCCUCUUA
	Anti-sense（5’-3’）	UAAGAGGGUGUCACAAUAC
silncAMPC-2	Sense（5’-3’）	CACAGAAUCCACUAAUACU
	Anti-sense（5’-3’）	AGUAUUAGUGGAUUCUGUG
silncAMPC-3	Sense（5’-3’）	GAAACAAAACAGCAGCAAU
	Anti-sense（5’-3’）	AUUGCUGCUGUUUUGUUUC

Table S4. Primer sequence for PCR of the RACE analysis

Probe/Primer name	Sequence（5’-3’）
Probe-lncAMPC	ACCATTCACTGAGCTCTG
Probe-lncAMPC-F	GTGTTTGGCTCTGTGCGAAA
Probe-lncAMPC-R	GCACACAGCAGCGAGTTG

Table S5. Primer sequence for qRT-PCR

Gene name	Primer name	Sequence（5’-3’）
lncAMPC	lncAMPC-Forward	TGAGGGGTGAGTTGGTCTGT
	lncAMPC-Reverse	ACAGTGCAATGTACTCGGCT
E-Cadherin	E-Cadherin-Forward	GCCCCATCAGGCCTCCGTTT
	E-Cadherin-Reverse	ACCTTGCCTTCTTTGTCTTTGTTGGA
ZO-1	ZO-1-Forward	CACGCAGTTACGAGCAAG
	ZO-1-Reverse	TGAAGGTATCAGCGGAGG
N-Cadherin	N-Cadherin-Forward	TGGACCATCACTCGGCTTA
	N-Cadherin-Reverse	ACACTGGCAAACCTTCACG
Vimentin	Vimentin-Forward	CCTGAACCTGAGGGAAACTAA
	Vimentin-Reverse	GCAGAAAGGCACTTGAAAGC
miR-637	miR-637-Forward	ACACTCCAGCTGGGACTGGGGGCTTTCGGG
	miR-637-Reverse	CTCAACTGGTGTCGTGGAGTCGG
LIF	LIF-Forward	CCAACGTGACGGACTTCCC
	LIF-Reverse	TACACGACTATGCGGTACAGC
LIFR2125bp	LIFR2125bp-Forward	TTGAGGCCCACTACTTTGGT
	LIFR2125bp-Reverse	ACAGAAGTAGCTCGTGGGGT
LIFR2328bp	LIFR2328bp-Forward	GCTGGATTCCCTCTCCGTCA
	LIFR2328bp-Reverse	AAGCACCAAAGTAGTGGGCCT
LIFR2406bp	LIFR2406bp-Forward	AAGCCCATGAAATACTTGCCC
	LIFR2406bp-Reverse	TGACGGAGAGGGAATCCAGC
LIFR2677bp	LIFR2677bp-Forward	TGGGGAAAGTGGGTGAATTTCT
	LIFR2677bp- Reverse	AGGGGCAAGTATTTCATGGG
LIFR2918bp	LIFR2918bp-Forward	AGAGCACCGAGATTGCACCAT
	LIFR2918bp-Reverse	AGCTGCATCCAGGATTCACATG
LIFR3091bp	LIFR3091bp-Forward	AGGCAGGCGGATCACCTAAG
	LIFR3091bp-Reverse	CCCTCCCGAGTTGAAGCATTTC
LIFR3204bp	LIFR3204bp-Forward	GAAAAAGCTCAGTGGGTTGGG
	LIFR3204bp-Reverse	ACTTTCAATCTGGCTCCTGCC

Table S6. Antibody for western blotting

Antibody name	Catalog number (Vendor)
LIF	ab138002 (Abcam)
LIFR	sc-515337 (Santa Cruz)
STAT3	#4904 (Cell Signal Technology)
Phospho-STAT3	#9145 (Cell Signal Technology)
Histone H1.2	ab181973 (Abcam)
CD8 (human)	GB13068 (Servicebio)
CD8 (mouse)	GB13429 (Servicebio)
PD-L1	ab238679 (Abcam)

Supplemental Methods

Cell culture and cell transfection

The human PCa cell lines (LNCaP, 22Rv1, C4-2, PC-3, DU145) and a human embryonic kidney cell line (293T) were purchased from ATCC (Rockville, MD, USA). LNCaP, 22Rv1, C4-2, PC-3, DU145 were maintained in RPMI medium 1640 (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA), and 293T was cultured in DMEM medium (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA). Transfection of siRNAs (100 nM, GenePharma, China) or plasmids was performed by using Lipofectamine RNAiMAX Reagent (Invitrogen, USA) or Lipofectamine 2000 Reagent (Invitrogen, USA), respectively. Sequences of siRNAs are listed in Supplementary Table 3.

5' and 3' rapid amplification of cDNA ends (RACE)

The 5'-RACE and 3'-RACE analyses were performed to demonstrate the transcriptional initiation and termination sites of lncAMPC using a SMARTer RACE 5’/3’ cDNA Amplification Kit (Clontech, USA) according to the manufacturer’s instructions. The gene-specific primers used for the PCR of the RACE analysis are listed in Supplementary Table 4.

Isolation of cytoplasmic and nuclear RNA

Cytoplasmic and nuclear RNA were isolated and purified using the Ambion PARISTM Kit (Invitrogen, USA) according to the manufacturer’s instructions. The nuclear and cytoplasmic distributions of lncAMPC were evaluated by qRT-PCR. The β-actin mRNA was used as cytoplasmic RNA control, while the U6 RNA and lncRNA MALAT1 were used as nuclear RNA controls.

RNA *in situ* hybridization

In RNA *in situ* hybridization assays, the presence of lncAMPC was evaluated in human PCa cell lines with the RNAscope® 2.5 HD Reagent Kit (Advanced Cell

Diagnostics) and custom probe to lncAMPC according to the manufacturer's instructions.

RNA extraction, reverse transcription, and qRT-PCR

Total RNA was extracted using TRIzol (Invitrogen, USA), and reversely transcribed using ReverTra Ace qPCR RT Kit (TOYOBO, Japan) according to the manufacturer's instructions. qRT-PCR was performed on triplicate samples by using Power SYBR Green PCR Master Mix (Applied Biosystems, USA) on the StepOne Plus Real-Time PCR System (Applied Biosystems, USA). GAPDH or U6 was respectively used as the endogenous reference control for mRNA or microRNA detection. The processing of urine samples and the isolation of total RNA have been previously described[6]. Sequences of primers used for qRT-PCR are listed in Supplementary Table 5.

Western blotting

Total protein was extracted from cell lysates using RIPA buffer (Thermo Fisher Scientific, USA) containing protease inhibitors (Sigma-Aldrich, USA). Protein quantification was performed using a BCA protein assay kit (Thermo Fisher Scientific, USA). The protein samples were separated on 4%-15% SDS-PAGE gels (Bio-Rad, USA) and then transferred to nitrocellulose membranes (Bio-Rad, USA) on the wet transfer blotting system (Bio-Rad, USA). The antibodies used for western blotting analysis are listed in Supplementary Table 6.

Cell immunofluorescence

Cells were seeded into 24-well plates and allowed to grow to the 60% confluence. After being fixed with 4% paraformaldehyde solution for 30min, cells were permeabilized with 3% Triton X-100 in PBS for 30min at 37°C and then blocked with 1% BSA in PBS for 30min at room temperature. The blocked cells were incubated with primary antibody overnight at 4°C, followed by incubation with secondary IgG antibody for 30min at 37°C. Nuclear staining of cells was performed with DAPI. Representative images were acquired using an fluorescence microscope.

Immunohistochemistry

Formalin-fixed paraffin-embedded sections from specimens were deparaffinized and rehydrated, followed by antigen retrieval. A commercial kit (Lab vision, Fremont, CA) was applied for the staining procedure according to the manufacturer's instructions. After primary and secondary antibody incubation, the sections were visualized with diaminobenzidine (DAB) and counterstained with hematoxylin. The staining score was evaluated by using a microscope at a magnification of 200×.

Proliferation assay

Cell proliferation was evaluated using MTS CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, USA). Cells were trypsinized 24h after siRNA or plasmid transfection and seeded into 96-well plates (3-5×10^3/well). Prior to the assay, 20μl of MTS combined with 80μl of fresh medium were added to each well and the samples were incubated at 37°C for 1h. Relative absorbance at 490nm was measured using a microplate reader (BioTek, USA).

Colony assay

Cells were trypsinized 24h after siRNA or plasmid transfection and seeded into 6-well plates at low confluence (2-5×10^4/well) and grown for up to 2 weeks for colony formation. Cell culture medium was renewed every 3 days. Colonies were fixed with cold methanol and then stained with 0.5% crystal violet solution. The number of colonies was counted and imaged under a microscope.

Migration and Invasion assay

For the cell migration assay, after siRNA or plasmid transfection for 24h, 3-6×10^4 cells in 100μl culture medium without fetal bovine serum were seeded on a fibronectin-coated polycarbonate membrane transwell insert (Corning, USA). Meanwhile, 500μl culture medium with 10% fetal bovine serum was added into the lower chamber. After incubation for 24h-72h at 37°C in a 5% CO_2 incubator, the cells

adhering to the lower surface of transwell insert were fixed with methanol, stained with crystal violet solution and counted under a microscope. The procedure for the cell invasion assay was similar to the cell migration assay, except that the transwell inserts precoated with matrigel (Corning, USA) were used instead of the conventional ones.

RNA immunoprecipitation (RIP)

RIP was performed in several approaches. The MS2 binding system based RIP assay using anti-GFP antibody was conducted to evaluate the ability of lncAMPC to directly bind miR-637. In addition, RIP assay using anti-Ago2 antibody was performed to measure the relative levels of Ago2 binding to lncAMPC and LIF mRNA. After transfection for 48h, PC-3 cells were harvested to perform RIP assays using Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) according to the manufacturer's instructions. The co-precipitated RNAs were detected by qRT-PCR. To demonstrate that the detected signals were from the RNA specifically binding to indicated antibody, total RNAs (input controls) and IgG controls were assayed simultaneously.

Luciferase reporter assay

The psiCHECK2-lncAMPCwt or psiCHECK2-lncAMPCmt was co-transfected with miR-637 mimics or control mimics into 293T cells for 48h. Then the luciferase reporter activity was measured using the Dual Luciferase Assay Kit (Promega, USA) according to the manufacturer's instructions. The relative luciferase activity was normalized to Renilla luciferase activity.

Microarray analysis

The Whole Human Genome Oligo Microarray (Agilent Technology, Palo Alto, CA) was used for gene expression profiles of the PC-3 cell line with or without lncAMPC-knockdown. Sample labeling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis

protocol. Acquired array images were analyzed using Agilent Feature Extraction software (version 11.0.1.1). Microarray data were normalized using the robust multiple-array average normalization method. GO analysis and Pathway analysis were performed in the standard enrichment computation method. Differentially expressed genes between the two samples were identified through Fold Change filtering.

RNA pull-down

lncAMPC was in vitro transcribed from vector pcDNA3.1-lncAMPC and biotin-labeled using Biotin RNA Labeling Mix (Roche, USA) and T7 RNA polymerase (Roche, USA), then treated with RNase-free DNase I (Roche, USA), and finally purified with an RNeasy Mini Kit (Qiagen, USA). Biotinylated lncAMPC was incubated with whole-cell lysates from PC-3 cells at room temperature for 1h, then the complexes were isolated with streptavidin agarose beads (Invitrogen, USA). The RNA present in the pull-down material was detected by qRT-PCR after purification. The associated proteins were resolved by SDS-polyacrylamide gel electrophoresis and visualized by silver staining, and then the specific bands were excised and analyzed by mass spectrometry or subjected to western blotting assay.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed using the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signal Technology, USA) according to the manufacturer's instructions. PC-3 cell chromatin was immunoprecipitated using anti-histone H1.2 antibody. ChIP-derived DNA was quantified by qRT-PCR, using 7 pairs of primers which encompassed the 2000bp-3200bp distal promoter regions from TSS of LIFR. The fold enrichment value was presented as a percentage of the co-precipitated DNA by anti-IgG antibody.