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Supplemental Information

Lysine-5 Acetylation Negatively Regulates Lactate Dehydrogenase A and Is Decreased in Pancreatic Cancer

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Inventory of Supplemental Information

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Supplemental Experimental Procedures

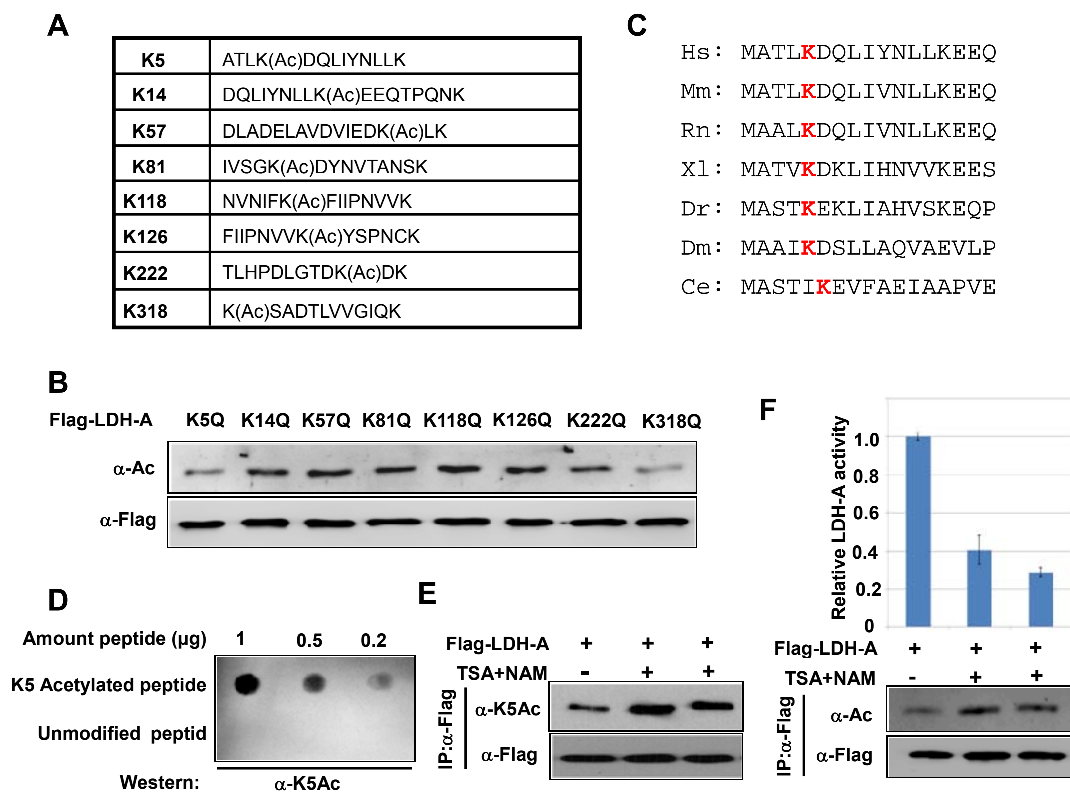


Figure S1. related to Figure 1

A. Identification of acetylated LDH-A peptides by mass spectrometry.

B. Analysis of acetylation of individual LDH-A mutants. The indicated plasmids were transfected into 293T cells and protein was immunoprecipitated for acetylation analysis.

C. Alignment of protein sequences surrounding K5 of LDH-A from different organisms. Hs: *Homo Sapient* (human); Mm: *Mus musculus* (mouse); Rn: *Rattus norvegicus* (Norway rat); Xl: *Xenopus laevis* (frog); Dr: *Danio rerio* (zebrafish); Dm: *Drosophila melanogaster* (fruit fly); At: *Arabidopsis thaliana* (mouse ear cress).

D. Characterization of anti-acetyl-LDH-A(K5) antibody. Specificity of antibody against acetyl-K5 of LDH-A was determined by dot blot assay. Nitrocellulose membrane was spotted with different amounts of acetyl-K5 peptide or unmodified peptide and probed with anti-acetyl-LDH-A(K5) antibody.

E, F. Inhibition of deacetylases increases LDH-A acetylation level and decreases LDH-A enzyme activity. Flag-tagged wild-type LDH-A protein was expressed in 293T and transfected cells were untreated or treated NAM and TSA, then purified by immunoprecipitation using an anti-Flag antibody. The acetylation at K5 (E) or total acetylation (F) of LDH-A were determined by western blotting with anti-acetyl-LDH-A(K5) antibody or anti-pan-acetylation, respectively. Enzyme activity was measured and normalized against protein levels (F). Mean values with standard deviation (±SD) of relative enzyme activity of triplicated experiments are presented.

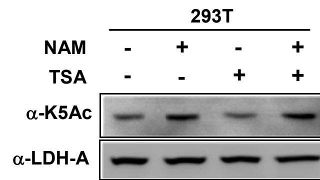


Figure S2. related to Figure 2

NAM but not TSA treatment increases LDH-A K5 acetylation. 293T cells were either untreated or treated with SIRT deacetylase inhibitor nicotinamide (NAM) and HDAC inhibitor trichostatin A (TSA) as indicated. Acetylation at K5 of LDH-A was measured by direct western blotting using the anti-acetyl-LDH-A(K5) antibody.

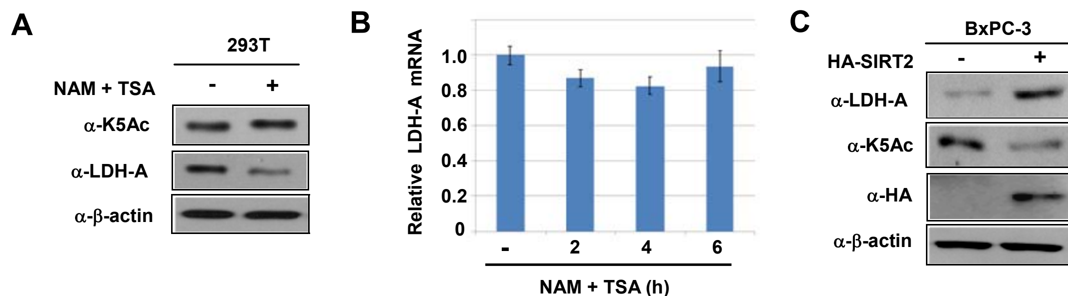
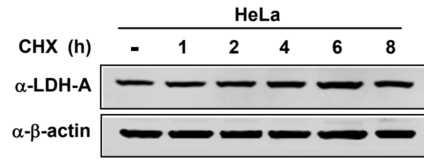


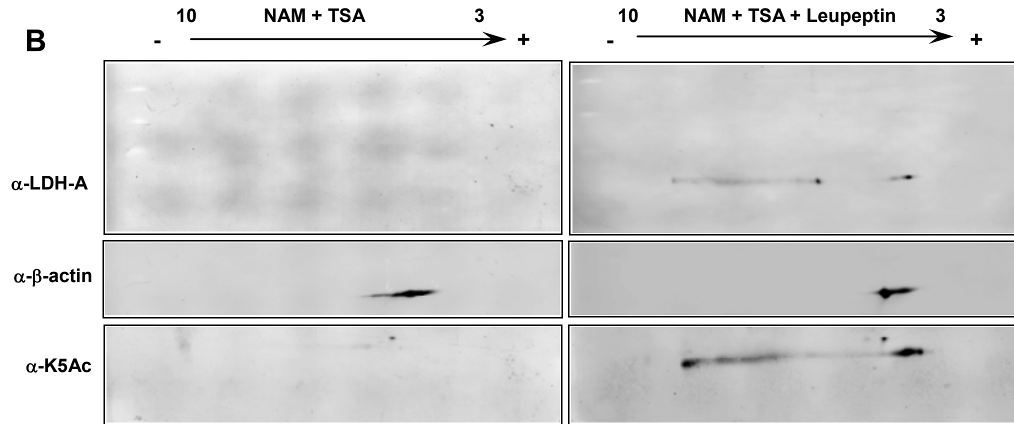
Figure S3. related to Figure 3

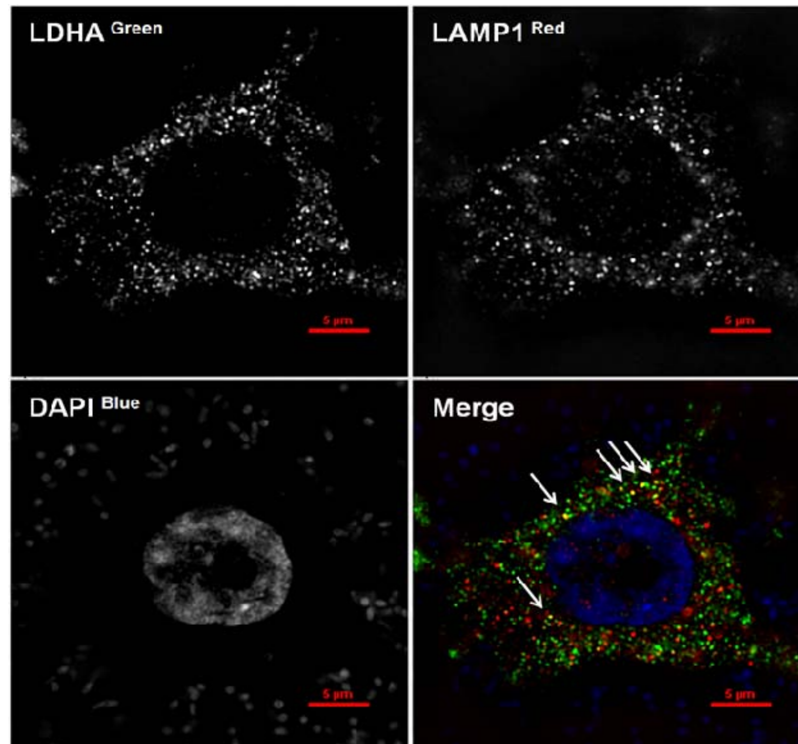
A. NAM and TSA treatment decreases LDH-A protein level. HEK293T cells were treated with NAM and TSA for 8 hours or left untreated. The levels of endogenous K5 acetylation and LDH-A protein were determined by immunoblotting using anti-acetyl-LDH-A(K5) antibody and anti-LDH-A antibody, respectively.

B. NAM and TSA treatment do not decrease the LDH-A mRNA level. HeLa cells were either untreated or treated with deacetylase inhibitors NAM and TSA for indicated hours. The levels of *LDH-A* mRNA were determined by qRT-PCR using GAPDH as an internal control. The ratios of *LDH-A* mRNA in the cells were normalized against GAPDH. Error bars represent ± SD for triplicated experiments.

C. SIRT2 overexpression decreases endogenous LDH-A K5 acetylation and increases LDH-A protein level. Plasmid expressing SIRT2 was transfected into BxPC-3 cells and endogenous K5 acetylation and LDH-A expression level were determined by western blotting.


A
HeLa
CHX (h)
-
1
2
4
6
8
α-LDH-A
α-β-actin


B
10
NAM + TSA
3
-
+
10
NAM + TSA + Leupeptin
3
-
+
α-LDH-A
α-β-actin
α-K5Ac


C
LDHA Green
LAMP1 Red
DAPI Blue
Merge

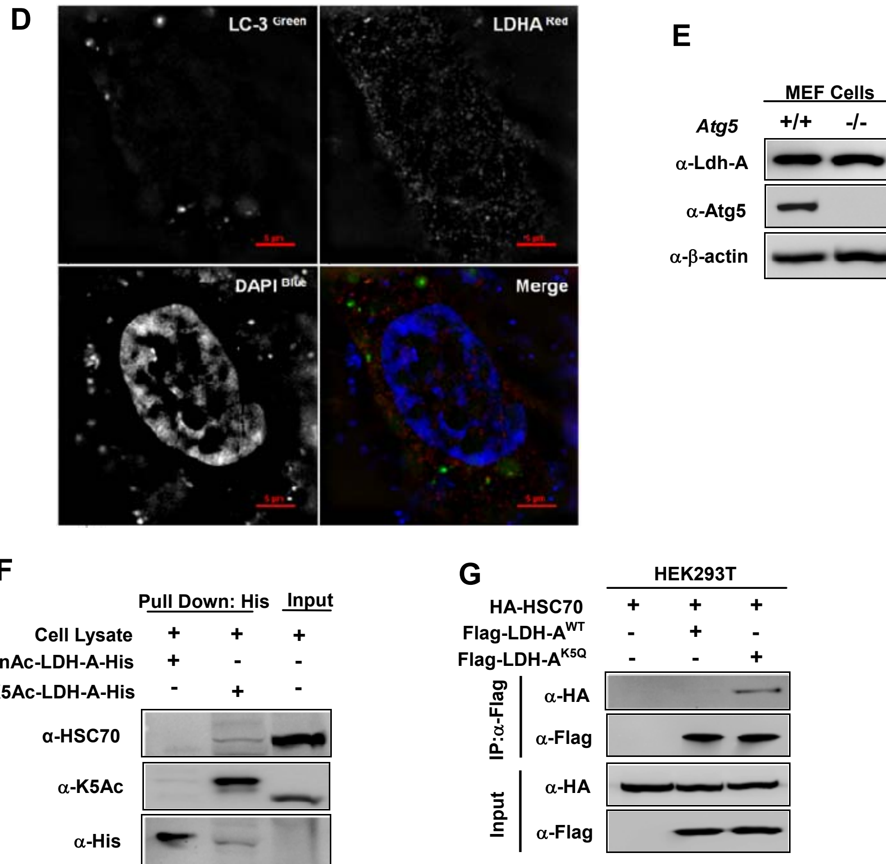


Figure S4. related to Figure 4

A. LDH-A is a stable protein. 293T cells were treated with protein synthesis inhibitor cycloheximide (CHX) and the LDH-A protein level was analyzed by western blotting.

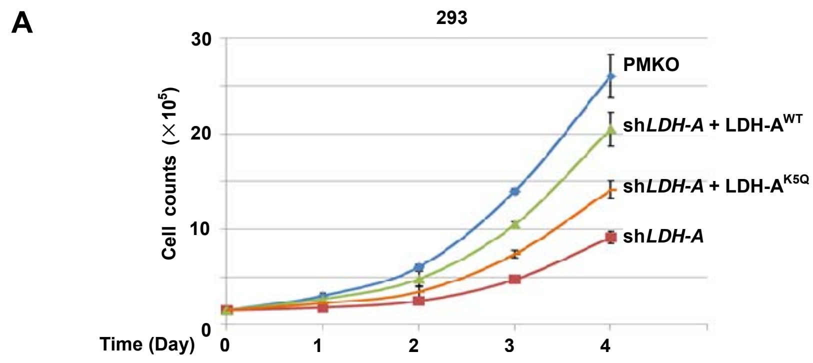
B. Leupeptin blocks NAM and TSA-induced LDH-A degradation**.** 293T cells were treated with deacetylase inhibitors NAM and TSA in the presence or absence of lysosomal proteases inhibitor leupeptin as indicated. IEF was employed to measure NAM and TSA induced K5 acetylation and LDH-A degradation.

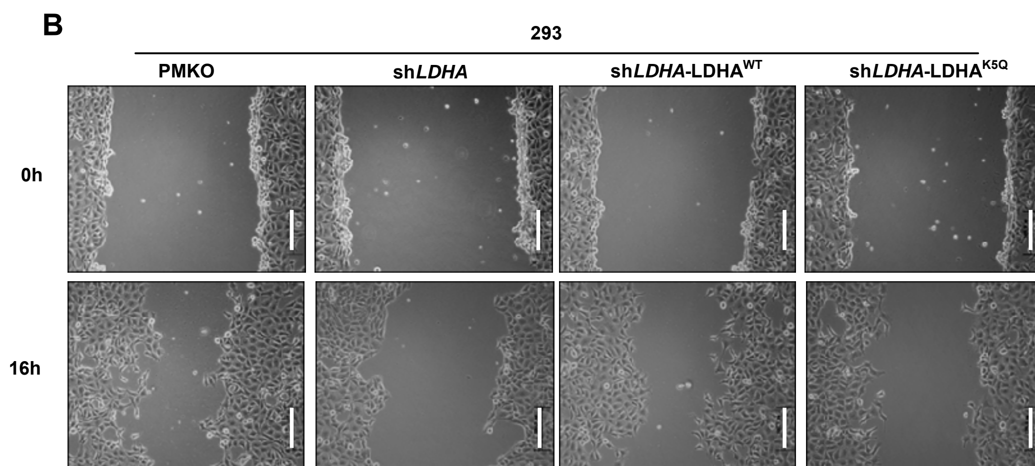
C. Co-staining of LDH-A and lysosomal marker (LAMP1) using BxPC-3 cell line. Scale bars are 5μm.

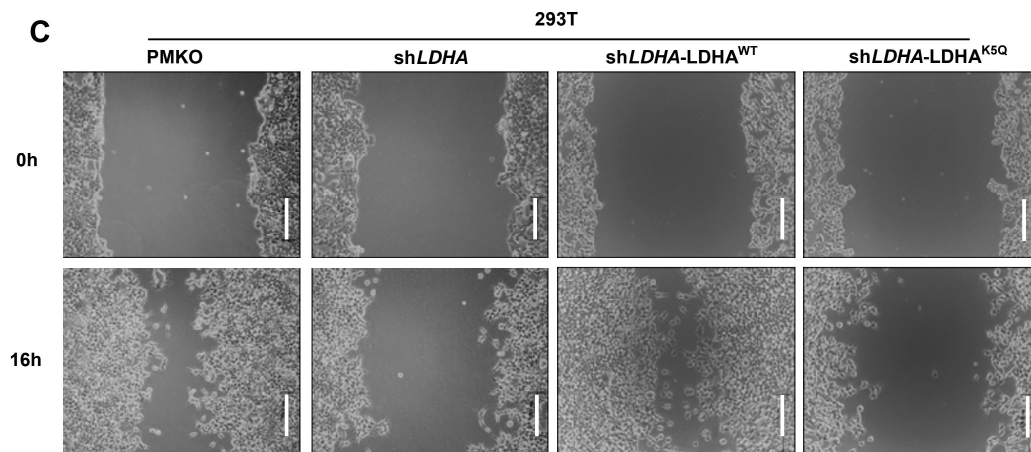
D, E. LDH-A targeted to lysosome is independent of macro-autphagy pathway. (D) Immuno-florence for LDH-A using H4 cells expressed GFP-LC-3. LC-3 is a marker for autophagosome in macro-autophagy pathway. Scale bars are 5μm. (E) LDH-A protein level is not affected by macro-autophagy. Ldh-A protein was detected by western blotting of wild type and *Atg5* knockout MEF, which is defective in macro-autophagy.

F. Acetylation at K5 increases LDH-A binding to HSC70. His tagged un-acetylated and K5 acetylated LDH-A protein was prepared by the system of genetically encoding Nε-acetyllysine in *E. coli.* The purified His-LDH-A was incubated with whole cell lysate to pulldown endogenous HSC70.

G. Acetyl mimetic K5Q mutation of LDH-A increases its binding with HSC70. Wild type or K5Q mutant of LDH-A was co-transfected with HSC70, and the binding between LDH-A and HSC70 were examined by immunoprecipitation-western analysis.


A
293
Cell counts (×10^5)
30
20
10
0
Time (Day)
0
1
2
3
4
PMKO
shLDH-A + LDH-A^WT
shLDH-A + LDH-A^K5Q
shLDH-A


B
293
PMKO
shLDHA
shLDHA-LDHA^WT
shLDHA-LDHA^K5Q
0h
16h


C
293T
PMKO
shLDHA
shLDHA-LDHA^WT
shLDHA-LDHA^K5Q
0h
16h

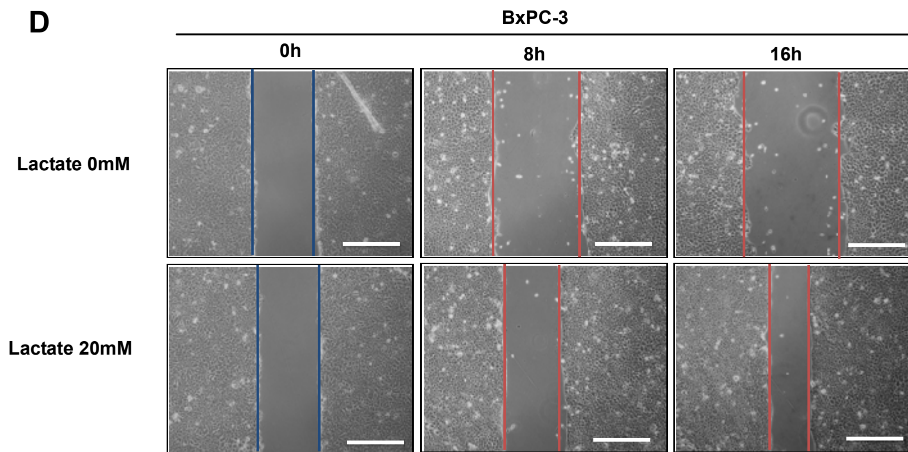
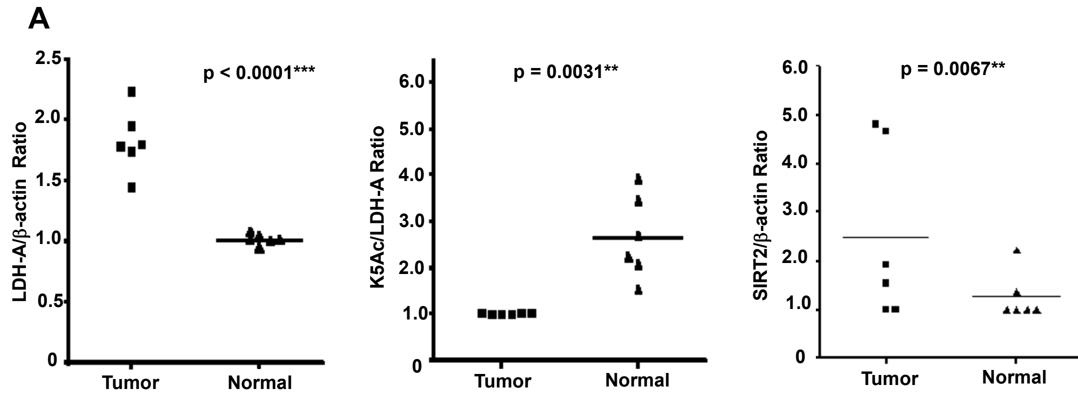


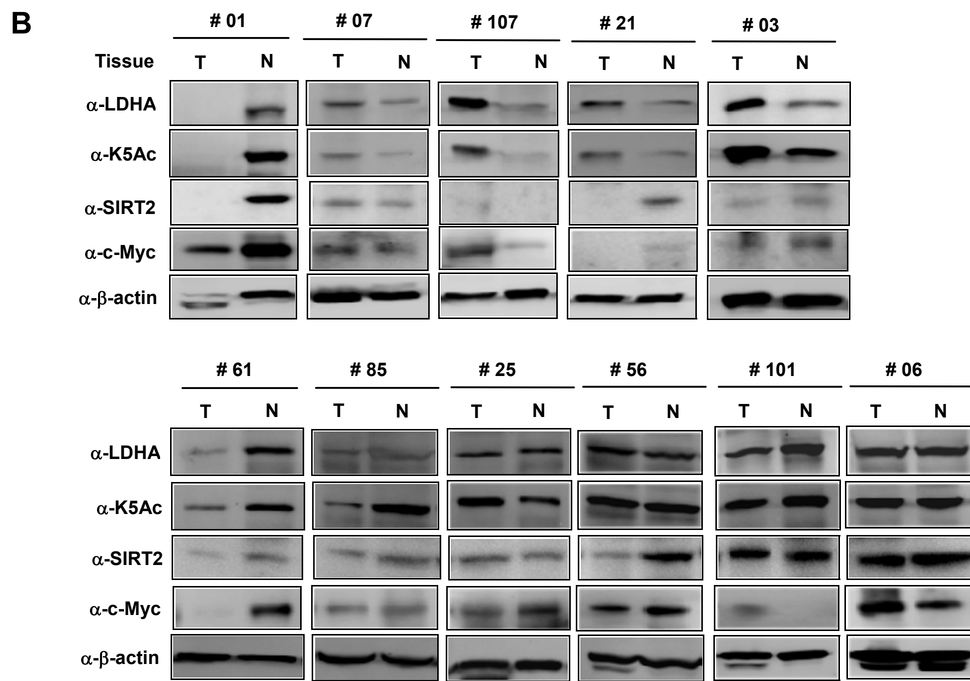
Figure S5. related to Figure 5

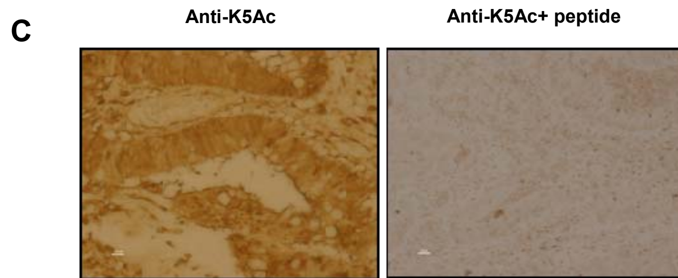
A. K5Q mutant inhibits cell growth in 293 cells. 293 cells were seeded in each well. Cell numbers were counted every day. Error bars represent ± SD for triplicate experiments. LDH-A K5Q mutant inhibits cell migration.

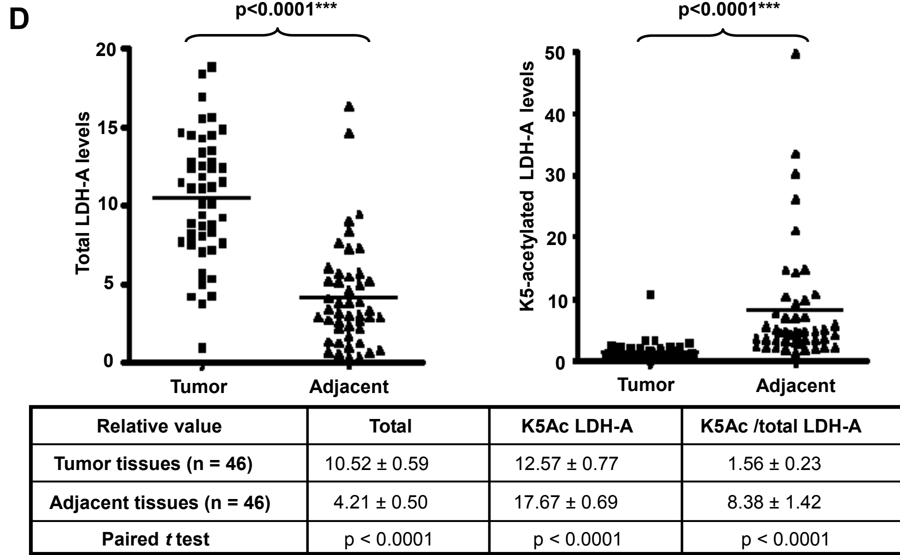
B, C. K5Q mutant inhibits migration of 293 and 293T cells. 293 and 293T stably knockdown LDH-A and expressing WT and K5Q mutant were analyzed for migration by a wound healing assay. Scale bars are 100μm.

D. Lactate promotes cell migration. BxPC-3 cells were cultured in 1.5mM glucose medium with or without 20mM lactate and medium were refreshed every 4 hours. Scale bars are 200μm.

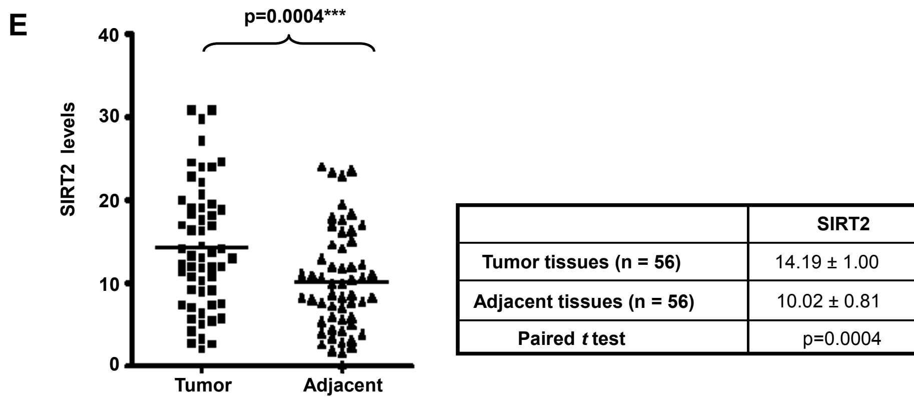

A
p < 0.0001***
LDH-A/β-actin Ratio
Tumor
Normal
p = 0.0031**
K5Ac/LDH-A Ratio
p = 0.0067**
SIRT2/β-actin Ratio


B
01
07
107
21
03
Tissue
T
N
α-LDHA
α-K5Ac
α-SIRT2
α-c-Myc
α-β-actin
61
85
25
56
101
06

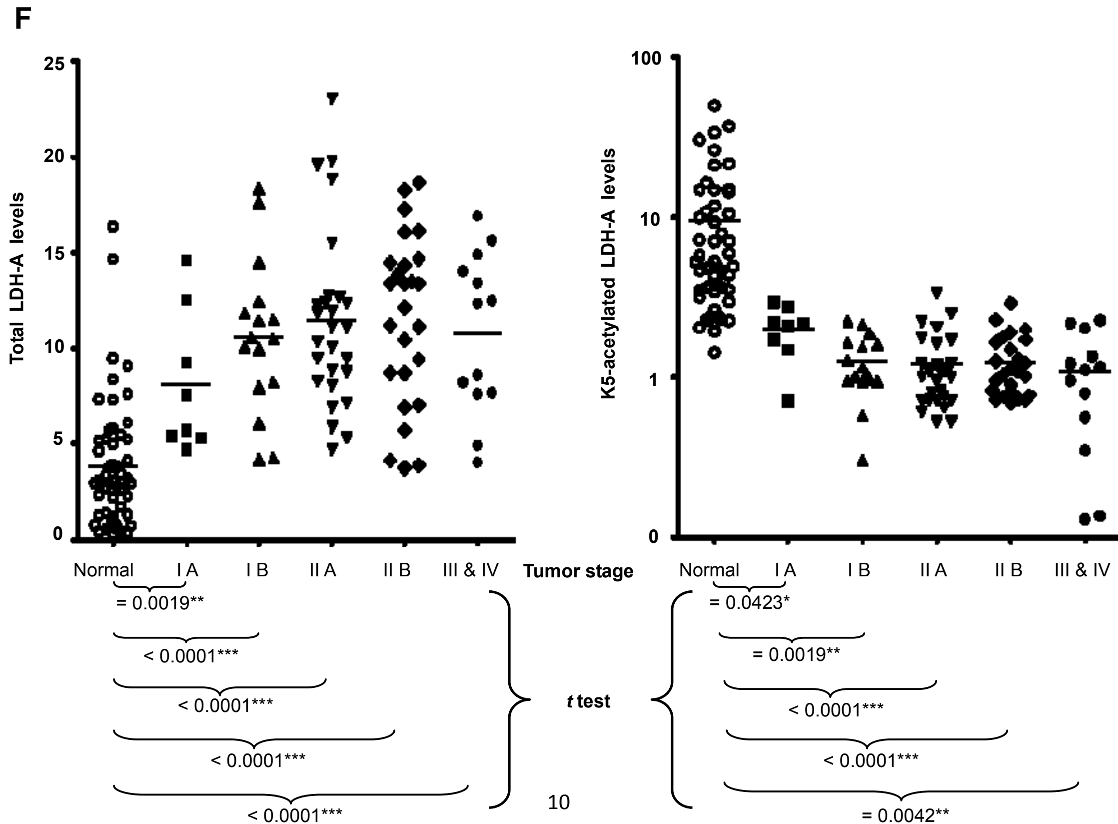

C
Anti-K5Ac
Anti-K5Ac+ peptide



Relative value	Total	K5Ac LDH-A	K5Ac /total LDH-A
Tumor tissues (n = 46)	10.52 ± 0.59	12.57 ± 0.77	1.56 ± 0.23
Adjacent tissues (n = 46)	4.21 ± 0.50	17.67 ± 0.69	8.38 ± 1.42
Paired *t* test	p < 0.0001	p < 0.0001	p < 0.0001



	SIRT2
Tumor tissues (n = 56)	14.19 ± 1.00
Adjacent tissues (n = 56)	10.02 ± 0.81
Paired *t* test	p=0.0004



G

Statistical analysis of total LDH-A proteins in different stages of pancreatic tumors					
Stages	Normal	I A	I B	II A	II B
I A	0.0019				
I B	<0.0001	0.1615			
II A	<0.0001	0.0708	0.5457		
II B	<0.0001	0.0547	0.4800	0.9201	
III & IV	<0.0001	0.1521	0.8843	0.6836	0.6173

H

Statistical analysis of K5-acetylated LDH-A proteins in different stages of pancreatic tumors					
Stages	Normal	I A	I B	II A	II B
I A	0.0423				
I B	0.0019	0.0090			
II A	<0.0001	0.0068	0.7794		
II B	<0.0001	0.0038	0.9130	0.8325	
III & IV	0.0042	0.0109	0.4734	0.6293	0.4811

Figure S6. related to Figure 6

A. Eight in 19 pairs of pancreatic cancer and adjacent normal tissues that exhibited clear inverse correlation between K5-acetylated and total LDH-A and positive correlation between SIRT2 and total LDH-A are shown. The quantifications were calculated from 6 pairs and the remaining 2 pairs expressing total LDH-A in normal tissue at a level too low to be reliably quantified.

B. Tumor samples with no increase in relative LDH-A acetylation over adjacent normal tissues.

C. Characterization of the anti-acetyl-LDH-A(K5) antibody for immunochemistry (IHC). Two image serial sections of a colorectal cancer were stained with the anti-acetyl-LDH-A(K5) antibody in the presence (right panel) or absence (left panel) of acetyl-K5 antigen peptide. Scale bars are 50μm.

D. Statistical analysis of total LDH-A and K5-acetylated LDH-A in 46 pairs of matched pancreatic tumors and adjacent normal tissues. The procedure was the same as Fig. 6B.

E. Immunohistochemical stainings of SIRT2 proteins in tumor and adjacent normal pancreatic cancer tissues. The statistical analysis of 56 pairs is shown. The intensities of SIRT2 proteins were quantified using the Motic Images Advanced software, followed by statistic analysis. The mean value of multiple samples and standard deviation are presented.

F. Immunohistochemical and statistical analyses of total (left panel) and K5-acetylated (right panel) LDH-A in pancreatic tumors of different stages. 108 cases of pancreatic cancer tissues were categorized into different stages based on clinical pathological data following WHO classification.

G, **H**. Statistical analysis (*t* test) of total (G) and K5-acetylated (H) LDH-A in different stages of pancreatic cancers.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell Culture and Transfection

HEK293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% newborn calf serum (HyClone), 100 units/ml penicillin and 100μg/ml streptomycin (Invitrogen). BxPC-3 and AsPC-1 cells were cultured in RPMI-1640 (GIBCO) with 10% fetal calfserum (HyClone), 100 units/ml penicillin and streptomycin (Invitrogen). Cell transfection was performed using Lipofectamine 2000(Invitrogen) or calcium phosphate methods.

Cell Lysis, Immunological Procedures and Antibodies

Cells were lysed in a NP40 buffer containing 50mM Tris-HCl (pH 7.5), 150mM NaCl, 0.3% Nonidet P-40, 1μg/ml aprotinin, 1μg/ml leupeptin, 1μg/ml pepstatin, 1mM Na_3VO_4 and 1mM PMSF. 500μl of cell lysate was incubated with anti-Flag M2-agarose for 3 h at 4 °C; the beads were washed three times with lysis buffer, and the Flag-tagged proteins were eluted by Flag peptides (Gilson Biochemical).

Proteins were blotted following standard protocol. Antibodies specific to Flag (Sigma), HA (Santa Cruz), LDH-A (Cell signaling), HSC70 (Abcam), LAMP2A (Abcam), LAMP1 (Abcam), SIRT2 (Novus) and β-actin (Sigma) were purchased. Polyclonal antibodies to Pan-acetyllysine (antigen: chemically modified acetylated chicken ovalbumin) were generated by immunizing rabbits at Shanghai Genomic Inc. To generate acetyl-lysine 5 specific polyclonal antibody of LDH-A, synthetic peptide MATLK(Ac)DQLIYN was coupled to KLH as antigen to immunize rabbit (Shanghai Genomic Inc). Antiserum was collected after four

doses of immunization and characterized by Western blotting under various conditions, such as peptide competition. Isoelectric focusing (IEF) analyses were carried out according to standard protocol (Awdeh et al, 1968), followed by immunoblotting with LDH-A antibody and acetyl-LDH-A(K5) antibody.

RNA Interference

SIRT2 knockdown was carried out by using synthetic siRNA oligonucleotides (target sequences: ATGACAACCTAGAGAAGTA) synthesized by Genepharma Inc. Shanghai. Cell transfections were performed using Lipofectamine 2000 (Invitrogen). To knock down Sirt2 in mouse liver, siRNA oligonucleotides (target sequences: CCTGGAGAAGTACCACCTT) were injected into tail vein of 6- to 8-week-old male BALB/c mouse. 48 hours later, mice were sacrificed. Livers were removed and whole-cell homogenates were made with NP-40 buffer, and used for western blot analysis or pyruvate and lactate detection.

Lysosome Uptake Assay

Lysosomes were isolated by following previous described procedures (Cuervo et al., 1995). Briefly, male Long-Evans rats were fasted for 24 hours before sacrifice. Livers were removed, washed with cold PBS and homogenized in the extraction buffer using a Lysosomal Isolation kit (Sigma, Cat. #069K4048). After separation by density gradient centrifugation (150,000×g for 4 hours), lysosomes were isolated from lysosomal fraction and tested for LAMP2A levels by immunoblotting. The integrity of the lysosomes was assessed using the Neutral Red dye (Sigma).

Lysosomal uptake assays were carried out as described previously (Cuervo et al., 2004). Briefly, isolated lysosomes were either untreated or treated with a cocktail from the lysosomal isolation kit for 10 minutes on ice and then incubated with the immunopurified Flag-LDH-A fusion proteins (0.1μg) for 20 minutes at 37ºC in a MOPS buffer [10 m M3-(N-morpholino) propanesulfonic acid (MOPS) pH 7.3, 0.3 M sucrose]. The mixture was washed for four times with cold PBS, followed by SDS-PAGE and western blot.

LDH-A Knocking Down and Putting Back

A shRNA retrovirus targeting human *LDH-A* was constructed using following sequences:

(59-CCGGGCTACACATCCTGGGCTATTGCTCGAGCAATAGCCCAGGATGTGT AGCTTTTTC-39). A control shRNA retrovirus that had no effect on LDH-A expression was constructed and used as a negative control using following sequences

(59-CCGGGAGGCTTCTTATAAGTGTTTACTCGAGTAAACACTTATAAGAAGCC TCTTTTTG-39) (Christofk et al., 2008). Retroviruses were produced using a two-plasmid packaging system. Briefly, the pMKO.1-puro vector expressing the shRNA sequence was co-transfected into 293T cells together with vectors expressing the *gag* and *vsvg* genes. Retroviral supernatant was harvested 36h after initial plasmid transfection and mixed with polybrene (8μg/ml) to increase the infection efficiency. For transduction, subconfluent BxPC-3, 293 and 293T cells were infected with 5ml retrovirus and 5ml fresh medium for 48h, and then

selected in puromycin (2 μg/ml) for 1 week.

To re-introducing LDH-A, Flag-tagged human LDH-A^{WT} and LDH-A^{K5Q} containing two silent nucleotide substitutions in the sequence corresponding to the shRNA targeted region were cloned into the retroviral vector (pQCXIH) and were co-transfected into 293T cells together with vectors expressing the *gag* and *vsvg* genes. Retroviral supernatant infect *LDH-A* knocking down BxPC-3, 293 and 293T cells. The infected cells were selected in hygromycin (2μg/ml) for 4 week.

Cell Proliferation and Wound Healing Assays

5×10^4 cells were seeded in triplicate in plates and cell numbers were counted every two days over a 10-day period for BxPC-3 cells and everyday over 4-day period for 293 cells. For wound healing assay, monolayer cells were wounded with a sterile plastic tip. Cell migration was observed by microscopy 16 and 24 hours later.