Molecular Cell, Volume *51*

Supplemental Information

Acetylation Stabilizes ATP-Citrate Lyase to Promote Lipid Biosynthesis and Tumor Growth

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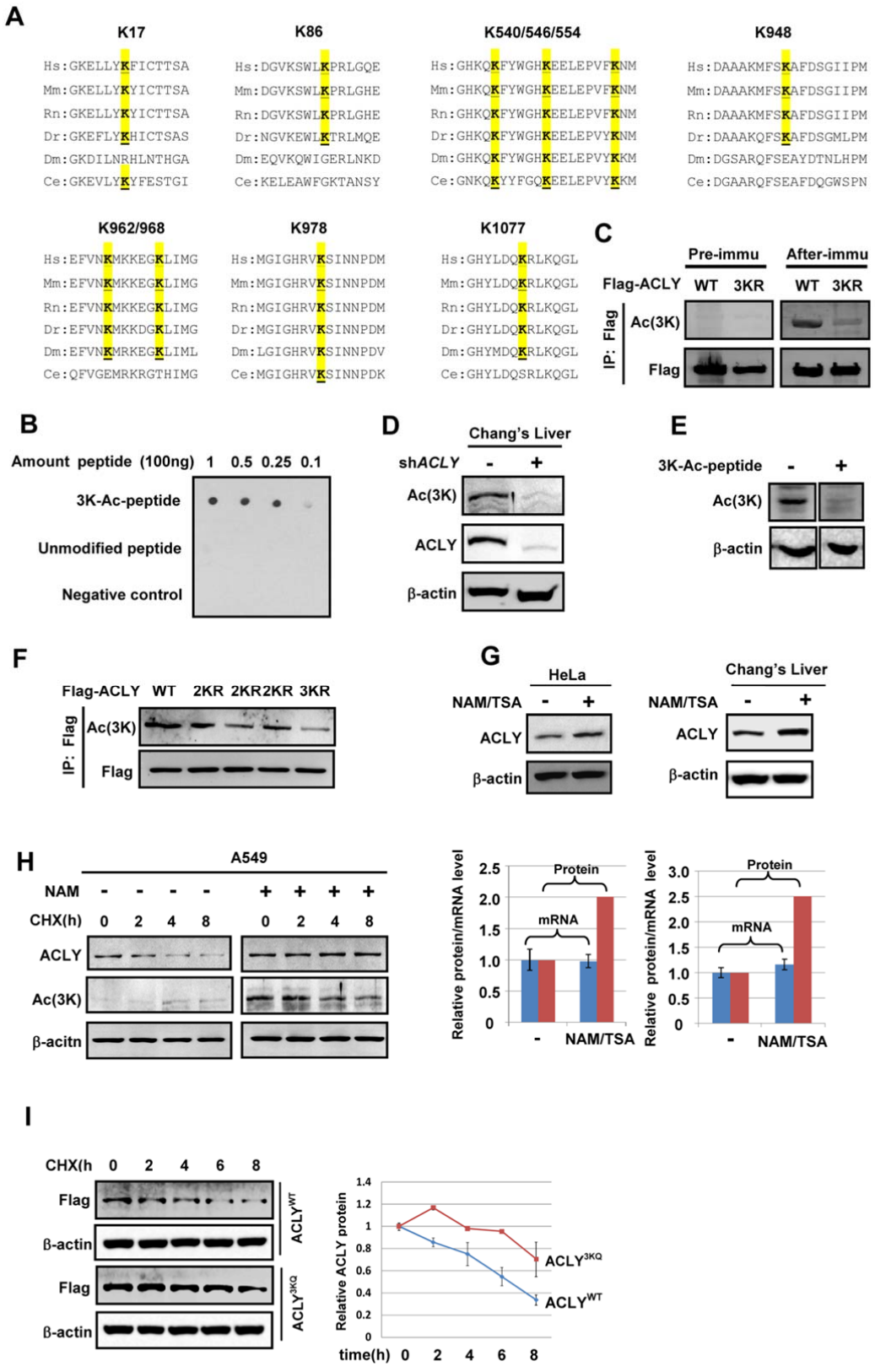
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Ce:GHYLDQSRLKQGL
C
Pre-immu
After-immu
Flag-ACLY WT 3KR WT 3KR
IP: Flag
Ac(3K)
Flag
B
Amount peptide (100ng) 1 0.5 0.25 0.1
3K-Ac-peptide
Unmodified peptide
Negative control
D
Chang's Liver
shACLY - +
Ac(3K)
ACLY
β-actin
E
3K-Ac-peptide - +
Ac(3K)
β-actin
F
Flag-ACLY WT 2KR 2KR2KR 3KR
IP: Flag
Ac(3K)
Flag
G
HeLa
NAM/TSA - +
ACLY
β-actin
Chang's Liver
NAM/TSA - +
ACLY
β-actin
H
A549
NAM - - - - + + + +
CHX(h) 0 2 4 8 0 2 4 8
ACLY
Ac(3K)
β-acitn
Relative protein/mRNA level
Protein
mRNA
- NAM/TSA
I
CHX(h 0 2 4 6 8
Flag
β-actin
ACLY^WT
Flag
β-actin
ACLY^3KQ
Relative ACLY protein
ACLY^3KQ
ACLY^WT
time(h) 0 2 4 6 8

Figure S1. Related to Figure 1

A. Multiple alignments of the protein sequences surrounding all identified acetylated sites of ACLY by MS spectrometry from different organisms. Hs: Homo Sapiens (human); Mm: Mus musculus (mouse); Dr: Danio rerio (zebrafish); Dm: Drosophila melanogaster (fruit fly); Rn: Rattus norvegicus (Norway rat); Ce: Caenorhabditis elegans (nematode).

B-F. Characterization of anti-acetyl-3K specific antibody. (B) The specificity of the antibody against Ac(3K) was determined by dot blot. Nitrocellulose membrane was spotted with different amounts of either acetyl-3K peptide or unmodified peptide and probed with anti-Ac(3K) antibody. (C) Flag tagged ACLY WT or 3KR was transfected into HEK293T cells and acetylation of each purified protein was measured by western blot using anti-Ac(3K) antibody or pre-immune serum. (D) Chang's cells with or without sh*ACLY* were collected and endogenous Ac(3K) level was determined by western blot. (E) Acetyl-3K peptide blocking experiment. Acetyl-3K can compete with 3K acetylated ACLY. Cell lysate treated with NAM and TSA was analyzed by western blot. (F) Flag tagged ACLY WT, 2KR or 3KR was transfected individually into HEK293T cells and acetylation of each purified protein was measured by western blot using anti-Ac(3K) antibody.

G. ACLY protein level is increased under NAM/TSA treatment in multiple cell types. HeLa and Chang's cells were treated by NAM/TSA for indicated times and endogenous ACLY was determined by western blot.

H. Deacetylase inhibitors NAM stabilize endogenous ACLY protein under low glucose. A549 cells were treated with CHX with or without the pre-treatment of NAM for indicated time points. Endogenous ACLY and acetylation level were determined by western blot.

I. $ACLY^{3KQ}$ is more stable than $ACLY^{WT}$ under low glucose. A549 cells with stable ACLY knockdown and re-expression of the Flag tagged shRNA resistant wild-type and 3KQ mutant were treated with CHX for indicated time points. Stabilities of $ACLY^{WT}$ and $ACLY^{3KQ}$ protein were determined by western blot and quantified with error bars represents ± SDs for triplicate experiments.

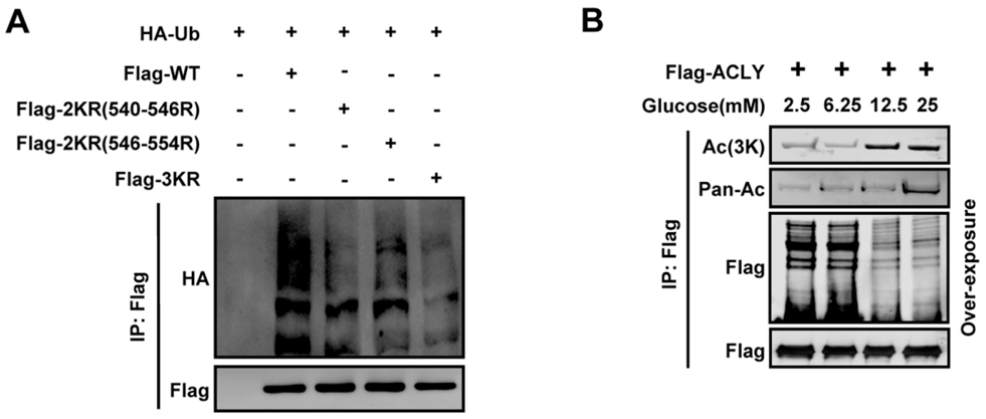


Figure S2. Related to Figure 2

A. Lysine 540,546, and 554 are the main ubiquitylation sites of ACLY. Flag tagged wild type and 2KR (540-546R, 546-554R) mutants were co-transfected with HA tagged ubiquitin. The *in vivo* ubiquitylation level of ACLY was determined by western blot.

B. Glucose up-regulates ACLY acetylation at 3K and decreases its ubiquitylation. Flag tagged ACLY was transfected into HEK293T cells. Acetylation and semi-endogenous ubiquitylation was detected by western blot using indicated antibodies.

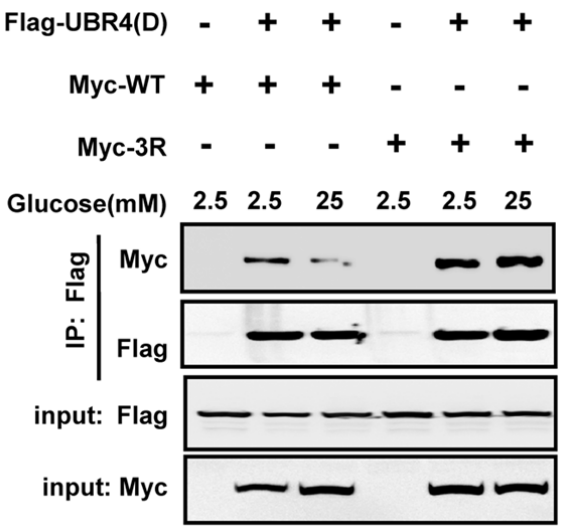


Figure S3. Related to Figure 3

High glucose decreases the interaction between UBR4-E3-ligase-domain (noted as UBR4(D)) and wild type ACLY but not 3KR. Flag tagged UBR4(D) was co-transfected with Myc tagged wild type, or 3KR of ACLY individually into HEK293T cells. Cells were treated with either 2.5mM or 25mM glucose 4 hours before harvested. The interactions between ACLY and UBR4(D) were determined by co-immunoprecipitation and western blot.

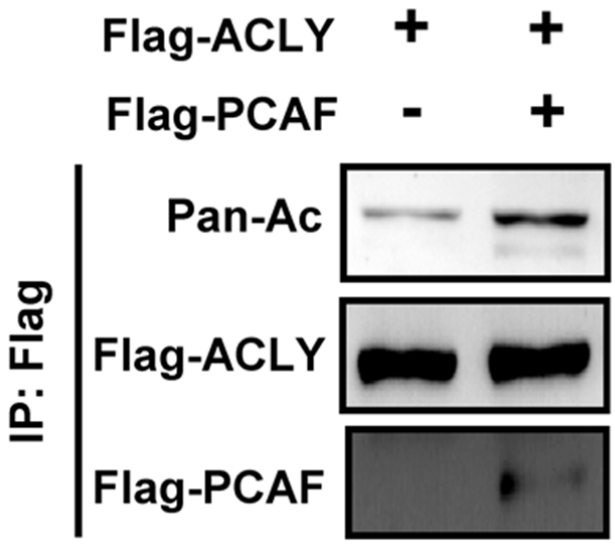


Figure S4. Related to Figure 4

PCAF over-expression increases ACLY acetylation. Flag tagged ACLY was co-transfected with Flag tagged PCAF into HEK293T cells. Pan-acetylation of ACLY was measured by western blot.

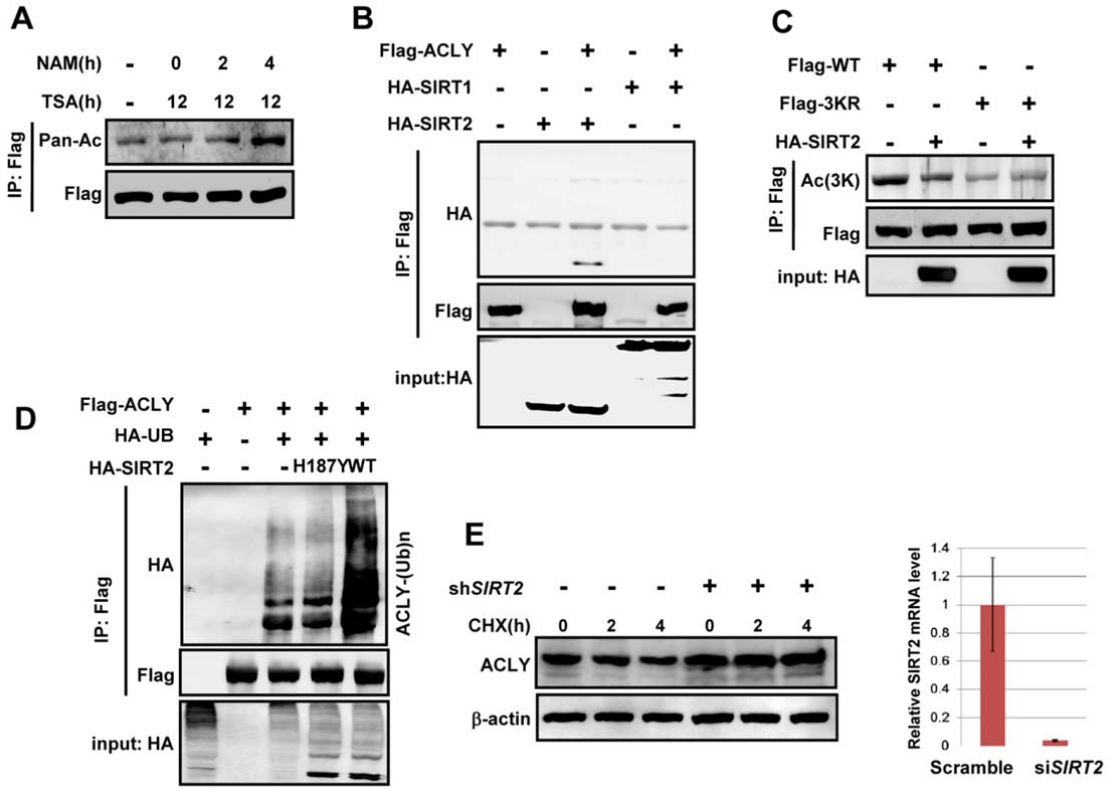


Figure S5. Related to Figure 5

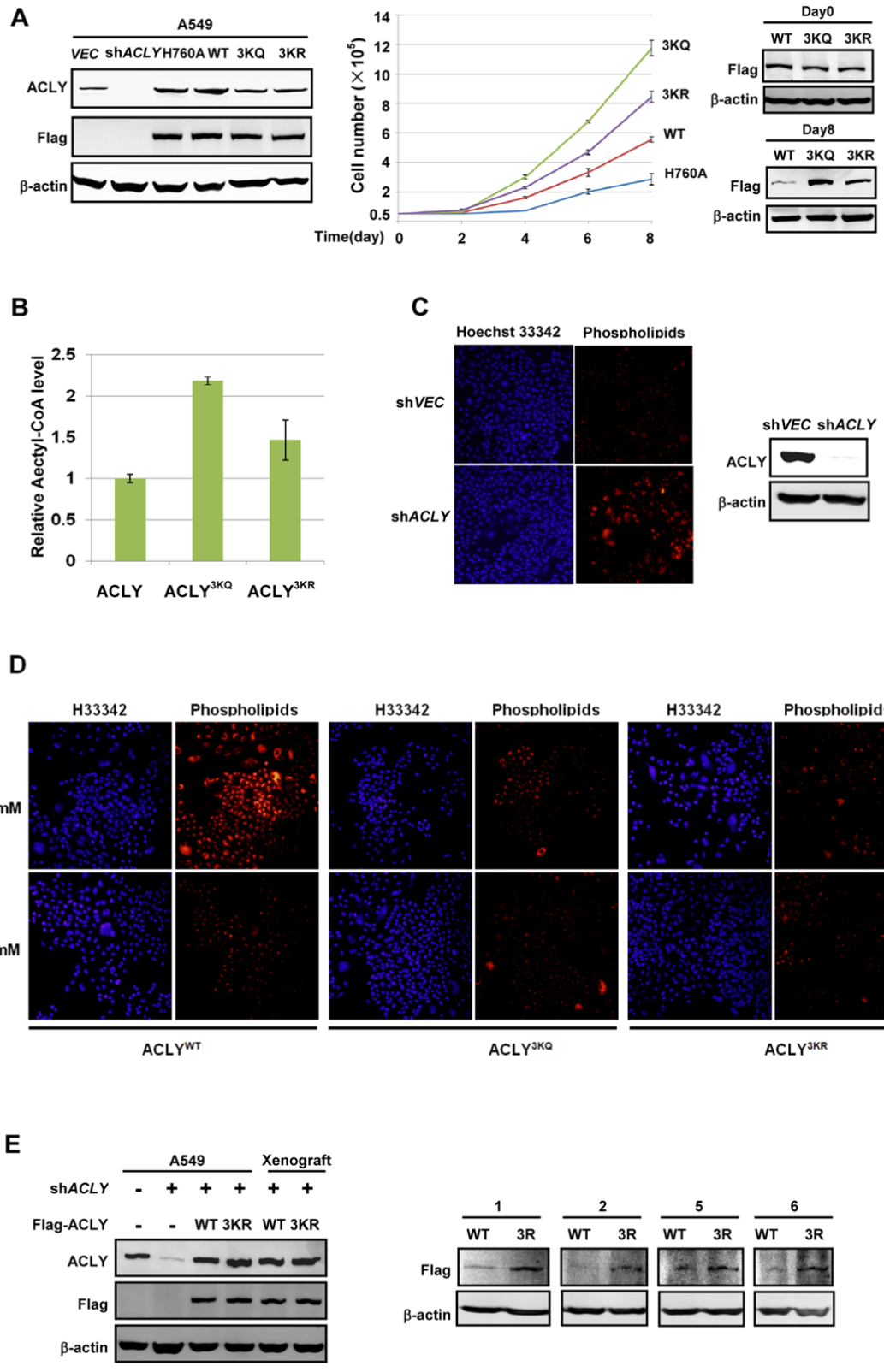
A. ACLY acetylation is accumulated by NAM/TSA treatment. Flag tagged ACLY was transfected into HEK293T cells with the treatment of TSA and NAM for indicated time. Protein acetylation was determined by western blot.

B. SIRT2 interacts with ACLY. Interactions between ACLY and SIRT1 or SIRT2 were determined by co-transfecting Flag tagged ACLY and HA tagged SIRT1 or SIRT2.

C. SIRT2 deacetylases ACLY at 3K *in vitro*. Flag tagged ACLY and 3KR mutant were co-transfected with HA tagged SIRT2. Protein acetylation was determined by western blot.

D. SIRT2, but not its mutant, promotes ACLY ubiquitylation. Flag tagged ACLY, HA tagged ubiquitin were co-transfected with HA tagged SIRT2 or SIRT2^{H187Y} into HEK293T cells. Ubiquitylation of purified proteins was determined by western blot.

E. Knocking down of *SIRT2* by shRNA stabilizes ACLY in low glucose. HEK293T cells with stable *SIRT2* knockdown were treated with CHX as indicated time points. Endogenous ACLY level was determined by western blot.


A
A549
VEC
shACLY
H760A
WT
3KQ
3KR
ACLY
Flag
β-actin
Cell number (×10^5)
Time(day)
3KQ
3KR
WT
H760A
Day0
Day8
B
Relative Aecty-CoA level
ACLY
ACLY3KQ
ACLY3KR
C
Hoechst 33342
Phospholipids
shVEC
shACLY
D
H33342
Phospholipids
2.5mM
25mM
ACLYWT
ACLY3KQ
ACLY3KR
E
A549
Xenograft
shACLY
Flag-ACLY
WT 3KR
WT 3KR
ACLY
Flag
β-actin
1
2
5
6
WT
3R

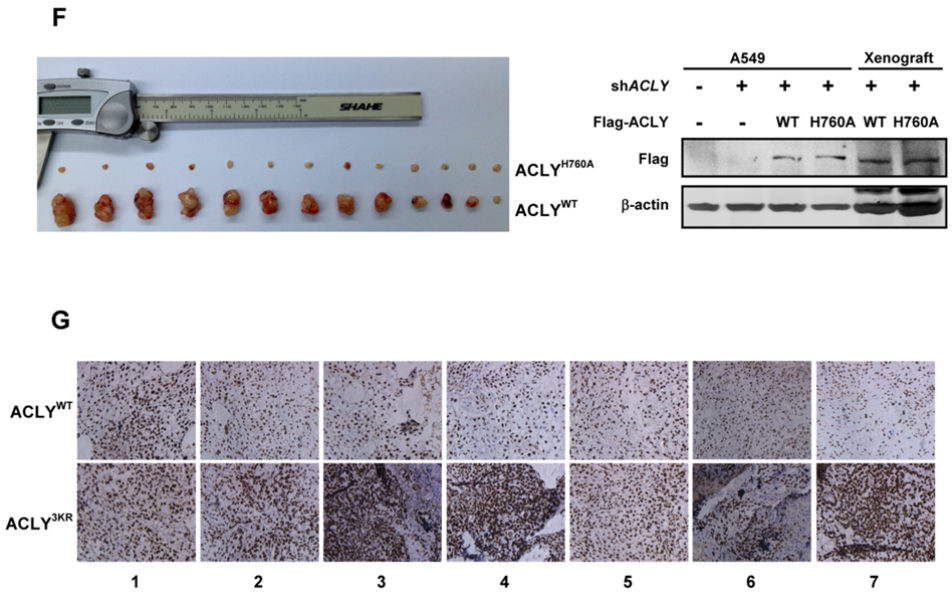


Figure S6. Related to Figure 6

A. Acetylation at 3K of ACLY promotes cancer cell proliferation. ACLY was stably knocked down with shRNA in A549 cells. shRNA resistant H760A (catalytic mutant) , WT, 3KQ, and 3KR mutant of ACLY were stably re-expressed in the knockdown cells to a level that is compatible with endogenous level. ACLY H760A, WT, 3KQ, or 3KR stable cells were seeded in each well of 6-well plate with the number of 50000. Cell numbers were counted every 48hrs. The cell lysate at day 0 and day 8 after counting were separated for measuring ACLY expression via western blot. Error bars represent cell numbers ±SDs for triplicate experiments.

B. 3KQ and 3KR of ACLY have increased cellular acetyl-CoA level under low glucose. Flag tagged ACLY WT, 3KQ, and 3KR were transfected into HEK293T cells maintained in 2.5mM glucose for indicated times. Total cellular acetyl-CoA was measured using PicoProbe™ Acetyl-CoA Assay Kit (Abcam). Error bars represent ±SDs for triplicate experiments.

C. Intracellular phospholipids are accumulated in sh*ACLY* cells. sh*VEC* or sh*ACLY* cells were seeded 4000 per well of 96-well plate maintained under serum free medium. Cells were maintained in the presence of fluorescence dyes-coupled phospholipids for indicated times. The accumulation of intracellular phospholipids was detected and captured using fluorescence microscope.

D. Reduced extracellular phospholipids uptake are shown in ACLY 3KQ/3KR expressing cells compared to WT. Intracellular phospholipids accumulation was measured using phospholipids conjugated to fluorescent dyes.

E. The expression of $ACLY^{WT}$ and $ACLY^{3KR}$ in xenograft. Whole cell lysate were prepared from either original stable A549/$ACLY^{WT}$ and A549/$ACLY^{3KR}$ pools or xenograft tumors, followed by western blot.

F. The H760A enzyme dead mutant of ACLY inhibits xenograft tumor growth. A549 cell stable cell lines of H760A and WT were injected subcutaneously to the flanks of nude mice. 7 weeks after injection, tumors from 13 mice were extracted and photographed (left). Expressions of $ACLY^{WT}$ and $ACLY^{H760A}$ in nude mice xenograft are indentified (right). Xenograft cell lysate was prepared from either original stable $ACLY^{WT}$ and $ACLY^{H760A}$ pools or xenograft tumors, followed by western blot.

G. Ki-67 staining of xenograft tumor sections of $ACLY^{WT}$ and $ACLY^{3KR}$. Tumor sections prepared from xenograft are shown in pairs.

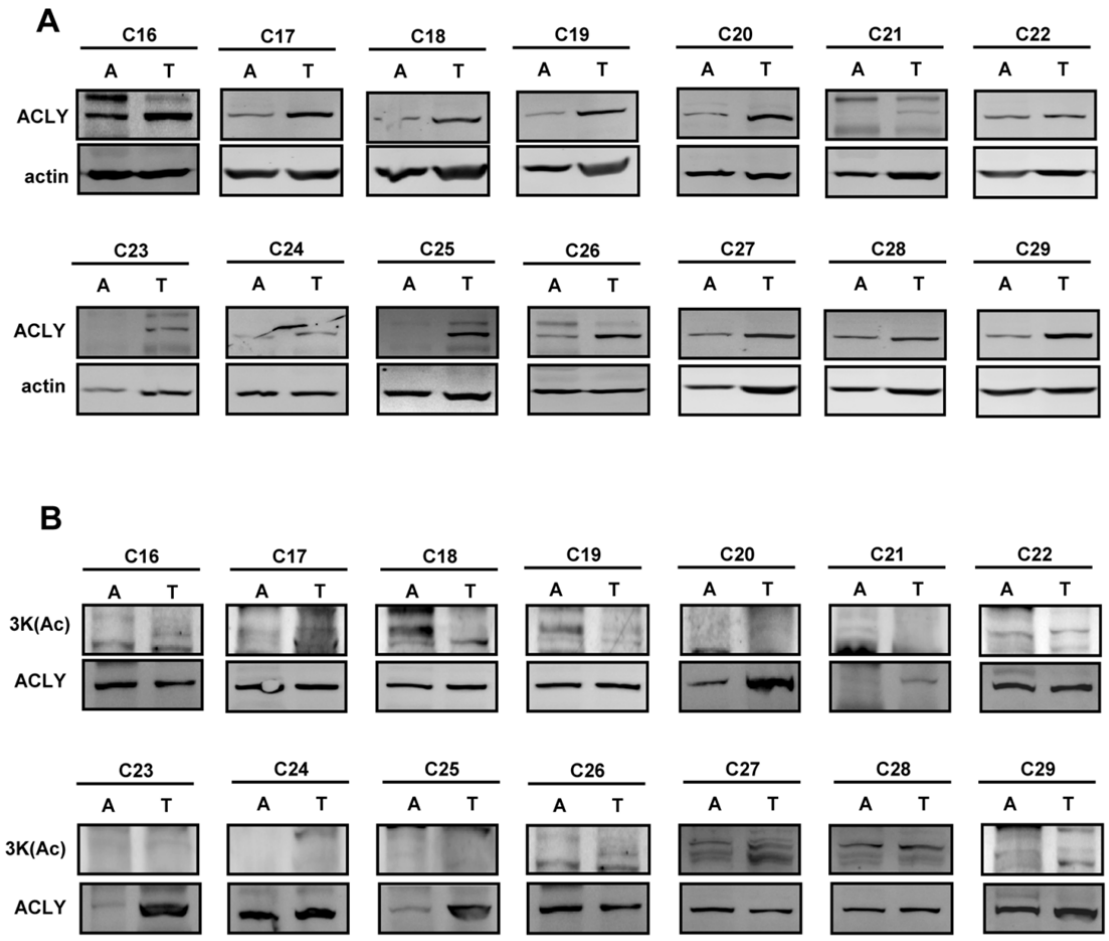


Figure S7. Related to Figure 7

A. The rest of clinical cases that with the increased of ACLY protein. Human lung carcinoma paired samples with carcinoma tissue (shown as C) and adjacent tissue (shown as A) were lyased and prepared. The ACLY protein levels were compared to β-actin under western blot. (Compared to Figure 7A)

B. The rest of up-regulated ACLY clinical cases without the increase of acetylation at 3K. (Compared to Figure 7B)

Table S1. Identification of Acetylated ACLY Peptides by Mass Spectrometry, Related to Figure 1

K17	KELLY**K**FICTTSAIQNRFKY
K86	SWL**K**PR
K540	KQ**K**FYWGHKE
K546	FYWGH**K**EILIPVFK
K554	EILIPVF**K**NMADAMR
K948	MFS**K**AFDSGIIPMEFVNK
K968	EG**K**LIMGIGHR
K978	V**K**SINNPDMR
K962	AFDSGIIPMEFVN**K**MK
K1077	SMGFIGHYLDQ**K**R

Table S2. Statistics of ACLY Upregulation at 3K in 54 Clinical Human Lung Carcinoma Samples, Related to Figure 7

Total	ACLY upregulation	Ac(3K) upregulation
54	**29**	**15/29**