Supplemental Tables

Table S1. ChIP-qPCR primer sequences 5 kb upstream of *ACSL4*

Primers	Forward sequence	Reverse sequence
primer 1	ATTCCTGTGCATAGATCTTTGTGT	TGCTGAGATTACAGGCGTGA
primer 2	AAGTTTCTTCATATTCTTGCCAAA	GACCCTACATGGCACCTGAT
primer 3	CCCATATGGTTTCCATCGTG	CCCTTTAACATACAAAAAGCTTCC
primer 4	TGGGAAGGTCACACAAACAA	TGCATGGAGTCAAGTTTTTCC
primer 6	ATTTGGCCTGGTTGTTGGGT	GCCTTTGCGTGTTAAACTTCAC
primer 7	GAAGCCCTGCTGCTTGTATT	AGATAGCCAGGCTGCTCTTG
primer 8	GCCTGGCTATCTGTTTCAGC	CAATGCAATTGACAGATTCAAAA
primer 9	GACAGCCAGAACTGTACACAC	AGCTTACTGAAGTTCCTCCAGC
primer 10	TCATCTTCATCCAGCACACTGAT	TGTCAGGTACGTAATTCACTGGG
primer 11	GTAAGTGGGTGGGTTTCTTGGA	TAAGCAGCCCAGGTGACACT
primer 12	CATTTCAACAAGCACCCCAGAT	GTGAACTAGCTTCCTGTTCCTGA
primer 13	AGCCCTCAGATAGTTCGCTG	TCACCGCAGCTTTGTGACAT
primer 14	AGACTGACAGAAGCGGATCG	AGACTGACAGAAGCGGATCG

Table S2. Primer sequences used for shRNAs, RT-PCR and ACSL4 primers 9 Luciferase assay

Gene	Direction	Sequence (5'-3')
ACSL4 shRNA 1	Forward	TCCCCTAGTGTTGAGCTTCTGGAAATTCAAGAGATTTCCAGAAGTTCAACACTGG
	Reverse	AAAACCAGTGTTGAACTTCTGGAAATCTCTTGAATTTCCAGAAGCTCAACACTAG
ACSL4 shRNA 2	Forward	TCCCGCAGTGGTTCATGGGTTAAATTTCAAGAGAATTTAGCCCATGAACTACTGC
	Reverse	AAAAGCAGTAGTTCATGGGCTAAATTCTCTTGAAATTTAACCCATGAACCACTGC
NMT1 shRNA 1	Forward	TCCCGCAGTTGTATGCTGTGGTTTCAGAGGAGCTTCAAGAGAGCTCCTCTGAAACCA CAGCATACAACTGC
	Reverse	AAAAGCAGTTGTATGCTGTGGTTTCAGAGGAGCTCTCTTGAAGCTCCTCTGAAACCA CAGCATACAACTGC
NMT1 shRNA 2	Forward	TCCCCTGGAGGCAATCAAGCAGACATAGAAGAGTTCAAGAGACTCTTCTATGTCTG CTTGATTGCCTCCAG
	Reverse	AAAACTGGAGGCAATCAAGCAGACATAGAAGAGTCTCTTGAACTCTTCTATGTCTG CTTGATTGCCTCCAG
ACSL3 shRNA 1	Forward	TCCCGTTGTGTAACAGTTGTGAGATTTCAAGAGAATTTCACAACTGTTACACAGC
	Reverse	AAAAGCTGTGTAACAGTTGTGAAATTCTCTTGAAATCTCACAACTGTTACACAAC
ACSL3 shRNA 2	Forward	TCCCCTTGGATGTGATACTTTGGATTTCAAGAGAATCTAAAGTATCACATCCAGG
	Reverse	AAAACCTGGATGTGATACTTTAGATTCTCTTGAAATCCAAAGTATCACATCCAAG
AR shRNA 1	Forward	TCCCGGGCATGCGTTTGGAGATTGTTCAAGAGACAGTCTCCAAACGCATGTCC
	Reverse	AAAAGGACATGCGTTTGGAGACTGTCTCTTGAACAATCTCCAAACGCATGCCC
AR shRNA 2	Forward	TCCCGGGCACTTGAACTGTCGTCTTTCAAGAGAAGACGGCAGTTCAAGTGTC
	Reverse	AAAAGGACACTTGAACTGCCGTCTTCTCTTGAAAGACGACAGTTCAAGTGCCC
ACSL4	Forward	CAGGCCAGTGTGAAAGAATACCT
	Reverse	TGGCTTCACACAGGAGACATTG
PSA	Forward	TCTGCGGCGGTGTTCTG
	Reverse	GCCGACCCAGCAAGATCA
ACSL3	Forward	ACTCCACTGTGCGACAGCTTT
	Reverse	CACCACACAACAGGAGACGAA
GAPDH	Forward	CCACATCGCTCAGACACCAT
	Reverse	ACCAGGCGCCCAATACG
Primer 9-1	Forward(SacI)	CATGAGCTCGACAGCCAGAACTGTACACAC
	Reverse(XhoI)	CATCTCGAGAGCTTACTGAAGTTCCTCCAGC
Primer 9-2	Forward(XhoI)	CATCTCGAGGACAGCCAGAACTGTACACAC
	Reverse(HindIII)	CATAAGCTTAGCTTACTGAAGTTCCTCCAGC

Supplemental Figure Legends

Figure S1. ACSLs expression levels in prostate cancer cell lines. (A-C) The relative mRNA levels of ACSL1, ACSL3, ACSL4, ACSL5, and ACSL 6 versus GAPDH were analyzed by Real-time RT-PCR in 22Rv1, PC-3 and DU145 cell lines. The relative expression levels of ACSL3 and ACSL4 are two abundant isoforms in the ACSLs family in prostate cancer cells. (**D**) ACSL3, ACSL4, and AR protein levels in LNCaP, 22Rv1, PC-3 and DU145 cell lines were analyzed by immunoblotting. **(E-F)** Negative correlation of ACSL4 with KLK2 or KLK3 in prostate cancer. Copy number (mRNA) of KLK2, KLK3, and ACSL4 were extracted from the cBioPortal for Cancer Genomics database (n=118)(21). ACSL4 levels were inversely correlated with KLK2 (E) and KLK3 levels (F) in prostate tumors.

Figure S2, related to Figure 3. Identification of AR binding sites in the promoter region of *ACSL4* by ChIP-qPCR. Based on the AR consensus sequence, fourteen potential AR binding sites were selected in the 5 kb upstream region of *ACSL4*. The genomic DNA derived from LNCaP cells treated with DMSO or R1881 were extracted for ChIP-qPCR. The enrichment of the examined *ACSL4* promoter regions covered by the primer pairs-1, 2, 4, 6, 8, 10, 11-14 was analyzed. The data were normalized using the % input method as described in the Materials and Methods with the IgG control in the DMSO treated sample normalized to 1%. The data represent the mean ± SEM (n=3). ns: no significance; *: $P<0.05$; **: $P<0.01$.

Figure S3. ACSL3 and 4 regulation of the biosynthesis of fatty acyl-CoAs and catalytic efficiency. (A) PC-3 cells were transduced with shRNA-control or shRNA-ACSL3 (shACSL3-1 or 2) by lentiviral infection. The cells were harvested for analysis of C12:0-CoA, C14:0-CoA, C16:0-CoA, C18:0-CoA, C18:1-CoA, C18:2-CoA and C20:4-CoA by LC-MS/MS. The data represent the mean ± SD (n=3). *: $P<0.05$; **: $P<0.01$. (**B**) ACSL3 or ACSL4 catalytic efficiency

of fatty acyl-CoAs biosynthesis was calculated based on the formula: percentage of the decrease of fatty acyl-CoA levels/percentage of the decrease of the relative mRNA levels. To compare ACSL4 catalytic efficiency with ACSL3, the above value was further divided by the ratio of ACSL4 to ACSL3 relative mRNA expression levels. The catalytic efficiency of ACSL3 was set as 1.

Figure S4. Verification of Myr-Src antibody. An antibody was developed using the myristoylated octapeptide myristoyl-GSNKSKPKC derived from Src kinase as the antigen. To verify the specificity of the antibody, SYF1($Src^{-/-}Yes^{-/-}Fyn^{-/-}$) cells were transduced with Src(WT) or Src(G2A), a mutant with loss of the myristoylation site by lentiviral infection. SYF1 cells or the above transduced cells were treated with different concentration of myristic acid (MA) overnight. Cell lysates were subjected to immunoblotting. Expression levels of Src, GAPDH, and myristoylated Src were analyzed by immunoblotting. The antibody was specifically recognized Src(WT), but not Src(G2A) at different MA concentrations.

Figure S5. Knockdown of ACSL3 has no effect on global protein myristoylation or myristoyl-Src in PC-3 and DU145 cells. PC-3 and DU145 cells transduced with shRNA-control (shCon) or shRNA-ACSL3 by lentiviral infection were cultured with/without 100 μM myristic acid-azide overnight. Global protein myristoylation in LNCaP **(A)** or PC-3 **(B)** expressing shRNA-control (shCon), shRNA-ACSL3 (shACSL3-1 or 2) was analyzed by Click Chemistry, and detected by streptavidin-HRP. ACSL3, Myr-Src, and GAPDH were detected by Western blot. (**C-D**) Three major bands of global protein myristylation and Myr-Src were analyzed by Image J. Knockdown of ACSL3 did not change Myr-Src levels, or global protein myristoylation. Some bands in global protein myristoylation had a mild increase.

Figure S6, related to Figure 5. Knockdown of ACSL4 regulates the cell cycle in PC-3 and DU145 cells. A-B) Knockdown of ACSL4 in PC-3 and DU145 cells. PC-3 (A) and DU145 (B) cells were transduced with shRNA-control or two shRNA-ACSL4 (shACSL4-1 or 2) by lentiviral infection. Knockdown of ACSL4 was measured by Real-time RT-PCR. The data represent the mean ± SEM (n=3). **: $P<0.01$. **(C-H)** PC-3 (C-E) and DU145 cells (F-H) expressing shRNA-Control (shCon) or shRNA-ACSL4 (shACSL4-1 or 2) were subjected to cell cycle analysis by the flow cytometry. The percentage of the cell population in G1, S, and G2 phases was recorded.

Figure S1

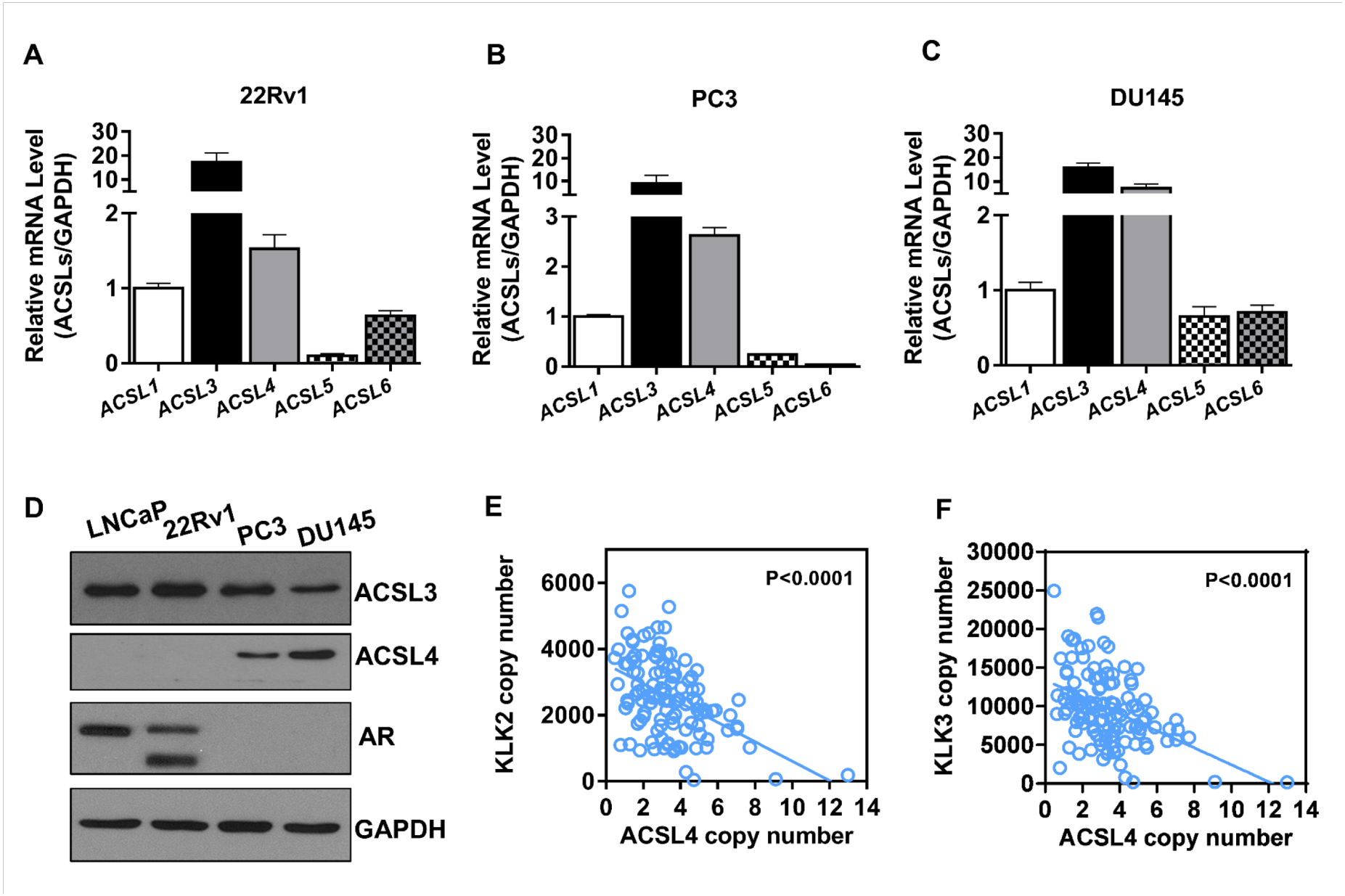

A
22Rv1
Relative mRNA Level (ACSLs/GAPDH)
30
20
10
2
1
0
ACSL1
ACSL3
ACSL4
ACSL5
ACSL6
B
PC3
Relative mRNA Level (ACSLs/GAPDH)
30
20
10
3
2
1
0
ACSL1
ACSL3
ACSL4
ACSL5
ACSL6
C
DU145
Relative mRNA Level (ACSLs/GAPDH)
30
20
10
2
1
0
ACSL1
ACSL3
ACSL4
ACSL5
ACSL6
D
LNCaP
22Rv1
PC3
DU145
ACSL3
ACSL4
AR
GAPDH
E
KLK2 copy number
6000
4000
2000
0
P<0.0001
0 2 4 6 8 10 12 14
ACSL4 copy number
F
KLK3 copy number
30000
25000
20000
15000
10000
5000
0
P<0.0001
0 2 4 6 8 10 12 14
ACSL4 copy number

Figure S2

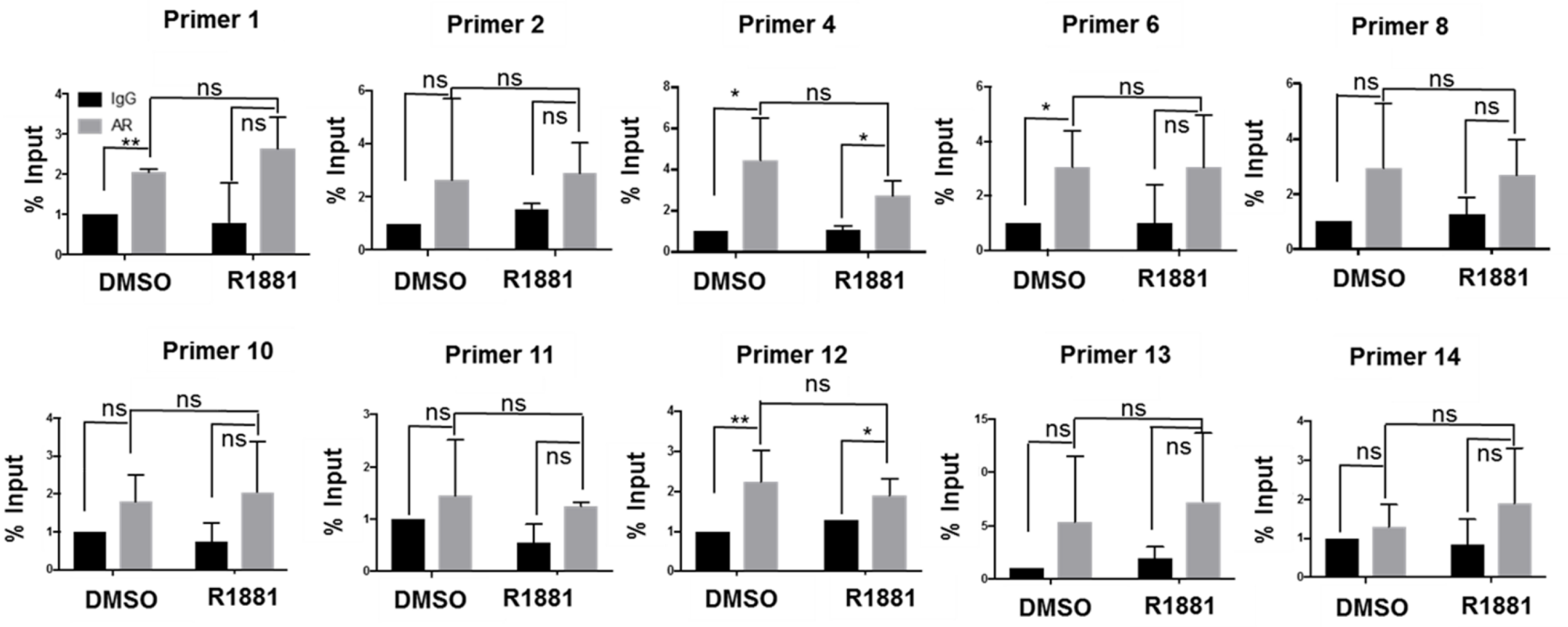

Primer 1
Primer 2
Primer 4
Primer 6
Primer 8
Primer 10
Primer 11
Primer 12
Primer 13
Primer 14
IgG
AR
% Input
DMSO
R1881
ns
*
**

Figure S3

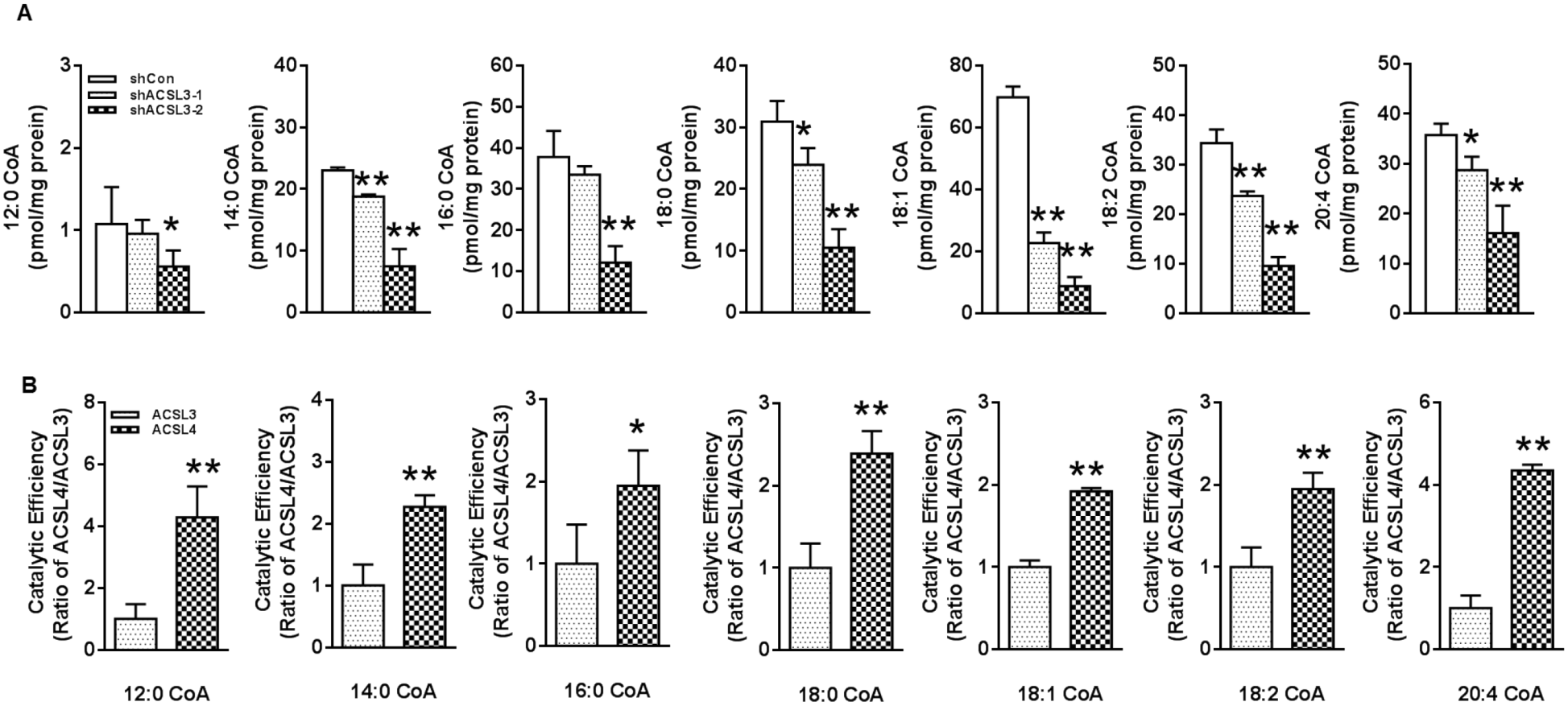
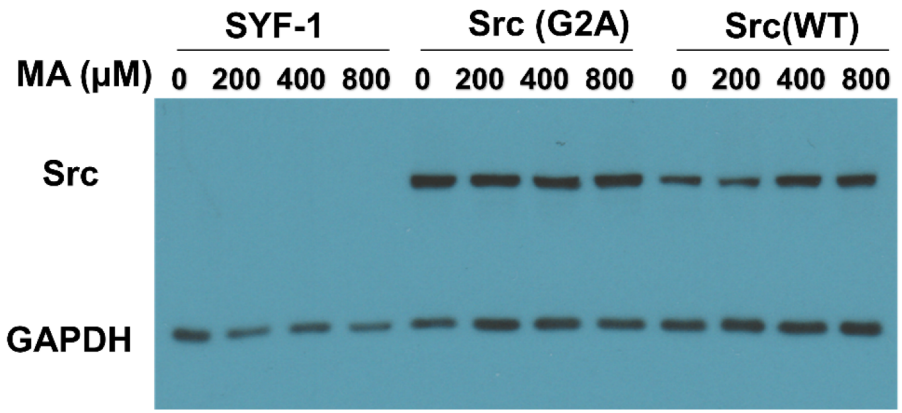

A
shCon
shACSL3-1
shACSL3-2
12:0 CoA (pmol/mg proein)
14:0 CoA (pmol/mg proein)
16:0 CoA (pmol/mg protein)
18:0 CoA (pmol/mg proein)
18:1 CoA (pmol/mg proein)
18:2 CoA (pmol/mg proein)
20:4 CoA (pmol/mg protein)
B
ACSL3
ACSL4
Catalytic Efficiency (Ratio of ACSL4/ACSL3)
12:0 CoA
14:0 CoA
16:0 CoA
18:0 CoA
18:1 CoA
18:2 CoA
20:4 CoA

Figure S4


SYF-1
Src (G2A)
Src(WT)
MA (μM) 0 200 400 800 0 200 400 800 0 200 400 800
Src
GAPDH

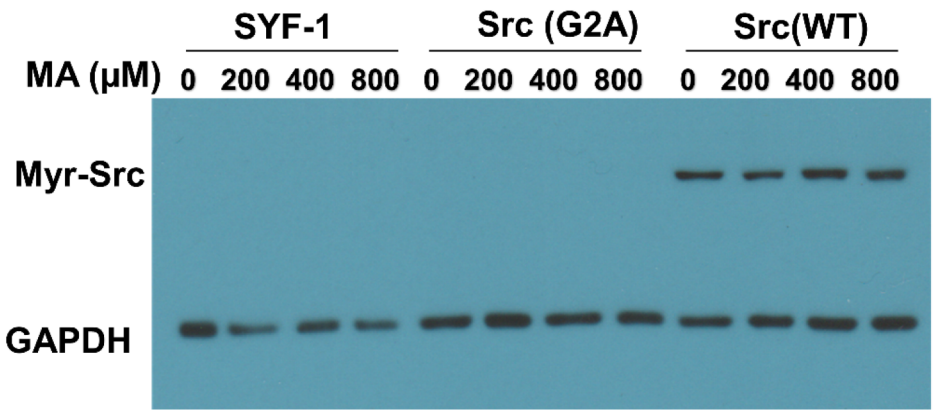

SYF-1
Src (G2A)
Src(WT)
MA (μM) 0 200 400 800 0 200 400 800 0 200 400 800
Myr-Src
GAPDH

Figure S5

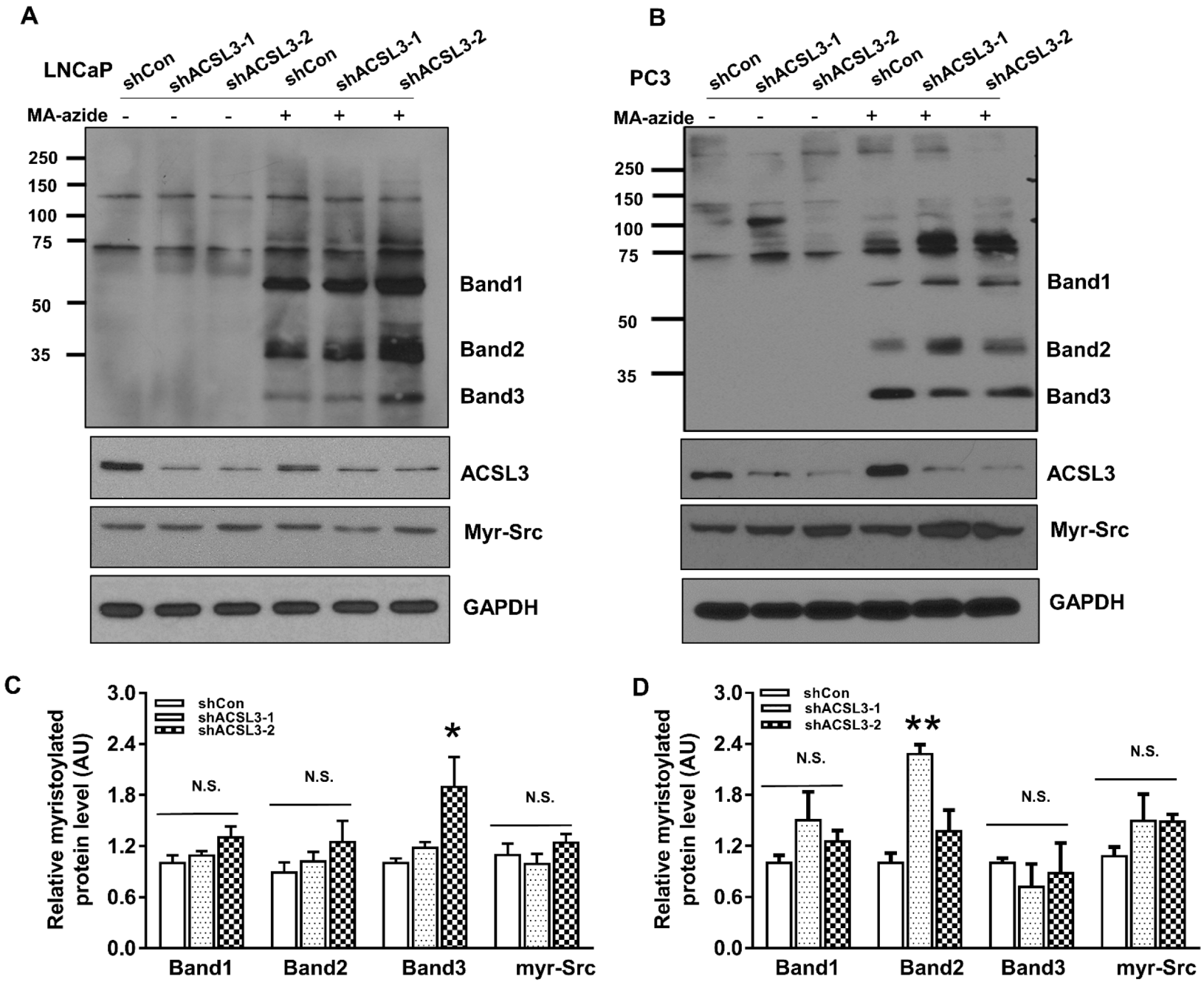
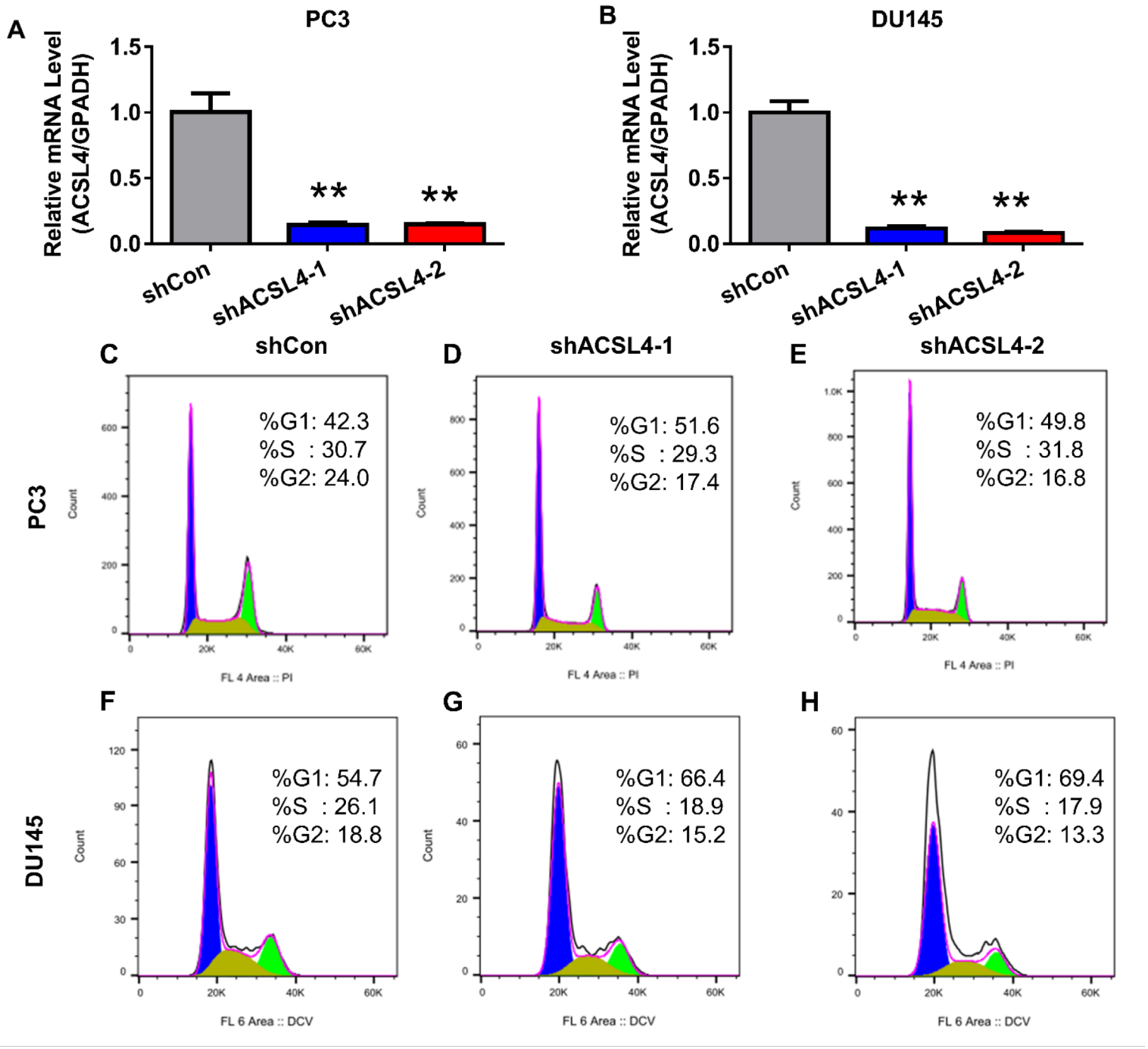

A
LNCaP
shCon
shACSL3-1
shACSL3-2
shCon
shACSL3-1
shACSL3-2
MA-azide - - - + + +
250
150
100
75
50
35
Band1
Band2
Band3
ACSL3
Myr-Src
GAPDH
B
PC3
shCon
shACSL3-1
shACSL3-2
shCon
shACSL3-1
shACSL3-2
MA-azide - - - + + +
250
150
100
75
50
35
Band1
Band2
Band3
ACSL3
Myr-Src
GAPDH
C
Relative myristoylated protein level (AU)
3.0
2.4
1.8
1.2
0.6
0.0
shCon
shACSL3-1
shACSL3-2
N.S.
N.S.
*
N.S.
Band1
Band2
Band3
myr-Src
D
Relative myristoylated protein level (AU)
3.0
2.4
1.8
1.2
0.6
0.0
shCon
shACSL3-1
shACSL3-2
N.S.
**
N.S.
N.S.
Band1
Band2
Band3
myr-Src

Figure S6


A
PC3
Relative mRNA Level (ACSL4/GPADH)
1.5
1.0
0.5
0.0
**
**
shCon
shACSL4-1
shACSL4-2
B
DU145
Relative mRNA Level (ACSL4/GPADH)
1.5
1.0
0.5
0.0
**
**
shCon
shACSL4-1
shACSL4-2
C
shCon
D
shACSL4-1
E
shACSL4-2
PC3
%G1: 42.3
%S : 30.7
%G2: 24.0
%G1: 51.6
%S : 29.3
%G2: 17.4
%G1: 49.8
%S : 31.8
%G2: 16.8
Count
FL 4 Area :: PI
F
G
H
DU145
%G1: 54.7
%S : 26.1
%G2: 18.8
%G1: 66.4
%S : 18.9
%G2: 15.2
%G1: 69.4
%S : 17.9
%G2: 13.3
Count
FL 6 Area :: DCV