Supplemental materials and methods

Cell culture and vectors

Human lung cancer H1299, H358, H23, H322 and A549 cells were obtained from ATCC (Manassas, VA). Primary NHBE cells were obtained from Lonza (Walkersville, MD). Metabolites supplementation assays were performed by treating cells with oleic acid (20 μM) and/or mevalonate (20 μM) as previously described (Freed-Pastor et al. 2012; Zaidi et al. 2012), or by treating cells with acetate (1 mM NaOAc; pH 7.0) as previously described (Wellen et al. 2009; Zaidi et al. 2012). The pLPCX-Myc-CUL3 retroviral vector was constructed by subcloning the CUL3 DNA fragment from pcDNA3-Myc-CUL3 (Addgene) into the pLPCX vector. The pLPCX-ACLY-HA and pLPCX-KLHL25-Flag vectors were constructed by using ACLY fragment and KLHL25 fragment from PANT7_cGST-ACLY and pDONR221-KLHL25 vectors (DNASU Plasmid Repository), respectively. pLPCX-ACLY-HA vectors with mutations at lysine 540, 546 and/or 554 were constructed by site-directed mutagenesis by using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) with the following primers: K540R: Forward 5'- GGGACCACAAGCAGAGGTTTTACTGGGGGCA -3'; Reverse 5'- TGCCCCCAGTAAAACCTCTGCTTGTGGTCCC -3'; K546R: Forward 5'- AAGTTTTAC TGGGGGCACAGAGAGATCCTGATCCC -3'; Reverse 5'- GGGATCAGGATCT CTCTGTG CCCCCAGTAAAACTT -3'; K554R: Forward 5'- CTGATCCCTGTCTTCAGGAACATGGC TGATGCC -3'; Reverse 5'- GGCATCAGCCATGTTCCTGAAGACAGGGATCAG -3'.

The WT or 3KR pLPCX-rACLY-HA vectors resistant to ACLY-shRNA #1 were constructed by site-directed mutagenesis by using the following primers: Forward: 5'-GGCAGCACTTC CCTGCCACTCCTCT**AT**T**A**GA**C**TA**C**GCACTGGAAGTAGAGAAGATT-3'; Reverse: 5'-AA TCTTCTCTACTTCCAGTGC**G**TA**G**TC**T**A**AT**AGAGGAGTGGCAGGGAAGTGCTGCC-3'.

The mutant sites are shown in bold. Lentiviral shRNA vectors against CUL3 (V3LHS_351781 and V3LHS_351782), and ACLY (V3LHS_395677 and V2LHS_94212) were purchased from Open Biosystems (Huntsville, AL). The shRNA vectors against KLHL25, UBR4 and KEAP1 were constructed by inserting the following targeting sequences into the GIPZ lentiviral shRNA vector as previously described (Liu et al. 2014). For KLHL25: 5'-CTGCATCACCACAGTGCCCTA-3' and 5'-CCGCTTTGACTGTGAGACCAA-3'; for UBR4: 5'-GCAGTACGAGCCATTCTAC-3' and 5'-GGAAAGAACATCATTGTTA-3'; for KEAP1: 5'-GGCCTTTGGCATCATGAAC-3' and 5'-CGAGTGGCGAATGATCACA-3'.

Supplemental figure legends

Supplemental Figure S1. KLHL25 expression levels in normal human lung tissues and lung cancer tissues. The same cohorts of human lung cancer in Figure *2A* were used for analysis. The expression levels of KLHL25 are expressed in terms of a log2 median-centered intensity which is calculated by normalizing the intensity of KLHL25 probe to the median of the probe intensities across the entire array.

Supplemental Figure S2. CUL3 or KLHL25 does not affect the mRNA levels of *ACLY* in human lung cells. (*A*) Ectopic expression of Myc-CUL3 or KLHL25-Flag did not affect the mRNA levels of *ACLY* in different human lung cells. (*B, C*) Knockdown of endogenous CUL3 (*B*) or KLHL25 (*C*) by 2 different shRNA vectors did not affect the mRNA levels of *ACLY* in different human lung cells. In (*A*)-(*C*), the mRNA levels of *ACLY* were measured by Taqman real-time PCR and normalized with *actin*.

Supplemental Figure S3. Knockdown of CUL3 and UBR4 upregulates ACLY protein levels in cells. (*A*) UBR4 knockdown by shRNA vectors in human lung cells analyzed by real-time PCR assays. Human lung H1299, H358 and NHBE cells were transduced with 2 different shRNA vectors against UBR4, and mRNA levels of *UBR4* were measured by Taqman real-time PCR and normalized with *actin*. (*B*) Knockdown of CUL3 and UBR4 simultaneously displayed a more pronounced effect on ACLY protein levels than knockdown of CUL3 or UBR4 individually in human lung cells. The efficiency of CUL3 knockdown was presented in Fig. 2E.

Supplemental Figure S4. KLHL25 reduces Acetyl-CoA levels and inhibits lipid synthesis through negative regulation of ACLY. (*A*) ACLY knockdown by shRNA vectors in human lung cancer cells analyzed by western blot assays. Human lung cancer cells were transduced with 2 different shRNA vectors, and ACLY protein levels were measured by western blot assays. (*B*) KLHL25 knockdown increased acetyl-CoA levels in human lung cancer cells, which was largely abolished by knockdown of endogenous ACLY. (*C*) KLHL25 knockdown increased lipid synthesis in human lung cancer cells, which was largely abolished by knockdown of endogenous ACLY. Data are presented as mean ± S.D. (n=3). *: $p<0.01$; **: $p<0.001$; Student *t*-tests.

Supplemental Figure S5. CUL3-KLHL25 inhibits cell proliferation through negative regulation of ACLY. (*A*) KLHL25 knockdown promoted the proliferation of human lung cells, which was greatly abolished by knockdown of endogenous ACLY. (*B*) Replacement of endogenous ACLY with WT or 3KR ACLY resistant to shRNA (rACLY) in H1299 and H358 cells. H1299 and H358 cells were transduced with ACLY-shRNA #1 to knock down endogenous ACLY, and were then transduced with vectors expressing WT or 3KR rACLY. The ACLY protein levels in cells were measured by western blot assays. (*C*) Ectopic expression of Myc-CUL3 greatly decreased the proliferation of cells expressing WT rACLY but not cells expressing 3KR rACLY. (*D*) Supplementation of oleic acid and/or mevalonate reduced the inhibitory effects of ACLY knockdown on the proliferation of human lung cancer cells. Cells transduced with the ACLY shRNA or control shRNA were treated with oleic acid and/or mevalonate (20 μM). (*E*) The conversion of acetate to acetyl-CoA by acetyl-CoA synthetase 2 (ACSS2) as an alternative pathway to produce acetyl-CoA in cells. (*F*) Supplementation of acetate reduced the inhibitory effect of ectopic Myc-CUL3 expression on the proliferation of human lung cancer cells. Cells

transduced with the myc-CUL3 or control vectors were treated with sodium acetate (1 mM). Cell proliferation was analyzed by MTT assays and the absorbance at 570 nm was measured at day 4. Relative cell number was quantified by comparing cell number of each group with the control group. Data are presented as mean ± S.D. (n=4). #: $p<0.05$; *: $p<0.01$; **: $p<0.001$; Student *t*-tests.

Supplemental Figure S6. CUL3-KLHL25 inhibits anchorage-independent cell growth through negative regulation of ACLY. (*A*) KLHL25 knockdown promoted anchorage-independent growth of human lung cancer cells, which was greatly abolished by ACLY knockdown. (*B*) Ectopic expression of Myc-CUL3 greatly decreased the anchorage-independent growth of cells expressing WT but not 3KR rACLY. (*C*) Supplementation of oleic acid and/or mevalonate (20 μM) reduced the inhibitory effect of ACLY knockdown on anchorage-independent growth in human lung cancer cells. (*D*) Supplementation of acetate (1 mM) reduced the inhibitory effect of ectopic Myc-CUL3 expression on anchorage-independent growth in human lung cancer cells. In (*A*)-(*D*), data are presented as mean ± SD (n = 4). *: $p<0.01$; **: $p<0.001$; Student *t*-tests.

Supplemental Figure S7. CUL3 inhibits cell proliferation and anchorage-independent cell growth in the absence of KEAP1. (*A*) Ectopic Myc-CUL3 expression inhibited the proliferation of A549 cells carrying a KEAP1 inactivation mutation, and this effect was much less pronounced in cells with ACLY knockdown. (*B*) CUL3 knockdown promoted the proliferation of A549 cells, which was greatly abolished by knockdown of endogenous ACLY. (*C*) Ectopic Myc-CUL3 expression inhibited anchorage-independent growth of A549 cells, and this effect was much less pronounced in cells with ACLY knockdown. (*D*) CUL3 knockdown promoted

anchorage-independent growth of A549 cells, which was greatly abolished by ACLY knockdown. (*E*) KEAP1 knockdown by shRNA vectors in H1299 and H358 cells analyzed by western blot assays. Cells were transduced with 2 different shRNA vectors against KEAP1, and KEAP1 protein levels were measured by western blot assays. (*F*) Ectopic Myc-CUL3 expression inhibited the proliferation of cells transduced with control or KEAP1 shRNA vectors. (*G*) CUL3 knockdown promoted the proliferation of cells transduced with control or KEAP1 shRNA vectors.

Supplemental Figure S8. CUL3 knockdown promotes the growth of xenograft tumors formed by A549 cells, which was greatly abolished by ACLY knockdown. Human lung cancer A549 cells carry a KEAP1 inactivation mutation. Presented are the growth curves of xenograft tumors formed by A549 cells with CUL3 and/or ACLY knockdown by shRNA vectors. Two shRNA vectors against CUL3 and ACLY, respectively, were used and similar results were observed. For the sake of clarity, only results from one shRNA vector were presented. Data are presented as mean ± SD (n=8); **: $p<0.001$; ANOVA followed by Student's *t*-tests.

Supplemental References

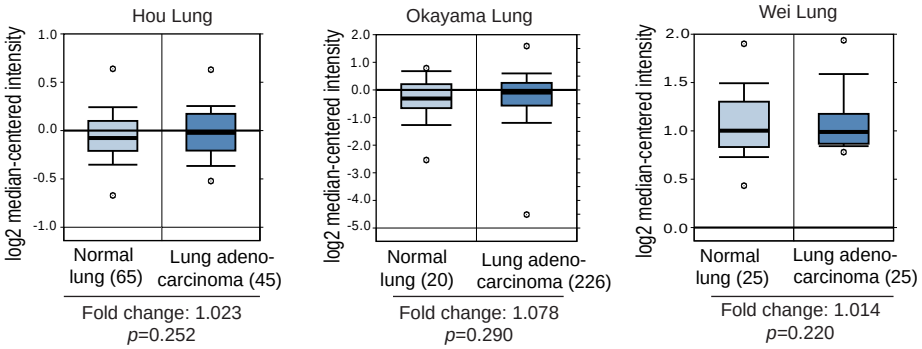
Freed-Pastor WA, Mizuno H, Zhao X, Langerod A, Moon SH, Rodriguez-Barrueco R, Barsotti A, Chicas A, Li W, Polotskaia A et al. 2012. Mutant p53 disrupts mammary tissue architecture via the mevalonate pathway. *Cell* **148**: 244-258.

Liu J, Zhang C, Wang XL, Ly P, Belyi V, Xu-Monette ZY, Young KH, Hu W, Feng Z. 2014. E3 ubiquitin ligase TRIM32 negatively regulates tumor suppressor p53 to promote tumorigenesis. *Cell death and differentiation* **21**: 1792-1804.

Wellen KE, Hatzivassiliou G, Sachdeva UM, Bui TV, Cross JR, Thompson CB. 2009. ATP-citrate lyase links cellular metabolism to histone acetylation. *Science* **324**: 1076-1080.

Zaidi N, Royaux I, Swinnen JV, Smans K. 2012. ATP citrate lyase knockdown induces growth arrest and apoptosis through different cell- and environment-dependent mechanisms. *Molecular cancer therapeutics* **11**: 1925-1935.

Zhang_Fig. S1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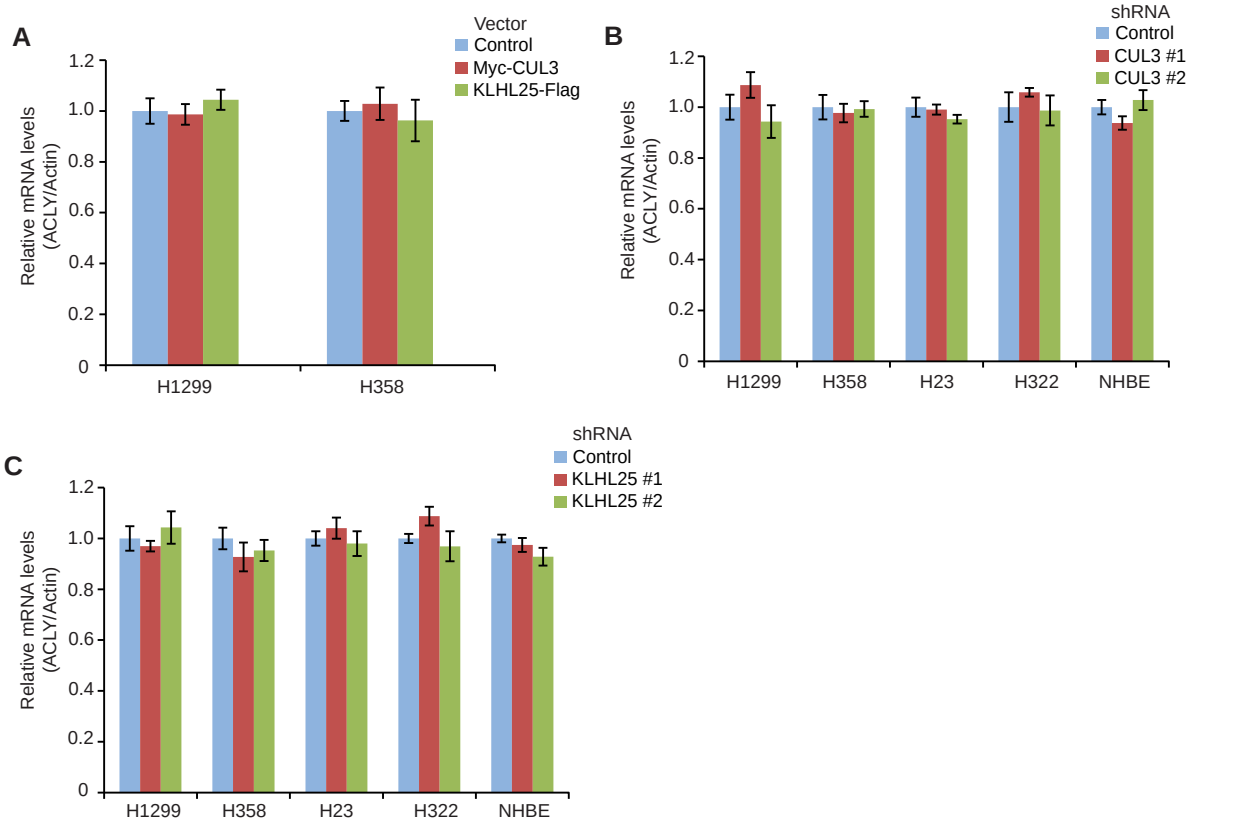
1.0
0.5
0.0
-0.5
-1.0
Normal lung (65)
Lung adeno-carcinoma (45)
Fold change: 1.023
p=0.252
Okayama Lung
log2 median-centered intensity
2.0
1.0
0.0
-1.0
-2.0
-3.0
-4.0
-5.0
Normal lung (20)
Lung adeno-carcinoma (226)
Fold change: 1.078
p=0.290
Wei Lung
log2 median-centered intensity
2.0
1.5
1.0
0.5
0.0
Normal lung (25)
Lung adeno-carcinoma (25)
Fold change: 1.014
p=0.220

Zhang_Fig. S2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0.4
0.2
0
H1299
H358
B
shRNA
Control
CUL3 #1
CUL3 #2
Relative mRNA levels (ACLY/Actin)
H1299
H358
H23
H322
NHBE
C
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KLHL25 #2
Relative mRNA levels (ACLY/Actin)
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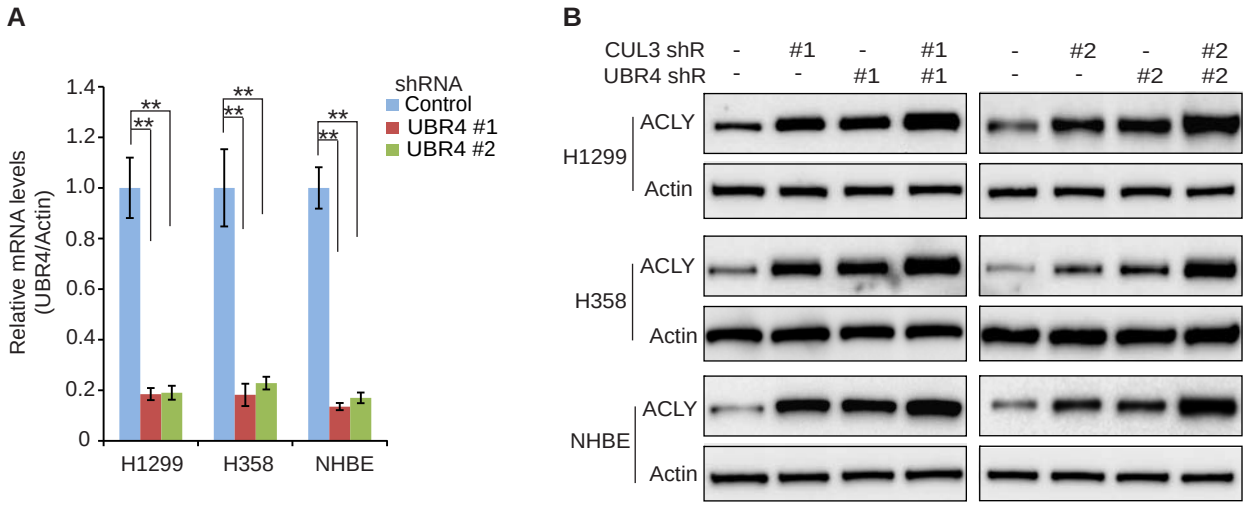
H358
H23
H322
NHBE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Relative mRNA levels (UBR4/Actin)
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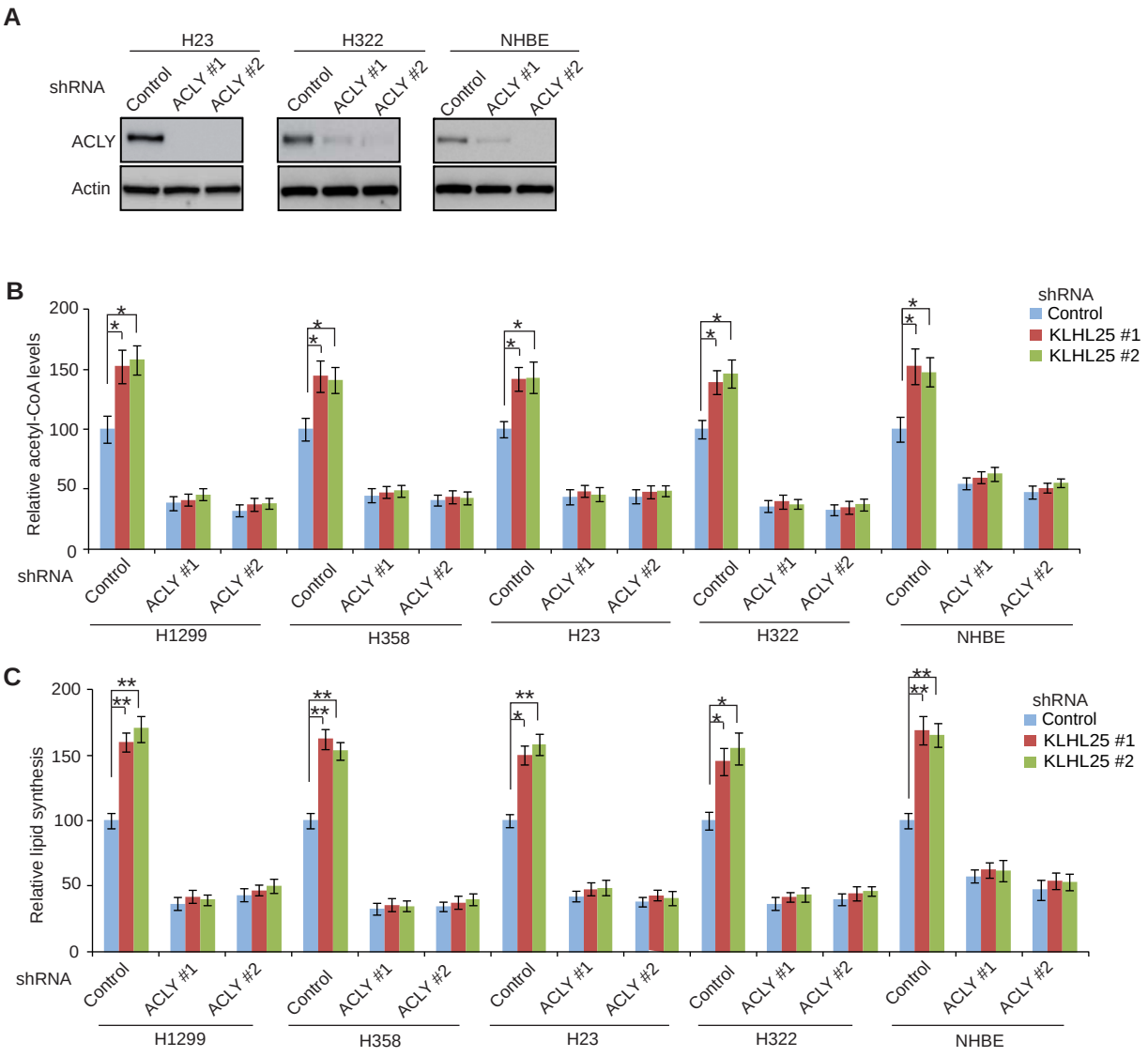
1.2
1.0
0.8
0.6
0.4
0.2
0
**
**
**
**
**
**
H1299
H358
NHBE
shRNA
Control
UBR4 #1
UBR4 #2
B
CUL3 shR - #1 - #1 - #2 - #2
UBR4 shR - - #1 #1 - - #2 #2
H1299
ACLY
Actin
H358
ACLY
Actin
NHBE
ACLY
Actin

Zhang_Fig. S4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H23
H322
NHBE
shRNA
Control
ACLY #1
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ACLY
Actin
B
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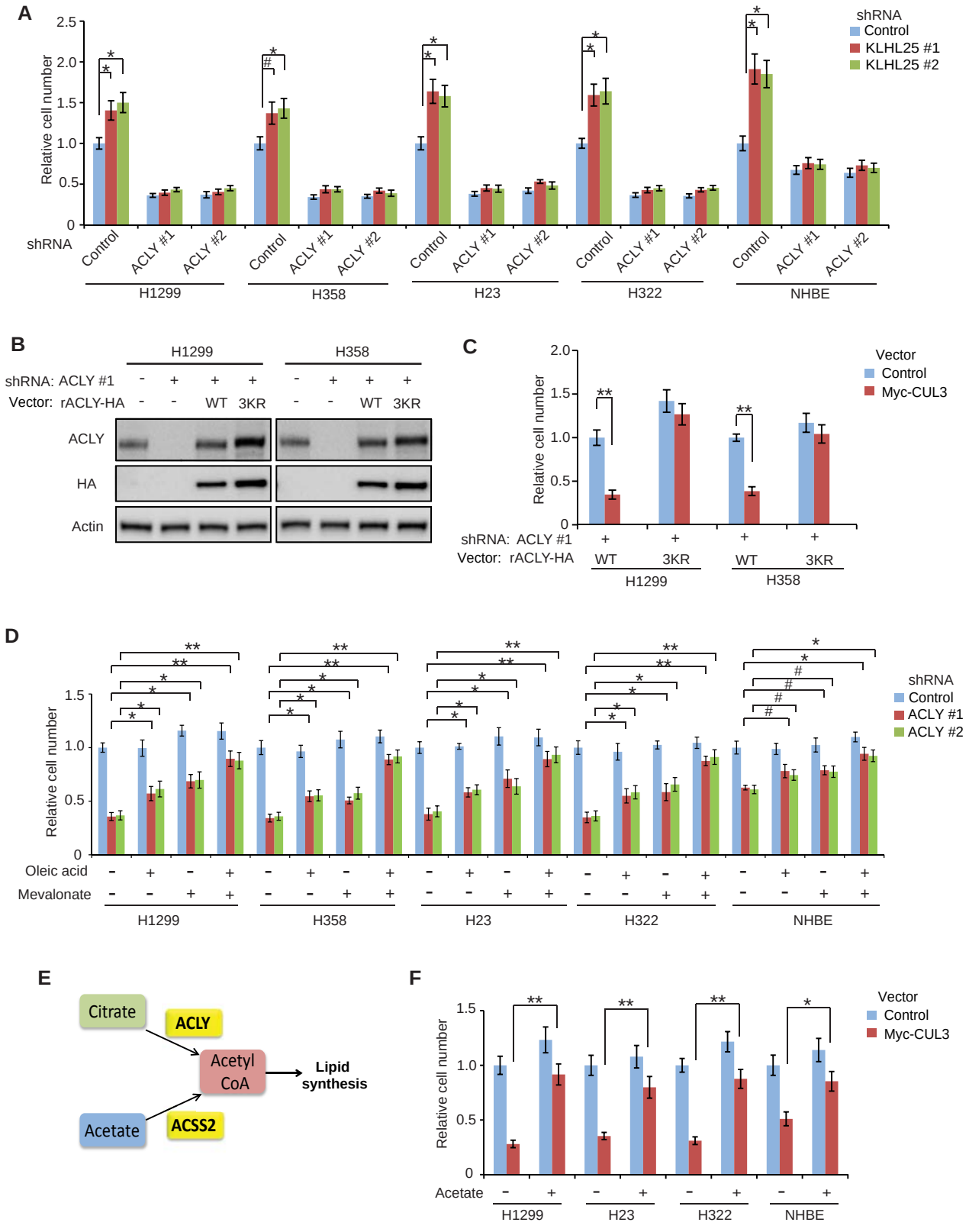
H1299
H358
H23
H322
NHBE
C
Relative lipid synthesis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H358
H23
H322
NHBE
B
H1299
H358
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Vector: rACLY-HA
WT
3KR
ACLY
HA
Actin
C
Relative cell number
Vector
Control
Myc-CUL3
shRNA: ACLY #1
Vector: rACLY-HA
WT
3KR
H1299
H358
D
Relative cell number
shRNA
Control
ACLY #1
ACLY #2
Oleic acid
Mevalonate
H1299
H358
H23
H322
NHBE
E
Citrate
ACLY
Acetyl CoA
Lipid synthesis
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ACSS2
F
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Vector
Control
Myc-CUL3
Acetate
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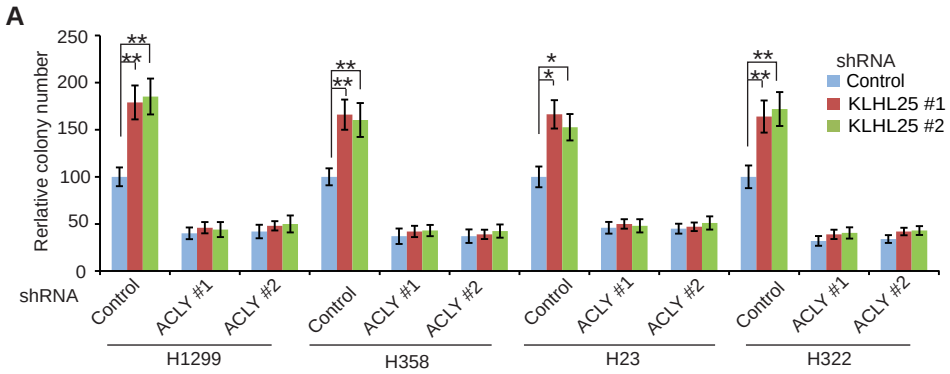
H23
H322
NHBE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200
150
100
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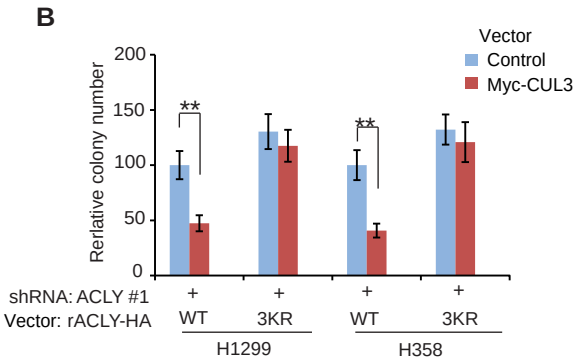
ACLY #2
H1299
H358
H23
H322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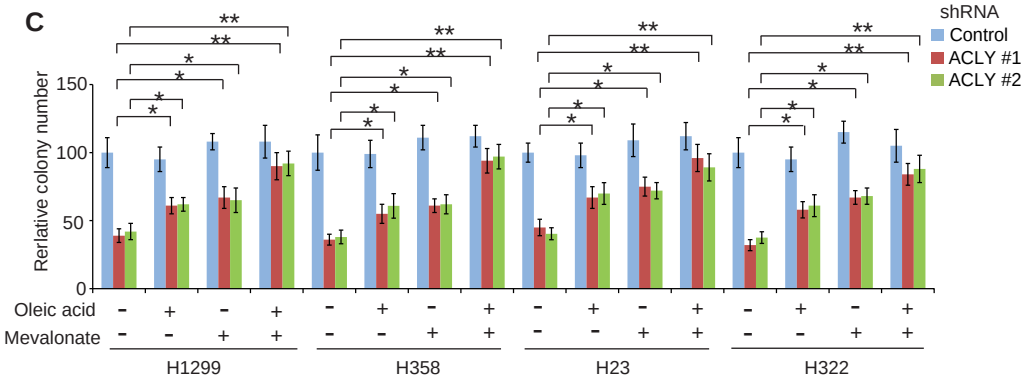
3KR
WT
3KR
H1299
H358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Mevalonate
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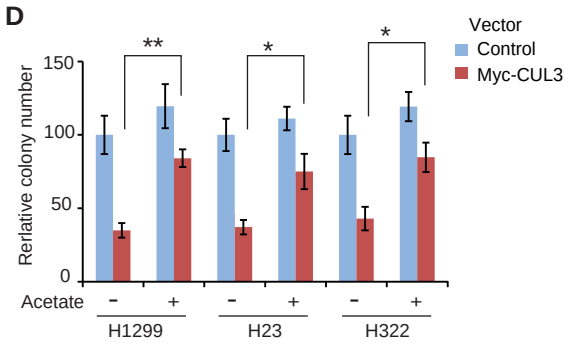
H322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H1299
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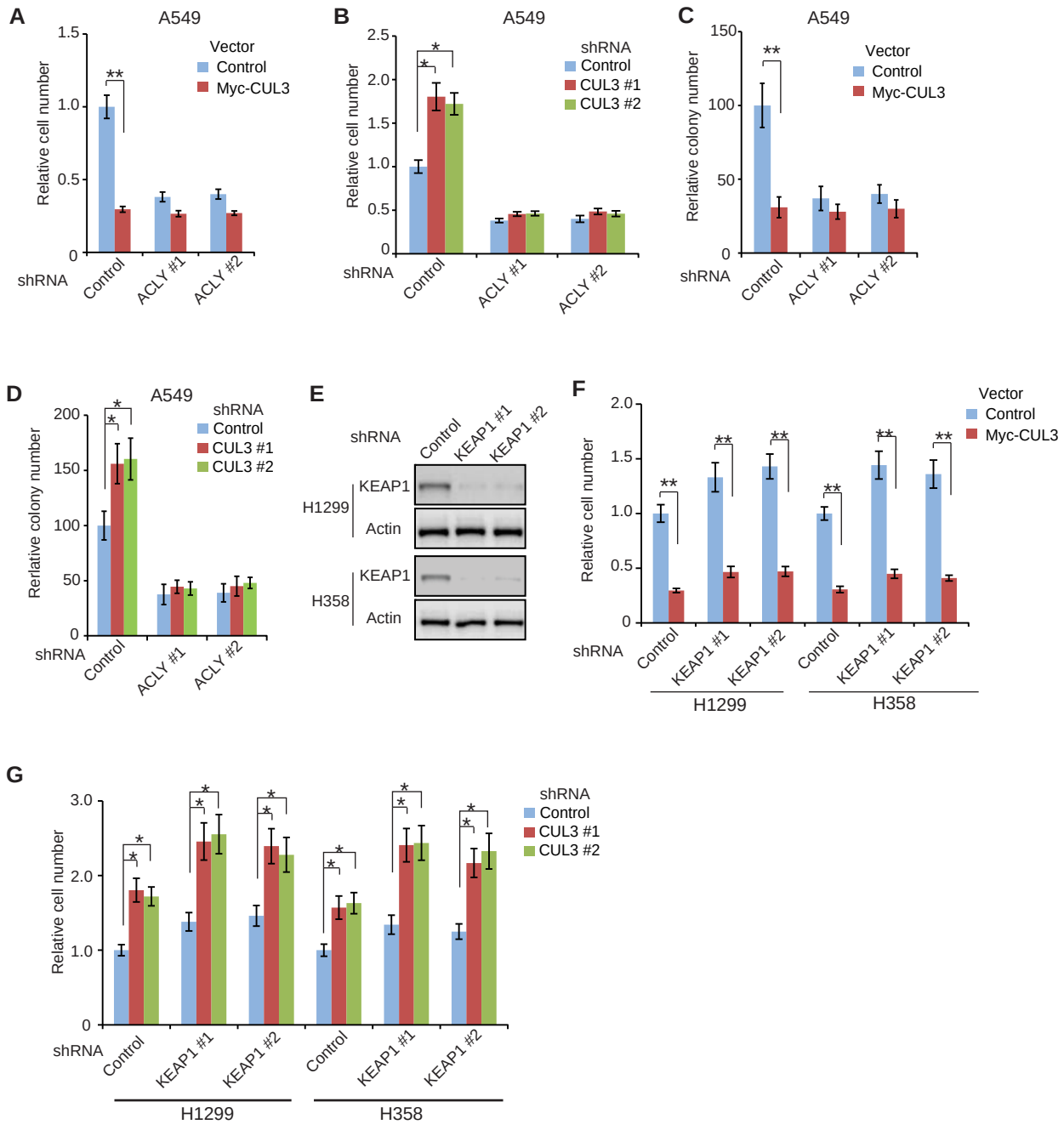
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CUL3 #1
CUL3 #2
C
A549
Rerlative colony number
Vector
Control
Myc-CUL3
D
A549
Rerlative colony number
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Control
CUL3 #1
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E
shRNA
Control
KEAP1 #1
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H1299
KEAP1
Actin
H358
KEAP1
Actin
F
Relative cell number
Vector
Control
Myc-CUL3
shRNA
Control
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KEAP1 #2
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H358
G
Relative cell number
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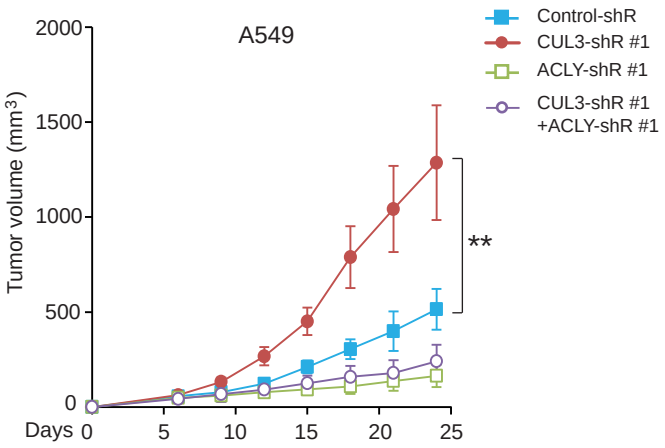
Zhang_Fig. S8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CUL3-shR #1
ACLY-shR #1
CUL3-shR #1
+ACLY-shR #1
Tumor volume (mm3)
2000
1500
1000
500
0
Days
0
5
10
15
20
25
**