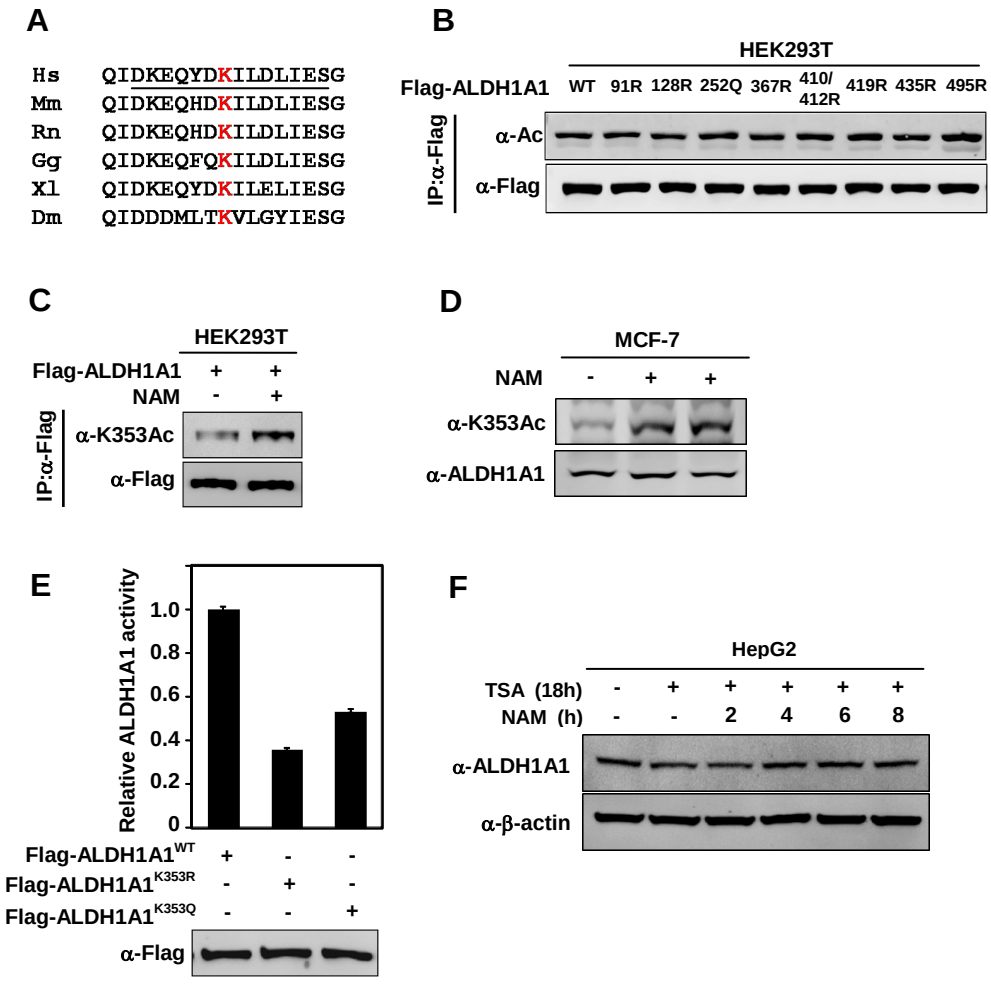
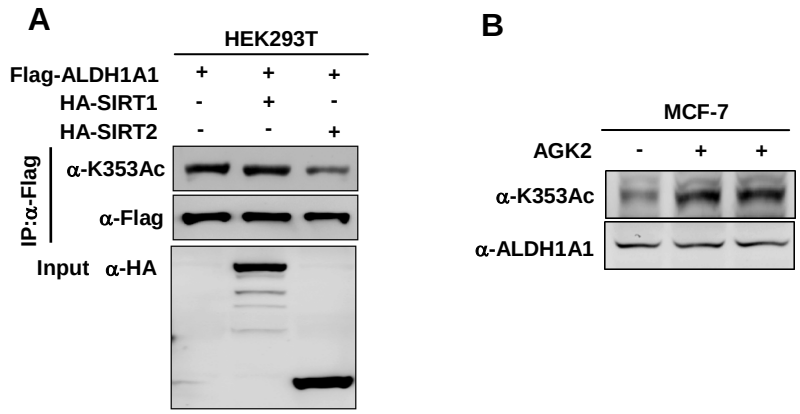
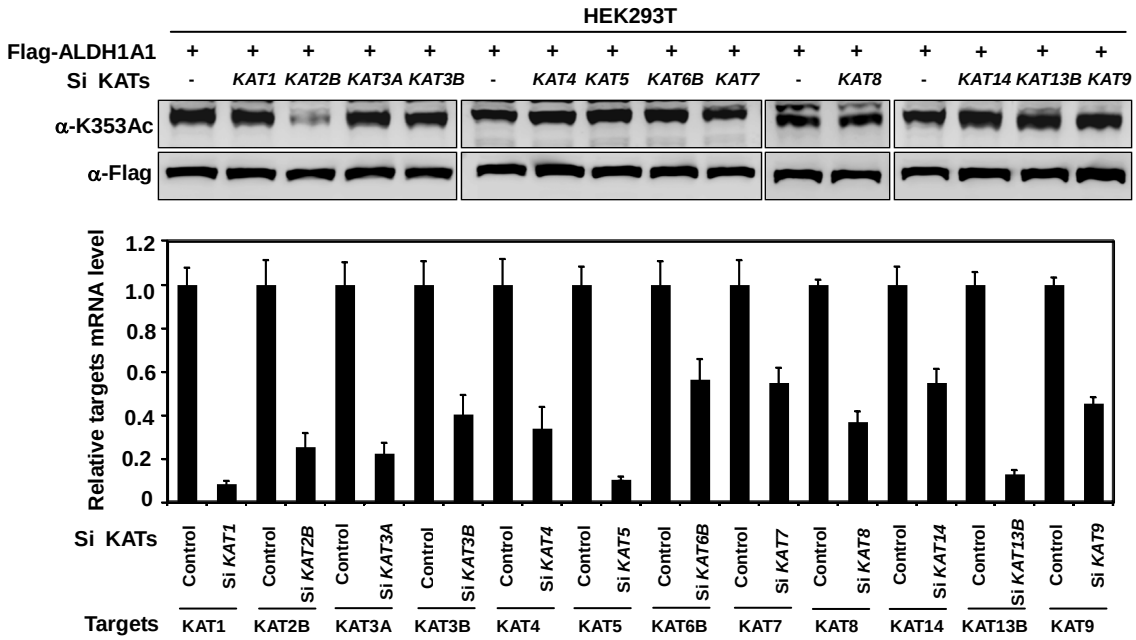
SUPPLEMENTAL FIGURES AND TABLES



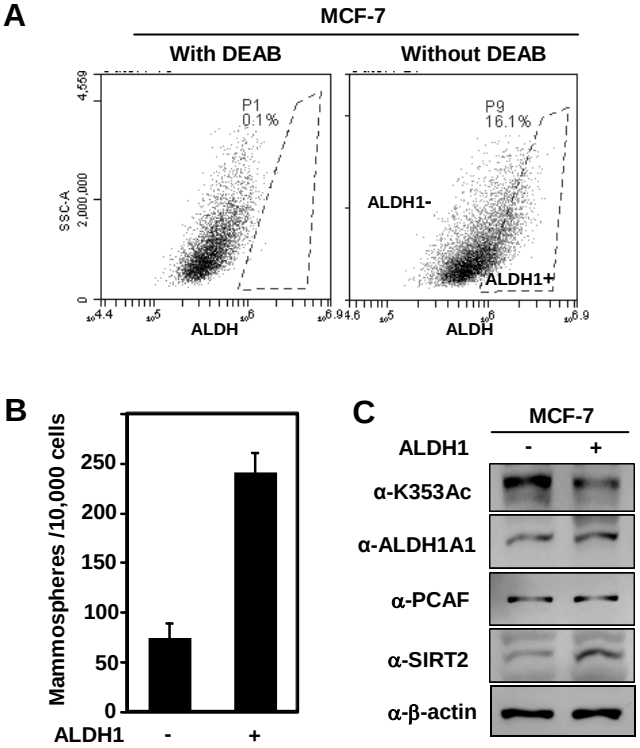
Supplemental Figure 1 Acetylation at Lys-353 decreases ALDH1A1 enzyme activity. (**A**) K353 of ALDH1A1 is conserved. Hs: Homo sapient (human); Mm: Mus musculus (mouse); Rn: Rattus norvegicus (Norway rat); Gg: Gallus gallus (chicken); Xl: Xenopus laevis (frog); Dm: Drosophila melanogaster (fruit fly). Sequence underlined in human ALDH1A1 indicates the peptide sequence used for generating the anti-AcALDH1A1(K353) antibody. (**B**) Mutations of other nine putative acetylation sites have no effect on ALDH1A1 acetylation. The indicated plasmids were transfected into 293T cells and proteins were immunoprecipitated for western blotting. (**C**) NAM treatment increases ALDH1A1 K353 acetylation. Flag-ALDH1A1 was transfected into 293T cells followed by treatment with NAM. ALDH1A1 acetylation was analyzed by western blot with the anti-AcALDH1A1(K353) antibody. (**D**) Treatment with NAM increases endogenous ALDH1A1 acetylation at K353. MCF-7 cells were treated with NAM. Endogenous ALDH1A1 protein levels and acetylation of K353 were determined. (**E**) K353R/Q mutant decreases ALDH1A1 enzyme activity. Flag-tagged wild-type and mutant ALDH1A1 protein were expressed in 293T cells and purified by immunoprecipitation. The enzyme activity was measured and normalized against protein level. Relative enzyme activities of triplicate experiments with standard deviation (±SD) are presented. (**F**) Acetylation has no effect on ALDH1A1 protein level. HepG2 cells were treated with NAM and TSA for indicated time, and endogenous ALDH1A1 protein level was determined.



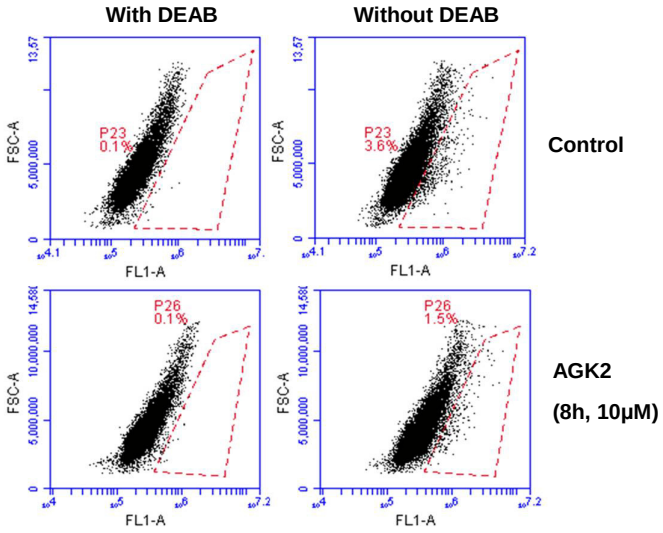
Supplemental Figure 2 SIRT2 deacetylates ALDH1A1 at K353. (**A**) SIRT2, not SIRT1, overexpression decreases ALDH1A1 acetylation. HEK293T cells were transfected with indicated plasmids and ALDH1A1 acetylation was determined by western blotting. (**B**) Inhibition of SIRT2 increases K353 acetylation. MCF-7 cells were treated with 10μM AGK2 for 48h, and K353 acetylation was detected.



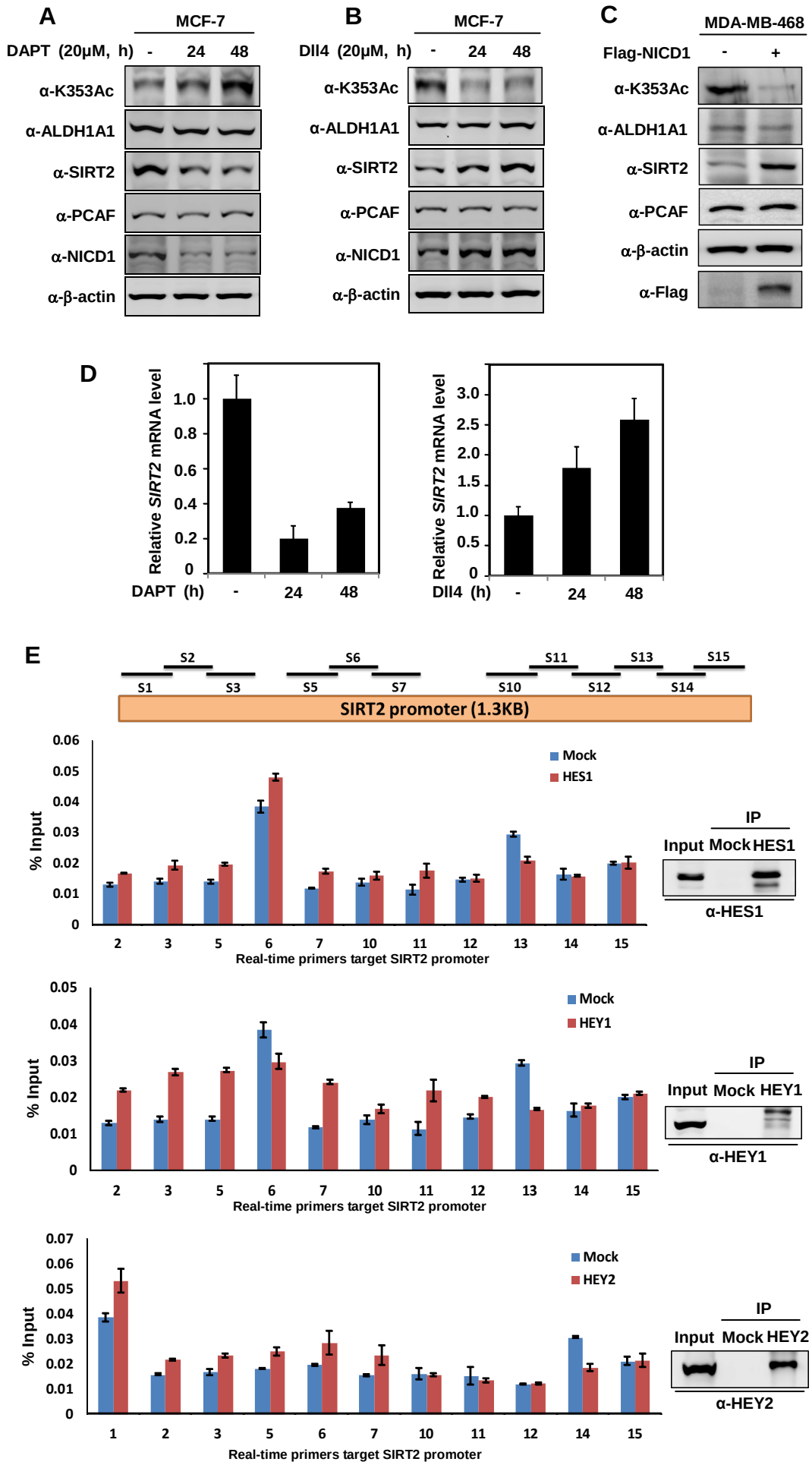
Supplemental Figure 3 Screen for lysine acetyltransferase (KAT) of ALDH1A1. 293T cells were transfected with indicated siRNA and Flag-tagged ALDH1A1 plasmid. The K353 acetylation of ectopically expressed ALDH1A1 was determined by western blotting, and KATs knockdown efficiency was detected by quantitative PCR using β-actin as an internal control. Error bars represent ± SD for triplicate experiments.



Supplemental Figure 4 K353 acetylation of ALDH1A1 negatively correlates with ALDH1 activity and mammosphere forming potential in MCF-7 cells. (**A**) ALDEFLUOR FACS analysis of breast cancer cell line MCF-7. Cells incubated with ALDEFLUOR substrate and DEAB were used to establish the baseline fluorescence (ALDH1-) and to define the ALDH1+ region (ALDH1+) (Left). Incubation of MCF-7 cells with ALDEFLUOR substrate in the absence of DEAB induces a fluorescence shift defining the ALDH1+ population (right). (**B-C**) ALDH1+ population (top 10% cells with high fluorescence) and ALDH1- population (bottom 10% cells with low fluorescence) of MCF-7 cells were sorted. The ALDH1+ and ALDH1- cells were measured for mammosphere-formation (**B**) and ALDH1A1 K353 acetylation (**C**).

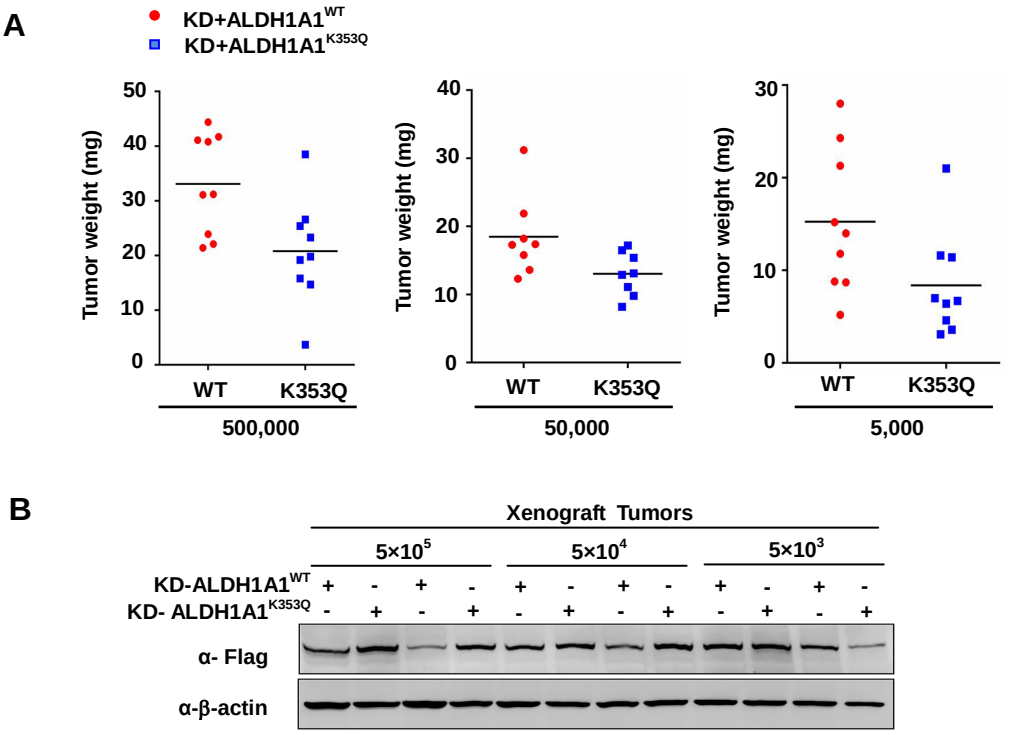


Supplemental Figure 5 Inhibition of SIRT2 decreases ALDH1+ population of breast cancer cells. Primary breast cancer cells were treated by 10 μM AGK2, an SIRT2 inhibitor, for 8 hours. ALDH1+ populations were determined.


A
MCF-7
DAPT (20μM, h) - 24 48
α-K353Ac
α-ALDH1A1
α-SIRT2
α-PCAF
α-NICD1
α-β-actin
B
MCF-7
Dll4 (20μM, h) - 24 48
α-K353Ac
α-ALDH1A1
α-SIRT2
α-PCAF
α-NICD1
α-β-actin
C
MDA-MB-468
Flag-NICD1 - +
α-K353Ac
α-ALDH1A1
α-SIRT2
α-PCAF
α-β-actin
α-Flag
D
Relative SIRT2 mRNA level
DAPT (h) - 24 48
Relative SIRT2 mRNA level
Dll4 (h) - 24 48
E
S1 S2 S3 S5 S6 S7 S10 S11 S12 S13 S14 S15
SIRT2 promoter (1.3KB)
% Input
Mock
HES1
Real-time primers target SIRT2 promoter
IP
Input Mock HES1
α-HES1
% Input
Mock
HEY1
Real-time primers target SIRT2 promoter
IP
Input Mock HEY1
α-HEY1
% Input
Mock
HEY2
Real-time primers target SIRT2 promoter
IP
Input Mock HEY2
α-HEY2

Supplemental Figure 6 Notch pathway induces deacetylation of ALDH1A1. (**A-B**) Notch signaling increases SIRT2 and decreases endogenous ALDH1A1 K353 acetylation. MCF-7 cells were treated with DAPT or Dll4 for indicated time. Endogenous NICD1, PCAF, SIRT2, ALDH1A1 protein levels and K353 acetylation were determined by western blotting. (**C**) Over-expression of NICD1 decreases endogenous K353 acetylation of ALDH1A1. NICD1 was transfected into MDA-MB-468 cells, and endogenous PCAF, SIRT2, ALDH1A1 protein levels and K353 acetylation were determined by western blotting. (**D**) Notch signaling regulates *SIRT2* mRNA levels. Primary breast cancer cells were treated with 20μM DAPT or 10nM Dll4 for indicated time. The levels of endogenous *SIRT2* mRNA were determined by quantitative PCR using β-actin as an internal control. Error bars represent ± SD for triplicate experiments. (**E**) Notch signaling transcriptional factor (HES1, HEY1 or HEY2) doesn't bind the promoter region of SIRT2. ChIP was performed with antibodies specific to HES1, HEY1 or HEY2 in MCF-7 cells followed by quantitative PCR. Eleven pairs of primers covering the promoter region of SIRT2 were used in quantitative PCR. The sequences of real-time primers target SIRT2 promoter are shown as below:

S1-F: TGGAACGTGGTAATGTGTAAAGAA; **S1-R**: GTAAGCTCTTCTATGTTGAATTCC
S2-F: CATAGAAGAGCTTACATTCTCTC; **S2-R**: GATCTTTGATTCTTAGTTCCCGA
S3-F: ACTCTAGGGCTCAAATCGGGAAC; **S3-R**: CACTGATACCCAAACCTGACTCC
S5-F: AAAAGTTAGGACTCGGGGG; **S5-R**: CCCGGCGTCACATTCCTCATTA
S6-F: GGAGTATGAGGTTAGGAAGCC; **S6-R**: AGCTACGTCACTGAGGATGGG
S7-F: CGGGTCCTAGGGCTCCGTTTGG; **S7-R**: ACCTGGGTTGGGCGCGGAGTT
S10-F: GCAGTCGCCCGCCTGTAGC; **S10-R**: CGAGGGCGGGGTGTGAGCGAT
S11-F: CTCACACCCCGCCCTCGTA; **S11-R**: GCCATGTTGGTAAAGGGCGCCCGG
S12-F: CATTTTCCGGGCGCCCTTTACC; **S12-R**: GACGCGCTTTCGTACAACACCCAG
S13-F: GGGGCGCTCTGGGTGTTGTA; **S13-R**: GTCACCGACTGCTCTGTCCCGT
S14-F: CCACTCCCATTTCTGGTGCCGTC; **S14-R**: TTGAGGCTGTCACCGACCGCT
S15-F: CAGGACAGAGCAGTCGGTG; **S15-R**: GACTCACGGTCTGGCTCTGC



Supplemental Figure 7 Lys-353 acetylation inhibits tumor growth of breast cancer. (**A**) ALDH1A1^{K353Q} is defective in supporting tumor growth in vivo. Xenograft in mammary fat pads was performed using the different concentrations of MDA-MB-468 cells (5x10^5, 5x10^4, 5x10^3, 1x10^3, 200, 40 cells per mouse) in which endogenous ALDH1A1 was knocked down and the wild-type or K353Q mutant ALDH1A1 was reintroduced. Seven weeks later, mice were sacrificed and tumor weight was measured, and shown are tumors weights derived from mice injected with 5×10^3, 5×10^4 and 5×10^3 cells. P value was calculated by *t* test. (**B**) The protein levels of re-expressing ALDH1A1 of xenograft tumors were determined by western blotting.

Supplemental Table 1

K91	LLYK(Ac)LADLIER
K128	LYSNAYLNDLAGCIK(Ac)TLR
K252	VAFTGSTEVGK(Ac)LIK
K353	EQYDK(Ac)ILDLIESGK
K367	EGAK(Ac)LECGGGPWGNK
K410	EEIFGPVQQIMK(Ac)FK
K412	EEIFGPVQQIMKFK(Ac)
K419	SLDDVIK(Ac)R
K435	RANNTFYGLSAGVFTK(Ac)DIDK
K495	TVTVK(Ac)ISQK

Supplemental Table 1 Putative acetylation sites in ALDH1A1 (colored in red) identified by mass spectrometry studies.

Supplemental Table 2

Cells injected	Tumor weight (mg)		Paired t test
	KD+ALDH1A1	KD+ALDH1A1^{K353Q}	
5×10^5	33.08 ± 3.062	20.78 ± 3.190	**P=0.039** *
5×10^4	18.46 ± 2.093	13.03 ± 1.038	**P=0.029** *
5×10^3	15.26 ± 2.582	8.378 ± 1.872	**P=0.019** *

Supplemental Table 2 ALDH1A1^{K353Q} is defective in supporting tumor growth in vivo. Xenograft in mammary fat pads was performed using the different concentrations of MDA-MB-468 cells in which endogenous ALDH1A1 was knocked down and the wild-type or K353Q mutant ALDH1A1 was reintroduced. Seven weeks later, mice were sacrificed and tumor weight was measured, and shown are tumors weights derived from mice injected with 5×10^3, 5×10^4 and 5×10^{3}cells. P value was calculated by *t* test.