

Targeting a Super-Enhancer induced Aldehyde Dehydrogenase metabolic loop mitigates CDK4/6 Inhibitor Resistance in Estrogen Receptor-Positive Cancers

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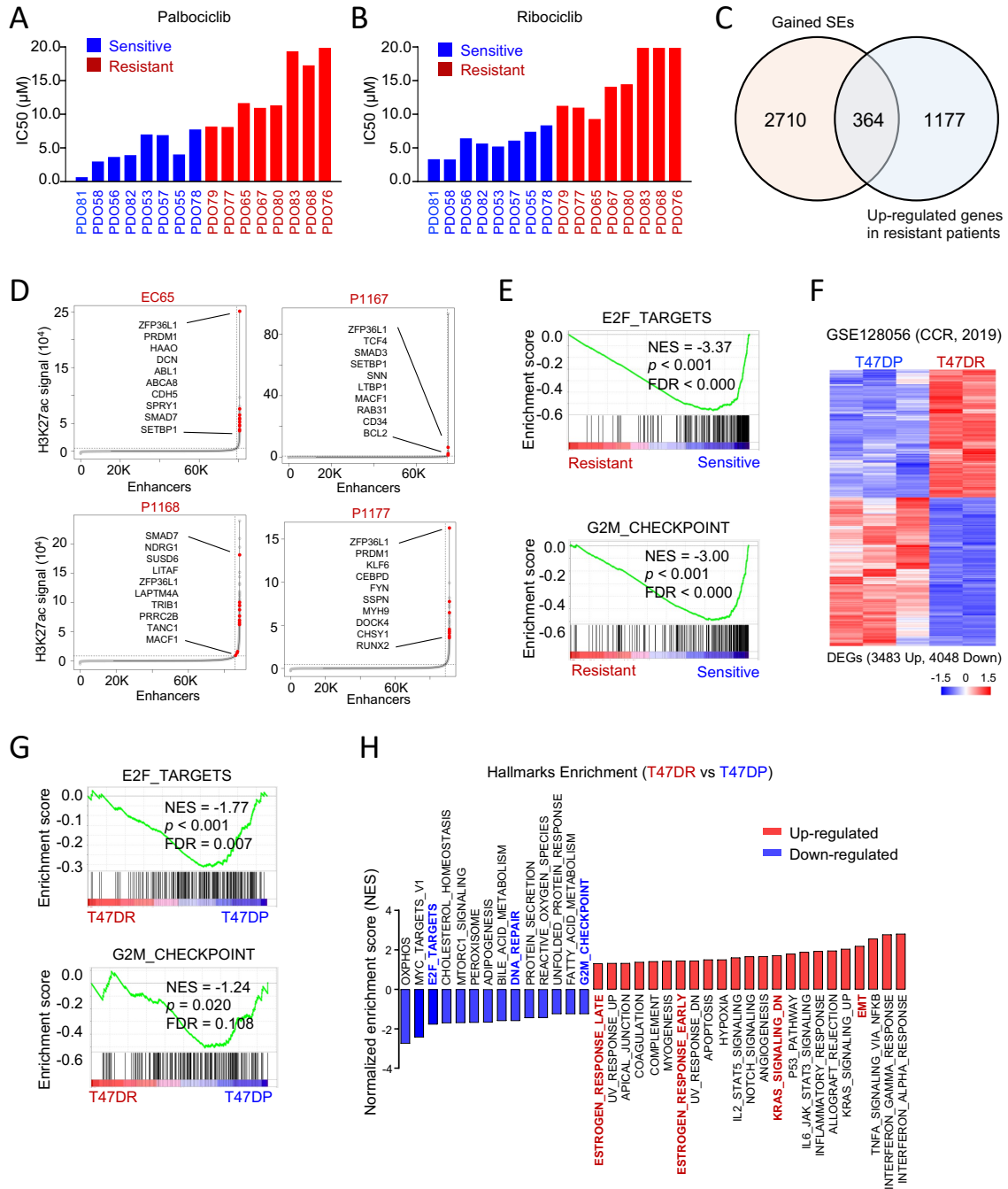
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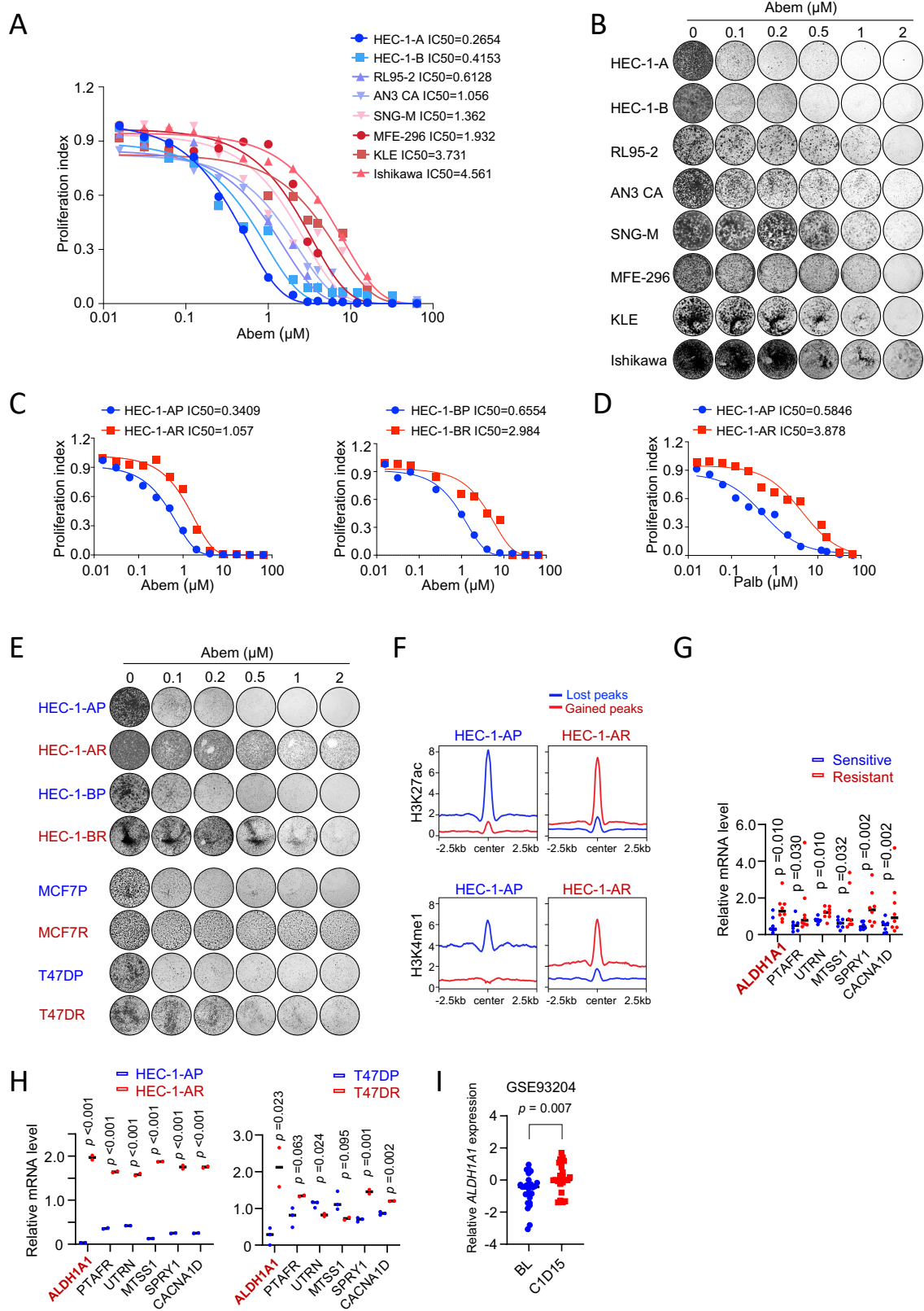
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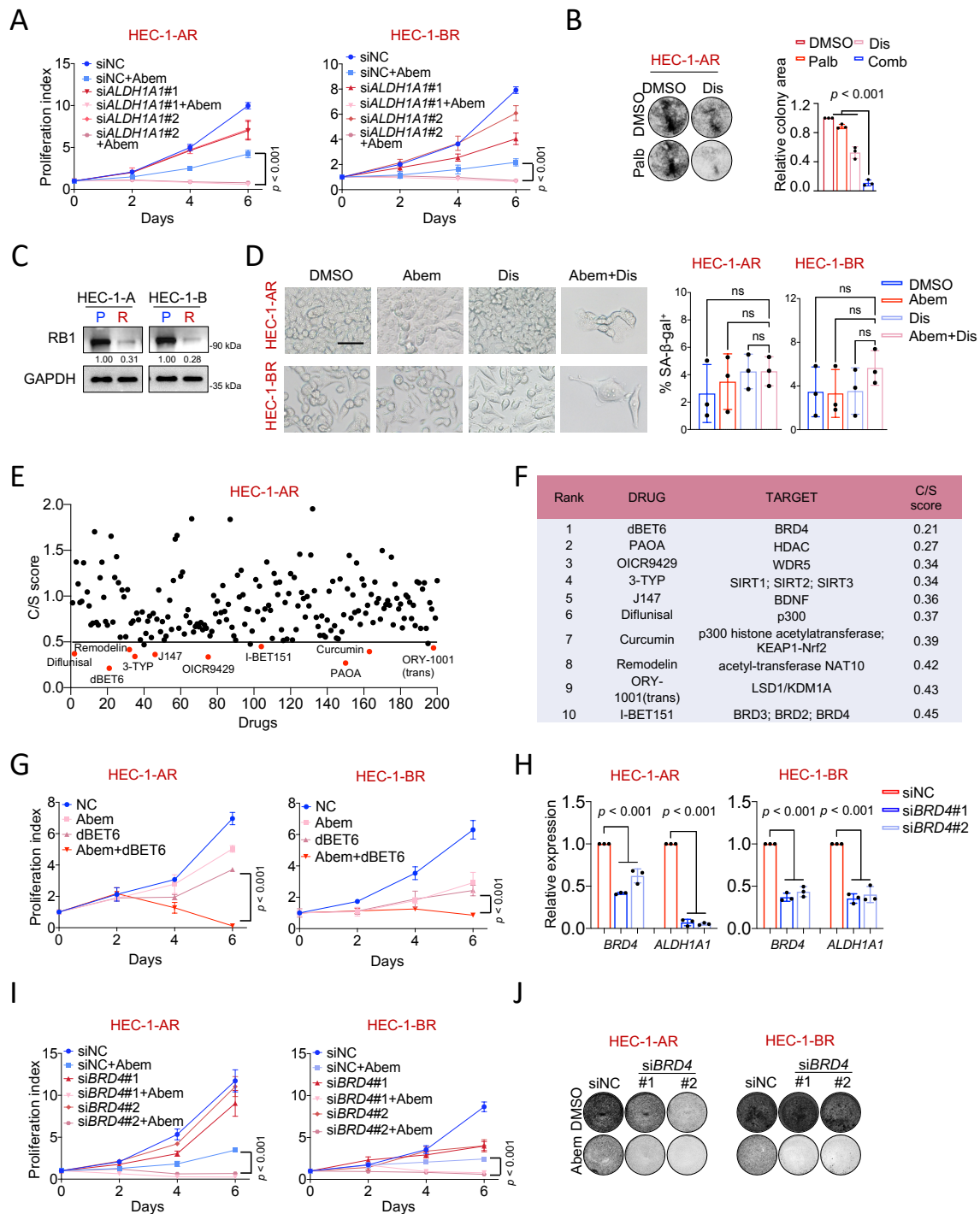


Supplementary Figure 1. The landscape of altered SEs associated with CDK4/6i resistance. (A-B) Sensitivity of 16 EC PDOs to Palbociclib and Ribociclib. Bar graph shows the IC50 values of Palbociclib (A) and Ribociclib (B) across a panel of 16 EC PDO lines. IC50 values were derived from dose-response curves. (C) Venn diagram shows the number and overlaps of SE related genes and up-regulated genes in patient samples. (D) Hockey-stick

plots display the rank order of H3K27ac ChIP-seq signals for SE-associated genes in resistant tumors. (E) GSEA pathway analysis in resistant EC tumors compared with sensitive EC tumors (Permutation test). (F) Heatmap shows differential expressed genes in RNA-seq between T47DP and T47DR from GEO database (GSE128056), of which 3483 up-regulated and 4048 down-regulated genes (Wald test, $p < 0.05$). (G) GSEA pathway analysis in T47DR compared with T47DP (Permutation test). (H) Pathway analysis using the Hallmark gene sets in T47DR versus T47DP.

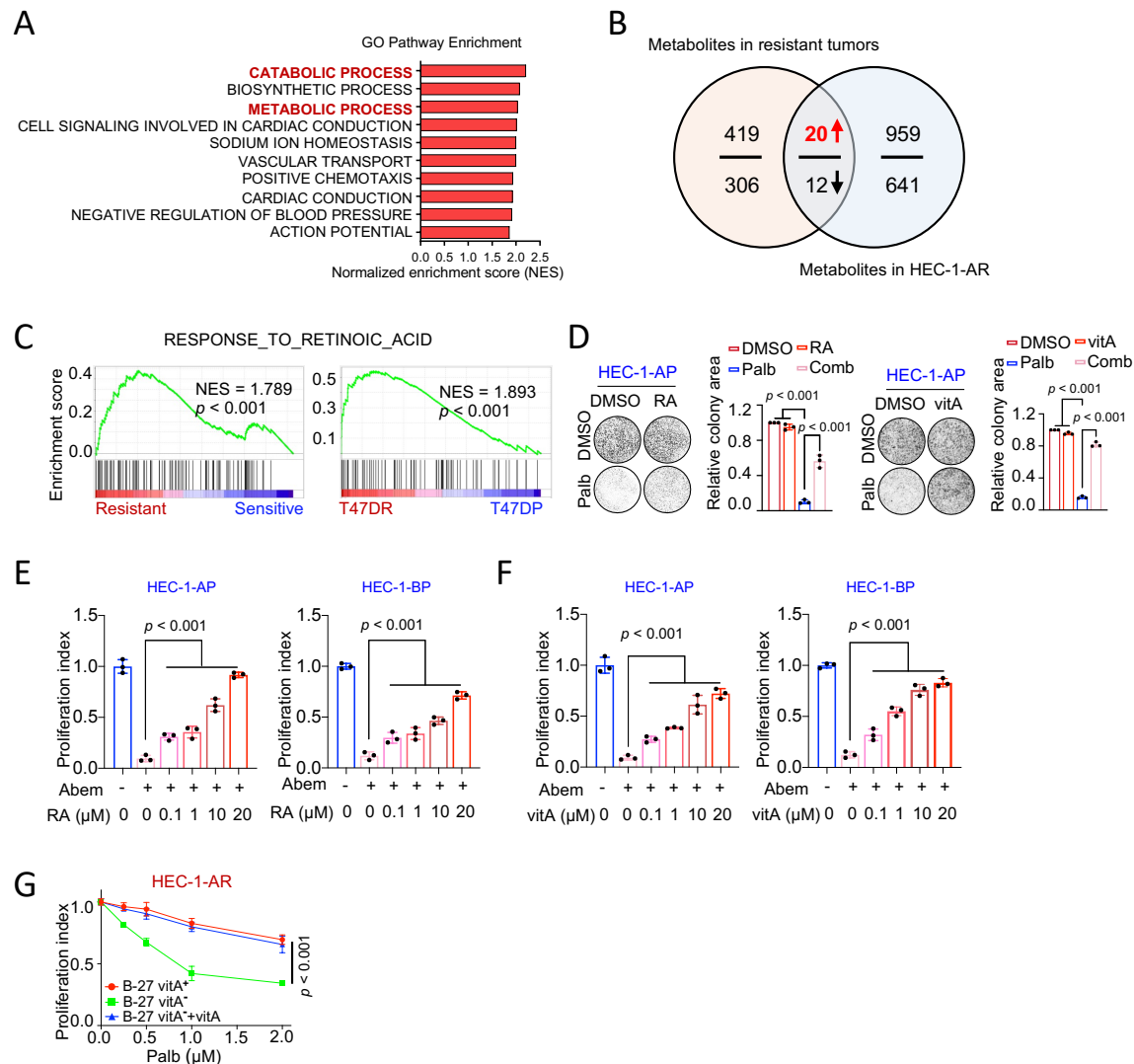


Supplementary Figure 2. *ALDH1A1* is a SE-associated gene in CDK4/6i resistance. (A) EC cell lines were treated with the indicated concentrations of Abemaciclib. The number of viable cells was measured at 96 hours. Data are represented the mean of three independent replicates. The IC50 values of Abemaciclib for EC cell lines after exposure durations of 96 hours are shown. (B) Colony formation assay of EC cell lines treated with the indicated concentrations of Abemaciclib. (C) Parental and resistant cells treated with the indicated concentrations of Abemaciclib. The number of viable cells was measured at 96 hours. Data are represented the mean of three independent replicates. The IC50 values of Abemaciclib for HEC-1-AP, HEC-1-AR, HEC-1-BP and HCE-A-BR after exposure durations of 96 hours are shown. (D) HEC-1-AP and HEC-1-AR cells treated with the indicated concentrations of Palbociclib. The number of viable cells was measured at 96 hours. Data are represented the mean of three independent replicates. IC50 values for HEC-1-AP and HEC-1-AR cells at exposure durations of 96 hours to Palbociclib are shown. (E) Colony formation assay of parental and resistant cells treated with the indicated concentrations of Abemaciclib. (F) Close-up view of the ChIP-seq profiles of H3K27ac and H3K4me1 at ± 2.5 kb of center between HEC-1-AP and HEC-1-AR cells. (G) Relative mRNA expression of the indicated SE-associated genes in resistant EC tumors compared with sensitive EC tumors from transcriptome data. (H) Relative mRNA expression of the indicated SE-associated genes in HEC-1-AP and HEC-1-AR cells, T47D-P and T47D-R cells from transcriptome data. (I) Expression of *ALDH1A1* in primary breast cancers of patients at baseline (BL) and 6 weeks after combination of anastrozole and palbociclib (C1D15) in the NeoPalAna trial. Means of relative microarray reads are shown. Figure G-I were analyzed by two-sided unpaired Student's t-test. All *p* values are shown in the figure.



Supplementary Figure 3. Inhibition of ALDH1A1 overcomes CDK4/6i resistance. (A) The growth curve of HEC-1-AR and HEC-1-BR treated with siNC, siALDH1A1, Abemaciclib (0.5 μ M), or combination. Data are presented as the mean $\pm$ SD of three independent replicates. (B) Representative images and quantification of colony formation assay of resistant cells treated with DMSO, Palbociclib (1 μ M), Disulfiram (1 μ M), or combination for 72 hours. Data are shown as mean $\pm$ SD of three independent experiments. (C) Immunoblotting of indicated

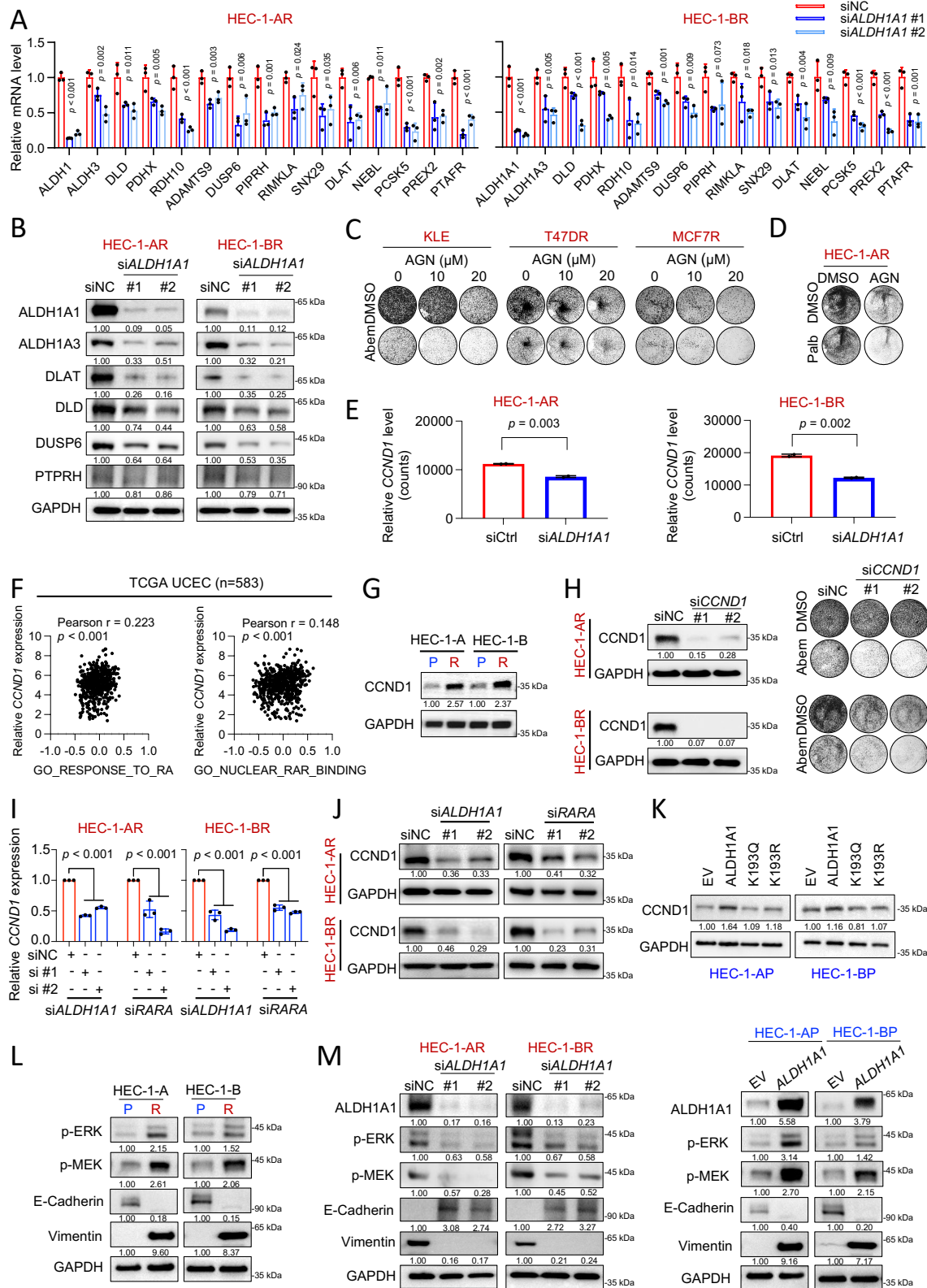
cells with RB1 and GAPDH. (D) Representative image and quantification of SA- β -gal positive cells in HEC-1-AR and HEC-1-BR cells treated with DMSO, Abemaciclib (0.5 μ M), Disulfiram (1 μ M for HEC-1-AR and 2 μ M for HEC-1-BR), or combination. Scale bar = 50 μ m. (E) Graph shows the results of a drug screening either singly or in combination with Abemaciclib. 199 drugs on-screen are ranked according to the C/S (combination/single) score. Top 10 sensitizers are highlighted in red. HEC-1-AR cells are seeded in 96-well plates and treated with 199 histone modification compounds (1.0 μ M) in the presence or absence of Abemaciclib (0.5 μ M). (F) The top 10 sensitizers and their targets. (G) The growth curve of cells treated with DMSO, Abemaciclib (0.5 μ M), dBET6 (0.5 μ M for HEC-1-AR and 2 μ M for HEC-1-BR), or combination. (H) RT-qPCR validation of *BRD4* or *ALDH1A1* in HEC-1-AR and HEC-1-BR cells transfected with control siRNA or *BRD4* siRNA. Data are shown as mean $\pm$ SD of three independent replicates. (I) The growth curve of HEC-1-AR and HEC-1-BR treated with siNC, si*BRD4*, Abemaciclib (0.5 μ M), or combination. Data are presented as the mean $\pm$ SD of three independent replicates. (J) Colony formation assay of cells infected with siNC, si*BRD4*, Abemaciclib (0.5 μ M), or combination. Figure A, G and I are analyzed with two-way ANOVA with Dunnett's multiple comparisons test. Figure B, D and H are analyzed with One-way ANOVA with Dunnett's multiple comparisons test. ns, not significant. All *p* values are shown in the figure. Source data of (A-D, G-J) are provided in Source Data file.



Supplementary Figure 4. ALDH1A1 promotes CDK4/6i resistance via vitA metabolism.

(A) Pathway enrichment analysis (GO) in resistant cells versus parental cells. (B) Venn diagram shows the number and overlaps of metabolites in resistance tissues and metabolites in HEC-1-AR cells. (C) GSEA pathway analysis shows that Vitamin A metabolic is enriched in both EC resistant tumors and T47D-R cells (Permutation test). (D) Representative images and quantification of colony formation assay of HEC-1-AP cells treated with DMSO, RA (1 μ M)/vitA (10 μ M), Palbociclib (0.5 μ M), or combination. Data are shown as mean $\pm$ SD of three independent experiments and analyzed by One-way ANOVA with Dunnett's multiple comparisons test. (E) HEC-1-AP and HEC-1-BP cells treated with combinations of RA (0 μ M, 0.1 μ M, 1 μ M, 10 μ M and 20 μ M) and Abemaciclib (0.5 μ M). The number of viable cells is measured at 96 hours. Data are shown as mean $\pm$ SD and analyzed by one-way ANOVA with Dunnett's multiple comparisons test. (F) HEC-1-AP and HEC-1-BP cells treated with combinations of vitA (0 μ M, 0.1 μ M, 1 μ M, 10 μ M and 20 μ M) and Abemaciclib (0.5 μ M). The

number of viable cells is measured at 96 hours. Data are shown as mean $\pm$ SD and analyzed by one-way ANOVA with Dunnett's multiple comparisons test. (G) Resistant cells are treated with the indicated concentrations of Palbociclib in B-27 supplement medium, vitA-deficient B-27 supplement medium or vitA-deficient B-27 supplement medium with vitA (10 μ M). The number of viable cells is measured at 96 hours. Data are analyzed by two-way ANOVA with Dunnett's multiple comparisons test. All *p* values are shown in the figure. Source data of (D-G) are provided in Source Data file.

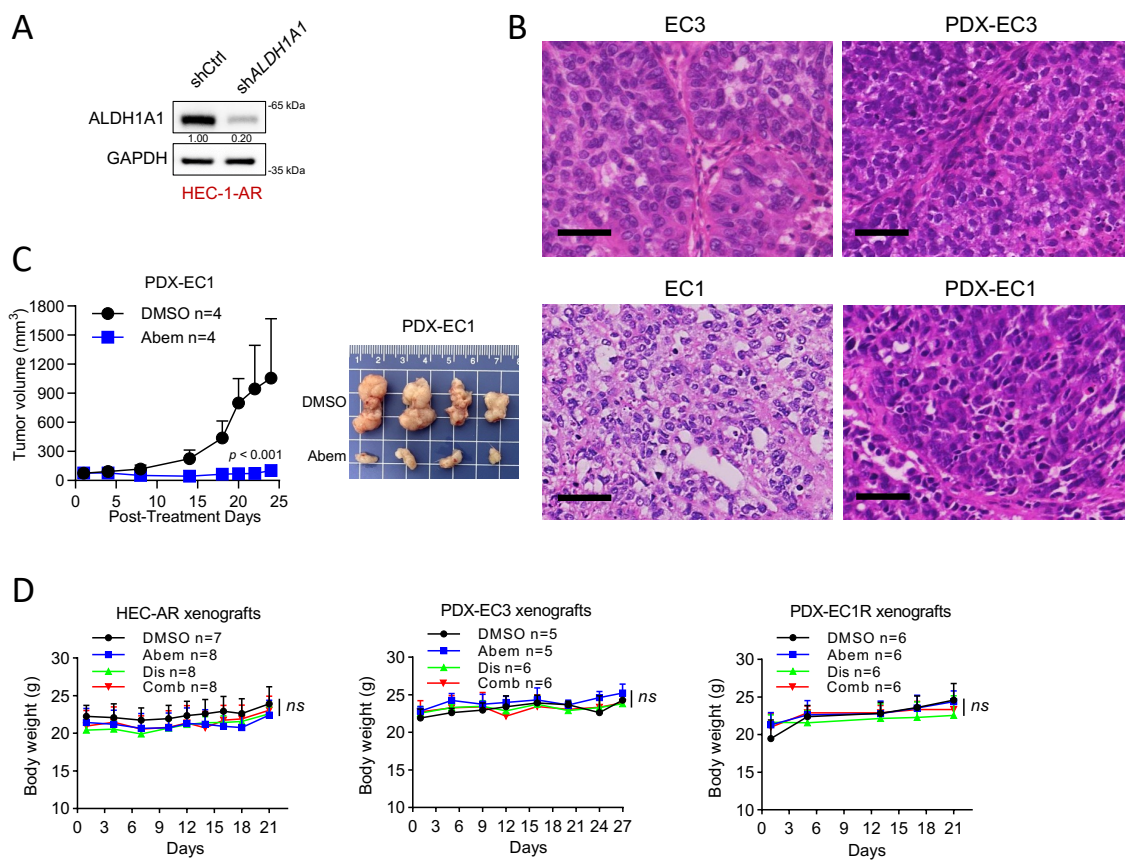


Supplementary Figure 5. RAR signaling is the downstream effector of ALDH1A1.

(A) RT-qPCR validation of the downstream genes of RAR signaling in resistant cells infected

with siNC or si*ALDH1A1*. Data are shown as mean $\pm$ SD and analyzed by one-way ANOVA with Dunnett's multiple comparisons test. (B) Immunoblotting of ALDH1A1, ALDH1A3, DLAT, DLD, DUSP6 and PTPRH in cells transfected with control siRNA or *ALDH1A1* siRNA. (C) Colony formation assay of resistant cells treated with DMSO, Abemaciclib (0.5 μ M), AGN (10 μ M and 20 μ M), or combination. (D) Colony formation assay of resistant cells treated with DMSO, Palbociclib (1 μ M), AGN (5 μ M), or combination. (E) Relative mRNA expression of *CCND1* in RNA-seq data and analyzed by two-sided unpaired Student's t-test. (F) Positive correlations (by Pearson's) between *CCND1* and NES values of RA and RAR signaling of EC from TCGA cohort (n = 583). (G) Immunoblotting of indicated cells with *CCND1* and GAPDH. (H) Immunoblotting of indicated cells with *CCND1* and GAPDH. (left). Colony formation assay of cells treated with siNC, si*CCND1*, Abemaciclib (0.5 μ M), or combination. (right). (I) RT-qPCR validation of *CCND1* in cells transfected with control siRNA or si*RARA*, si*ALDH1A1*. Data are shown as mean $\pm$ SD of three independent replicates and analyzed by one-way ANOVA with Dunnett's multiple comparisons test. (J) Immunoblotting of *CCND1* and GAPDH in HEC-1-AR and HEC-1-BR cells transfected with siNC, si*ALDH1A1* or si*RARA*. (K) Immunoblotting of *CCND1* and GAPDH in HEC-1-AP and HEC-1-BP cells infected with empty vehicle, ALDH1A1 or ALDH1A1 with a site mutation (K193Q/R). (L) Immunoblotting of p-ERK, p-MEK, E-cadherin and Vimentin in indicated cells. (M) Immunoblotting of p-ERK, p-MEK, E-cadherin and Vimentin in cells transfected with control siRNA or *ALDH1A1* siRNA (left), or in cells overexpressing empty vector (EV) or *ALDH1A1* (right). All *p* values are shown in the figure. Source data of (A-E, G-M) are provided in Source Data file.

TF family, Zinc- Finger proteins (Zf), nuclear receptors (NR) and T-box family (hypergeometric test). (B) Motif analysis shows significantly enriched motifs of RAR. *p* value is from HOMER based on binomial distribution (hypergeometric test). (C) GSEA pathway analysis shows ER signaling is enriched in resistant tumors compared with sensitive tumors, HEC-1-AR compared with HEC-1-AP and T47D-R compared with T47D-P (Permutation test). (D) Scatter diagrams show IC50 of Palbociclib in GDSC database between high and low activation of ER response. Data is analyzed by two pair t test. (E) Immunoblotting of RAR α and ALDH1A1 in cells infected with siNC and siRARA. (F) Immunoblotting of RAR α and ALDH1A1 in cells infected with siNC and siESR1. (G) Immunoblotting of ALDH1A1 expression in cells treated with DMSO or AGN for 72 hours. (H) Immunoblotting of ALDH1A1 in cells treated with DMSO or Fulvestrant (10 μ M or 20 μ M) for 72 hours. (I) RT-qPCR validation of *ALDH1A1* in cells treated with DMSO, RA (0.1 μ M, 1 μ M, 10 μ M and 20 μ M). Data are shown as mean $\pm$ SD. (J) RT-qPCR validation of *ALDH1A1* in cells treated with DMSO, vitA (0.1 μ M, 1 μ M, 10 μ M and 20 μ M). Data are shown as mean $\pm$ SD. (K) ChIP-qPCR analysis of H3K27ac, ER α and RAR α occupancy at genomic loci of *ALDH1A1* in cells overexpressing empty vector (EV) or ALDH1A1, or the latter treated with AGN (5 μ M) for 72 hours. (L) ChIP-qPCR analysis of H3K27ac, ER α and RAR α occupancy at genomic loci of *ALDH1A1* in cells expressing shCtrl or sh*ALDH1A1*, or the latter treated with RA (20 μ M) for 72 hours. One-way ANOVA with Dunnett's multiple comparisons test were used in Figure I-L. All *p* values are shown in the figure. Source data of (E-L) are provided in Source Data file.



Supplementary Figure 7. Targeting ALDH1A1 enhanced anti-tumor effects of Abemaciclib *in vivo*. (A) Immunoblotting of indicated cells with ALDH1A1 and GAPDH. (B) H&E staining comparison of patient primary tumors and PDXs, scale bar = 50 μ m. (C) The growth curve of tumor volume with DMSO or Abemaciclib in PDX-EC1 xenografts, error bars represent $\pm$ SD. (left). The image of tumors with indicated treatment in PDX-EC1 xenografts. (right). (D) The change of the body weight of the mice during the drug treatment. Two-way ANOVA with Dunnett's multiple comparisons test are used in Figure C and D. All *p* values are shown in the figure. ns, not significant. Source data of (A, C, D) are provided in Source Data file.

Table S1. Clinicopathologic features of study patients.

Characteristic	Sensitive	Resistant	<i>p</i>	All cases
Number of patients	8	8	-	16
Age at the time of surgery, yrs	55.50 ± 10.81	57.87 ± 10.56	0.663*	56.69 ± 10.40
2023 FIGO Stage, n (%)			0.426†	
Stage I	4 (25.00%)	7 (43.75%)		11(68.75%)
Stage II	1 (6.25%)	1 (6.25%)		2 (12.50%)
Stage III	2 (12.50%)	0 (0%)		2 (12.50%)
Stage IV	1 (6.25%)	0 (0%)		1 (6.25%)
Grade, n (%)			0.804†	
Grade 1	1 (6.25%)	3 (18.75%)		4(25.00%)
Grade 2	5 (31.25%)	3 (18.75%)		8 (50.00%)
Grade 3	2 (12.50%)	2 (12.50%)		4 (25.00%)
Histopathology, n (%)			1†	
Endometrioid carcinoma	7 (43.75%)	7 (43.75%)		14 (87.50%)
Others	1 (6.25%)	1 (6.25%)		2 (12.50%)
Estrogen receptor, ER, n (%)			-	
positive	8 (50.00%)	8 (50.00%)		16 (100%)
negative	0 (0%)	0 (0%)		0 (0%)
Progesterone receptor, PR, n (%)			-	
positive	8 (50.00%)	8 (50.00%)		16 (100%)
negative	0 (0%)	0 (0%)		0 (0%)

* Student's t-test (two-sided).

† Fisher's exact test (two-sided).

No adjustments were made for multiple comparisons.

Table S2. *KRAS* Mutation types in EC patients.

Patient	Response	Mutation type
UCEC1153	Sensitive	Wild-type
UCEC1155	Sensitive	<i>KRAS-G12C</i>
UCEC1156	Sensitive	Wild-type
UCEC1157	Sensitive	Wild-type
UCEC1158	Sensitive	Wild-type
UCEC1178	Sensitive	<i>KRAS-G12D</i>
UCEC1181	Sensitive	Wild-type
UCEC1182	Sensitive	Wild-type
UCEC1165	Resistant	Wild-type
UCEC1167	Resistant	<i>KRAS-G12V</i>
UCEC1168	Resistant	Wild-type
UCEC1176	Resistant	Wild-type
UCEC1177	Resistant	<i>KRAS-G12A</i>
UCEC1179	Resistant	Wild-type
UCEC1180	Resistant	Wild-type
UCEC1183	Resistant	Wild-type

Table S3. List of antibodies used in the study.

Antibodies used in western blot				
Cat. No	Antibody	Species	dilution	Company
15910-1-AP	ALDH1A1	Rabbit	1:5000	Proteintech
60004-1-Ig	GAPDH	Mouse	1:20000	Proteintech
9542S	PARP1	Rabbit	1:1000	CST
5625S	Cleaved-PARP1	Rabbit	1:1000	CST
14220S	Caspase 3	Rabbit	1:1000	CST
9664S	Cleaved- Caspase 3	Rabbit	1:1000	CST
ab32063	ER α	Rabbit	1:1000	abcam
ab275745	RAR α	Rabbit	1:1000	abcam
ab16663	CCND1	Rabbit	1:1000	CST
20874-1-AP	E-cadherin	Rabbit	1:1000	Proteintech
10366-1-AP	Vimentin	Rabbit	1:1000	Proteintech
28733-1-AP	p-ERK	Rabbit	1:1000	Proteintech
28930-1-AP	p-MEK	Rabbit	1:1000	Proteintech
25167-1-AP	ALDH1A3	Rabbit	1:2000	Proteintech
13426-1-AP	DLAT	Rabbit	1:5000	Proteintech
16431-1-AP	DLD	Rabbit	1:1000	Proteintech
83356-5-RR	DUSP6	Rabbit	1:1000	Proteintech
68635-1-Ig	PTPRH	Mouse	1:1000	Proteintech

Antibodies used in IP				
Cat. No	Antibody	Species	dilution	Company
ab275745	RAR α	Rabbit	1:100	abcam
ab32063	ER α	Rabbit	1:100	abcam
ab4729	H3K27ac	Rabbit	1:250	Abcam
13440S	BRD4	Rabbit	1:100	CST
2629S	Rpb1	Mouse	1:100	CST

Antibodies used in IF and IHC				
Cat. No	Antibody	Species	dilution	Company
15910-1-AP	ALDH1A1	Rabbit	1:5000	Proteintech

Table S4. List of quantitative real-time PCR primers and ChIP-qPCR primers.

qPCR sequence		
Target	Sense Primer (5'-3')	Antisense Primer (5'-3')
18S	AACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG
ALDH1A1	CCGTGGCGTACTATGGATGC	GCAGCAGACGATCTCTTTTCGAT
CCND1	GCTGCGAAGTGGAACCATC	CCTCCTTCTGCACACATTTGAA
RARA	AAGCCCGAGTGCTCTGAGA	TTCGTAGTGTATTTGCCCAGC
ESR1	CCCACTCAACAGCGTGTCTC	CGTCGATTATCTGAATTTGGCCT

ChIP-qPCR sequence		
Name	Sense Primer (5'-3')	Antisense Primer (5'-3')
ALDH1A1 P1	CCATGAAGCTGAGTAGTAAAGGT	GTTGGAGTGCAGTTATTCTCAAAG
ALDH1A1 P2	TGACAGGAAAGCAGTGTGAA	GAAACAAGAGCAGGAGCTAGA
ALDH1A1 P3	TTCCAACGTGGTGGGATTAC	GTAGCCAAGATAGTTTGAGGAGAG
ALDH1A1 P4	CGTGCCTGGGACGTATTATT	GCAGAGGACTTTGACATGGA