DLGAP1-AS2-Mediated Phosphatidic Acid Synthesis Confers Chemoresistance via Activation of YAP Signaling

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Supplementary figures

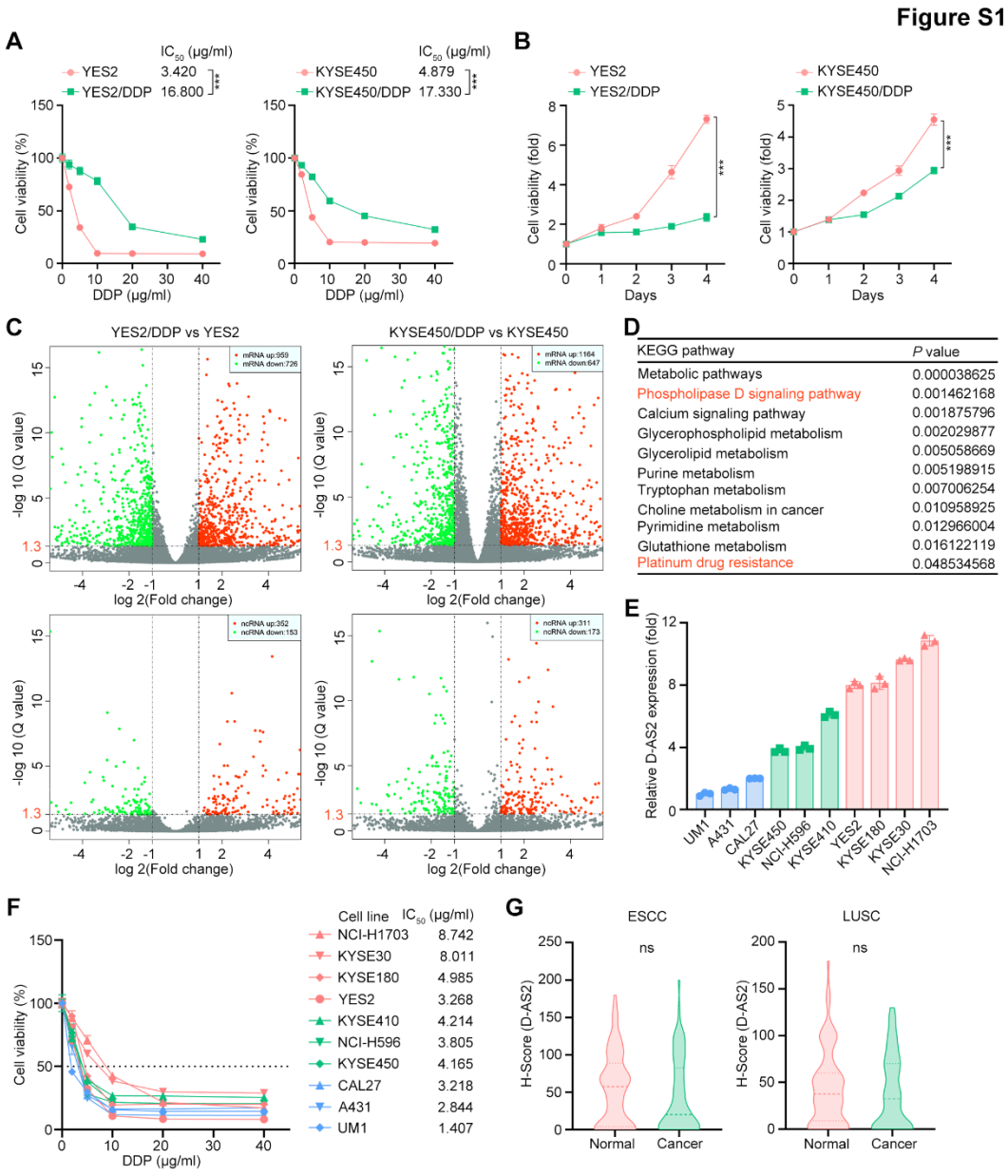

Figure S1. D-AS2 shows increased expression in chemoresistant SCC cells. **A,** Relative viability of parental and chemoresistant SCC cells after DDP treatment for 48 h. The data are presented as the mean ± s.d. values; two-way ANOVA, ***$P < 0.001$; n = 5 technical replicates. **B,** In vitro growth curves of parental and chemoresistant SCC cells. The data are presented as the mean ± s.d. values; two-tailed *t* test, ***$P < 0.001$; n = 5 technical replicates. **C,** Volcano plots showing upregulated and downregulated

mRNAs and ncRNAs in chemoresistant cells compared to the corresponding parental cells. **D,** KEGG enrichment analysis of the predicted target genes of the 38 ncRNAs upregulated and downregulated in both chemoresistant cell lines. **E,** RT–qPCR detection of D-AS2 expression in SCC cell lines. The data are presented as the mean ± s.d. values; n = 3 technical replicates. **F,** Relative cell viability and DDP IC_{50} values in SCC cell lines after DDP treatment for 48 h. The data are presented as the mean ± s.d. values; n = 5 technical replicates. **G,** Statistical analysis of D-AS2 expression in the two cohorts of esophageal and lung SCC tissues and adjacent normal tissues. The data are presented as the mean ± s.e.m. values; two-tailed *t* test, ns: not significant; n = 70 for esophageal SCC; n = 90 for lung SCC.

Figure S2

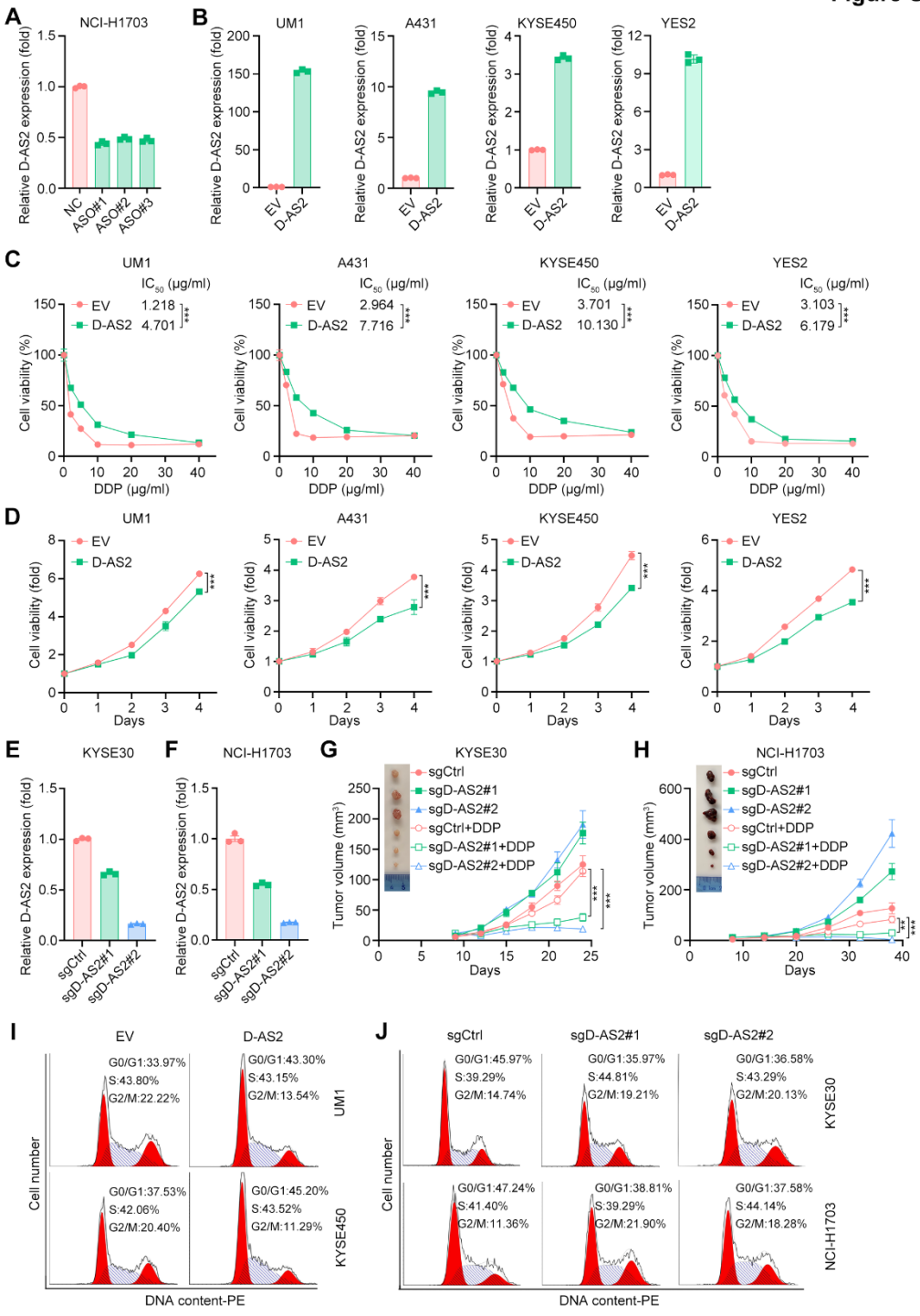

Figure S2. D-AS2 suppresses SCC cell proliferation and leads to chemoresistance. **A,** RT–qPCR detection of D-AS2 expression in NCI-H1703 cells transfected with negative control (NC) or D-AS2 ASOs (ASO#1, ASO#2, and ASO#3). The data are presented as the mean ± s.d. values; n = 3 technical replicates. **B,** RT–qPCR detection of D-AS2

expression in SCC cells expressing empty vector (EV) or D-AS2. The data are presented as the mean ± s.d. values; n = 3 technical replicates. **C,** Relative viability of SCC cells expressing EV or D-AS2 after DDP treatment for 48 h. The data are presented as the mean ± s.d. values; two-way ANOVA, ***$P < 0.001$; n = 5 technical replicates. **D,** In vitro growth curve of SCC cells expressing EV or D-AS2. The data are presented as the mean ± s.d. values; two-tailed *t* test, ***$P < 0.001$; n = 5 technical replicates. **E** and **F,** RT–qPCR detection of D-AS2 expression in KYSE30 and NCI-H1703 cells expressing control or D-AS2-targeting sgRNAs. The data are presented as the mean ± s.d. values; n = 3 technical replicates. **G** and **H,** Representative images and volumes of xenografts derived from KYSE30 and NCI-H1703 cells expressing control or D-AS2-targeting sgRNAs. The data are presented as the mean ± s.e.m. values; two-tailed *t* test, ***$P < 0.001$, **$P < 0.01$; n = 8 biological replicates. **I** and **J,** Representative images of the cell cycle analyses of the indicated cells.

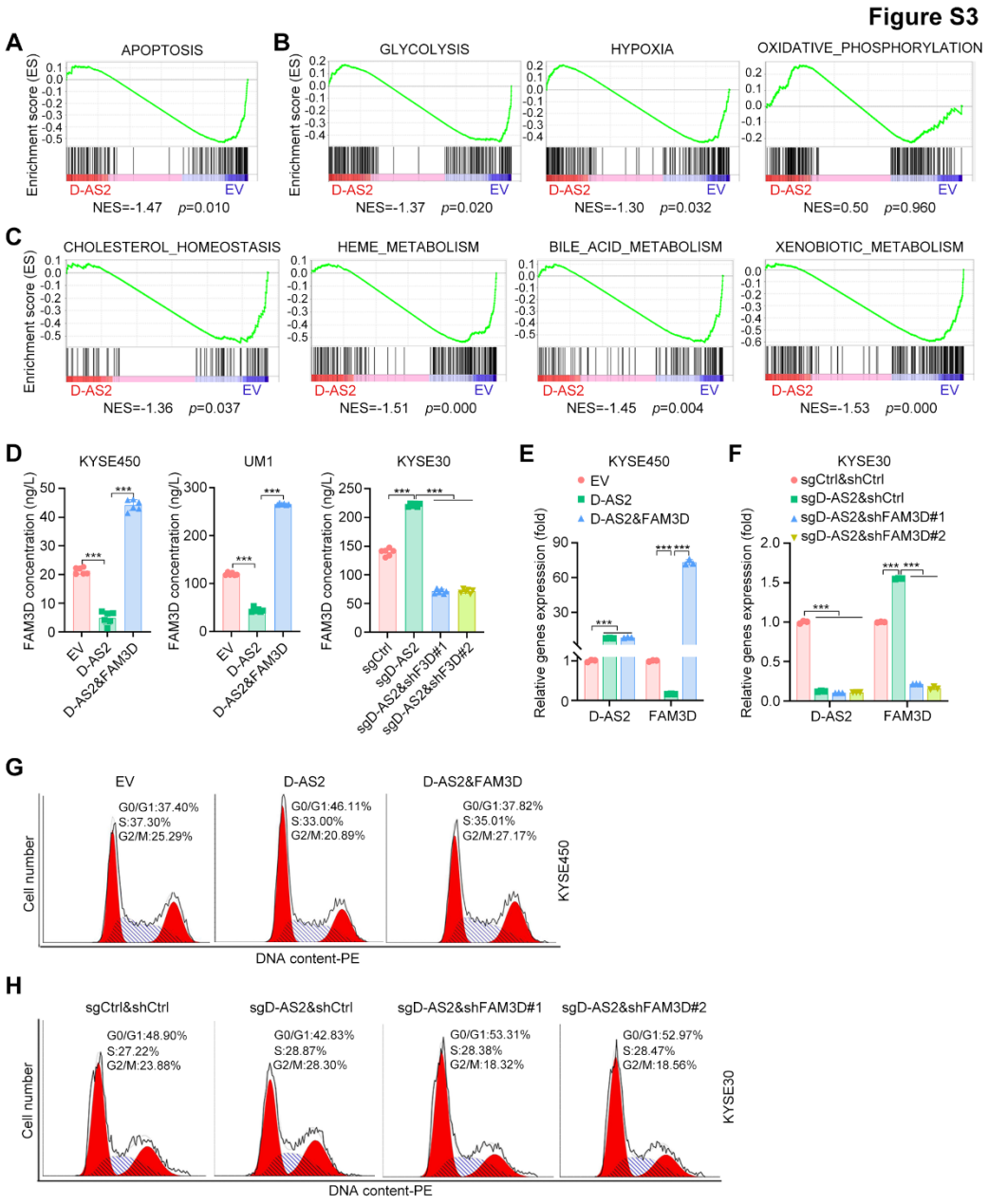

Figure S3. FAM3D mediates the function of D-AS2. **A-C,** GSEA of differentially expressed mRNAs in D-AS2-overexpressing KYSE450 cells compared to control cells. **D,** ELISA of the FAM3D concentration in the indicated cells. The data are presented as the mean ± s.d. values; two-tailed *t* test, ***$P < 0.001$; n = 6 technical replicates. **E** and **F,** RT–qPCR detection of D-AS2 and FAM3D expression in the indicated cells. The data are presented as the mean ± s.d. values; two-tailed *t* test, ***$P < 0.001$; n = 3

technical replicates. **G** and **H,** Representative images of the cell cycle analyses of the indicated cells.

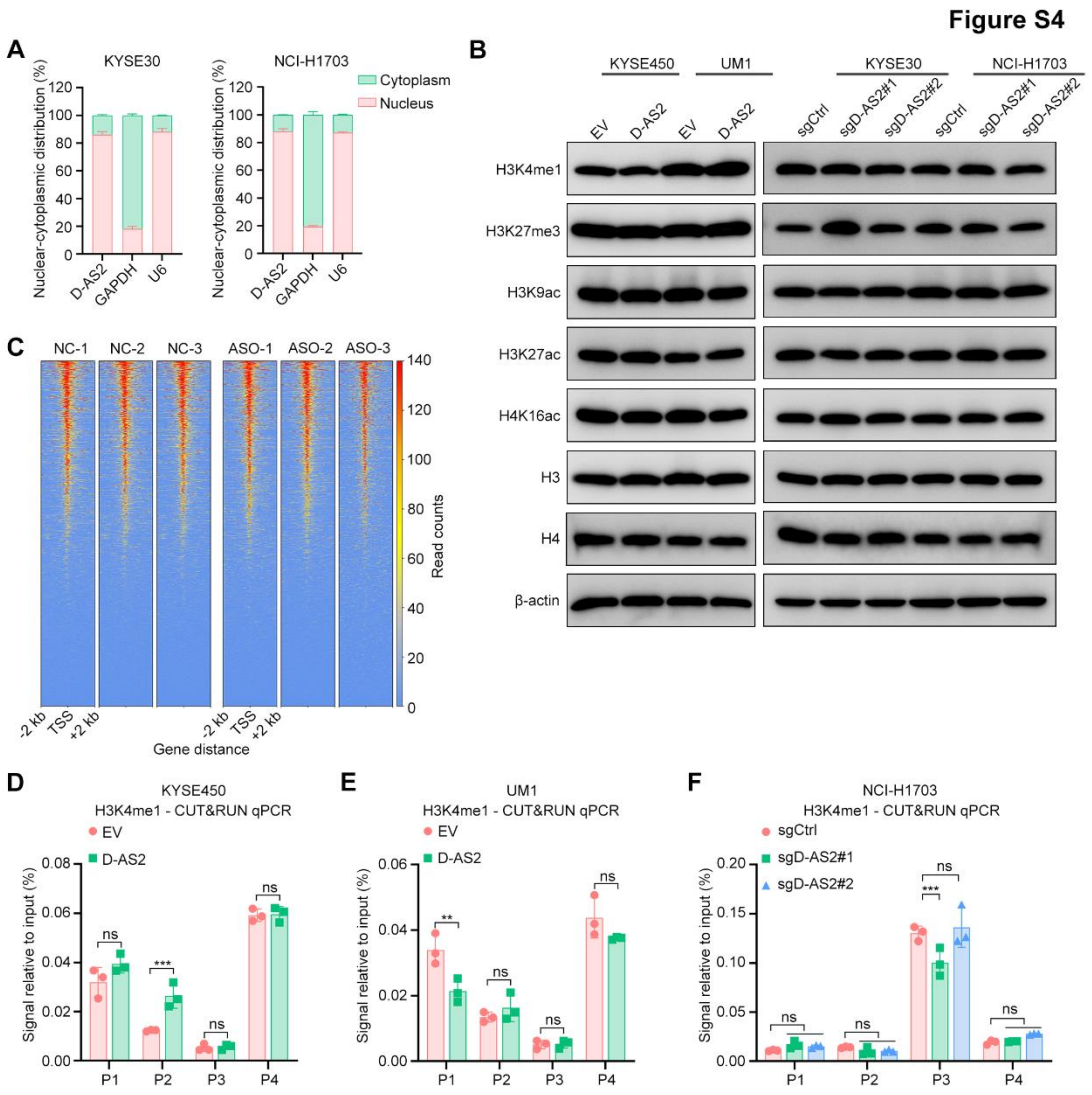

Figure S4. D-AS2 primarily regulates gene expression via non-promoter regulatory elements. **A,** RT–qPCR detection of D-AS2 expression in the cytoplasmic and nuclear fractions. **B,** Western blot analyses of histone marks in D-AS2-depleted and D-AS2-overexpressing SCC cells. Representative results of at least three biological replicates are shown. **C,** Heatmaps showing ATAC signals within the region ±2 kb around the TSS. **D-F,** CUT&RUN assay of the FAM3D enhancer region in D-AS2-depleted and D-AS2-overexpressing SCC cells with antibodies against H3K4me1. The data are presented as the mean ± s.d. values; two-tailed *t* test, ***$P < 0.001$, **$P < 0.01$, ns: not significant; n = 3 technical replicates.

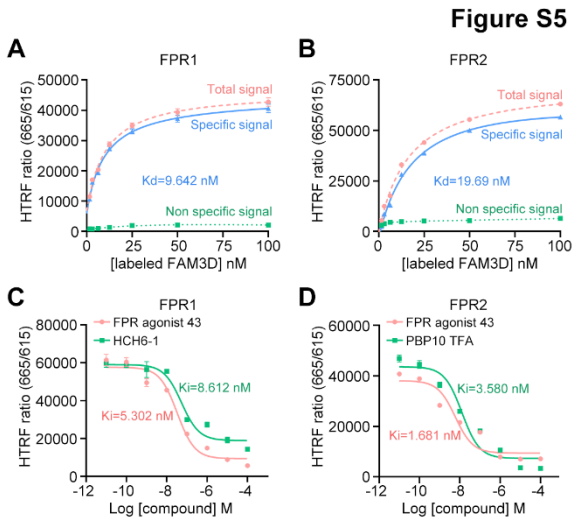

Figure S5. D-AS2 activates PLD through FAM3D-mediated FPR1 and FPR2 signaling. **A** and **B,** Saturation curve of FAM3D binding to FPR1 and FPR2. 293T cells transiently expressing SNAP-tagged FPR1 or FPR2 were incubated with increasing concentrations of labeled FAM3D. A prominent homogeneous time-resolved fluorescence (HTRF) signal is visible. Nonspecific binding was measured by adding 10 µM FPR agonist 43 to the wells. The dissociation constants (K_d values) are shown. The data are presented as the mean ± s.d. values; n = 3 technical replicates. **C** and **D,** Competitive binding curve for FAM3D to FPR1 and FPR2. 293T cells transiently expressing SNAP -tagged FPR1 or FPR2 were incubated with increasing concentrations of the indicated competitors. The concentration of labeled FAM3D used for FPR1 was 8 nM and that used for FPR2 was 16 nM. The inhibitory constants (K_i values) are shown. The data are presented as the mean ± s.d. values; n = 3 technical replicates.

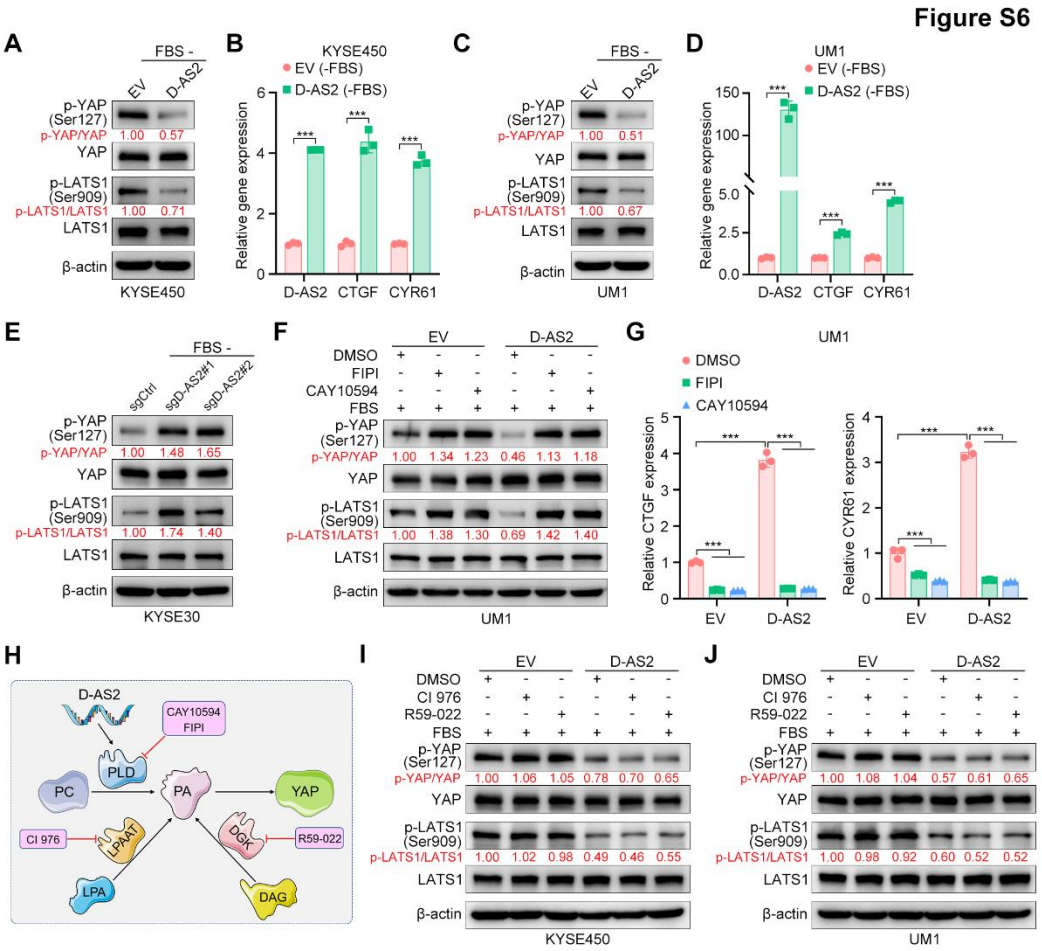

Figure S6. D-AS2 activates YAP signaling through PLD/PA. **A,** Western blot analyses of phosphorylated YAP and LATS1 in serum-starved control or D-AS2-overexpressing KYSE450 cells. Representative results of at least three biological replicates are shown. **B,** RT–qPCR detection of the YAP target genes CTGF and CYR61 in serum-starved control or D-AS2-overexpressing KYSE450 cells. The data are presented as the mean ± s.d. values; two-tailed *t* test, ***$P < 0.001$; n = 3 technical replicates. **C,** Western blot analyses of phosphorylated YAP and LATS1 in serum-starved control or D-AS2-overexpressing UM1 cells. Representative results of at least three biological replicates are shown. **D,** RT–qPCR detection of the YAP target genes CTGF and CYR61 in serum-starved control or D-AS2-overexpressing UM1 cells. The data are presented as the

mean ± s.d. values; two-tailed *t* test, ***$P < 0.001$; n = 3 technical replicates. **E,** Western blot analyses of phosphorylated YAP and LATS1 in serum-starved control or D-AS2-depleted KYSE30 cells. Representative results of at least three biological replicates are shown. **F,** Western blot analyses of YAP and LATS1 phosphorylation. UM1 cells expressing EV or D-AS2 were treated with FIPI (30 μM) or CAY10594 (20 μM) for 1 h. Representative results of at least three biological replicates are shown. **G,** RT–qPCR detection of the YAP target genes CTGF and CYR61. UM1 cells expressing EV or D-AS2 were treated with FIPI (30 μM) or CAY10594 (20 μM) for 1 h. The data are presented as the mean ± s.d. values; two-tailed *t* test, ***$P < 0.001$; n = 3 technical replicates. **H,** Schematic illustration of the three metabolic pathways for PA production. The inhibitors targeting each pathway or enzyme are indicated. **I** and **J,** Western blot analyses of phosphorylated YAP and LATS1 in control or D-AS2-overexpressing KYSE450 and UM1 cells. Cells were pretreated with CI 976 (20 μM) or R59-022 (20 μM) for 30 min. Representative results of at least three biological replicates are shown.

Supplementary tables

Table S4. D-AS2 probe sequences used for ISH.

Sequence (5’ to 3’)	Name
AATGGTATTTCCATTTATATAAGAAGCGCCTAAGAAATGC	Probe#1
AATTTGATGCCAACTTTATGTGTAAAGAAGCTAACTCCTG	Probe#2
AAGAAGAAACTGAATTTGAAGTGGATTCTTACAAAGGAAA	Probe#3

Table S5. RT–qPCR primers.

Sequence (5’ to 3’)	Name
CTCGCTTCGGCAGCACA	U6-F
AACGCTTCACGAATTTGCGT	U6-R
CCGGGAAACTGTGGCGTGATGG	GAPDH-F
AGGTGGAGGAGTGGGTGTCGCTGTT	GAPDH-R
GCGCCTAAGAAATGCCTGT	DLGAP1-AS2-F
AGCTGTTCATTCAGCCACGA	DLGAP1-AS2-R
CTGCCCAGCCAACTACTTTG	FAM3D-F
CTCCCGTGGTTCCATTCAC	FAM3D-R
CCAATGACAACGCCTCCTG	CTGF-F
TGGTGCAGCCAGAAAGCTC	CTGF-R
AGCCTCGCATCCTATACAACC	CYR61-F
TTCTTTCACAAGGCGGCACTC	CYR61-R

Table S6. shRNA and sgRNA sequences.

Sequence (5’ to 3’)	Name
GCACCTAGTGAAATTCCTTAA	shFAM3D#1
GCCCAGACACAAACAAATACG	shFAM3D#2
GCACGCTCTCTGACAGCATC	sgDLGAP1-AS2#1
GAACGTCACAGGCATTTCTT	sgDLGAP1-AS2#2

Table S7. CUT&RUN qPCR primers targeting the FAM3D enhancer region.

Sequence (5’ to 3’)	Name
TGGAAGGGATGTTGGGCAGTGA	Primer1-F
TAGCGAGAGGCAGCAGAGTGAA	Primer1-R
ACCTCCAGGGCACGATTCCTATAC	Primer2-F
CAGGCAACAGGAAGTGCTGACAT	Primer2-R
ACCCTGGTGGAGAAAGAAGTGACTAT	Primer3-F
CTGACTGTGAAGGCTGACAAGGTG	Primer3-R
ACCTTGTCAGCCTTCACAGTCAG	Primer4-F
GAAACCCACTTCCTTCCTTCCAACA	Primer4-R

Table S8. Fold changes in gene expression in D-AS2-overexpressing and chemoresistant cell models.

Gene symbol	D-AS2 vs EV		YES2/DDP vs YES2		KYSE450/DDP vs KYSE450	
	Log_2FC	padj	Log_2FC	padj	Log_2FC	padj
FAM3D	-1.9275	3.16E-23	-4.3598	0.007825	-3.3364	1.48E-10
ATP13A4	-2.0415	2.47E-37	-9.4870	5.10E-08	-1.6382	9.57E-06
LY6D	-1.3867	2.74E-13	-1.7933	0.002695	-5.6973	0.031811
CSTB	-1.2903	1.06E-24	-1.1249	3.73E-05	-1.1374	0.000112
PRMT8	-1.3118	1.57E-12	-5.0803	0.010046	-7.3156	0.009199
CALML5	-2.8248	8.61E-16	-5.0536	0.001981	-2.5444	5.27E-05

Table S9. Lists of D-AS2-interacting histones identified using MS in KYSE30 and NCI-H1703 cells.

KYSE30#1	KYSE30#2	NCI-H1703
Histone H1.3	Histone H1.5	Histone H1.3
Histone H1.5	Core histone macro-H2A.1	Core histone macro-H2A.1
Histone H2A type 1	Histone H2A.V	
Histone H2B	Histone H3	
Histone H4		