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Supplemental information

Modification of lysine-260 2-hydroxyisobutyrylation destabilizes ALDH1A1 expression to regulate bladder cancer progression

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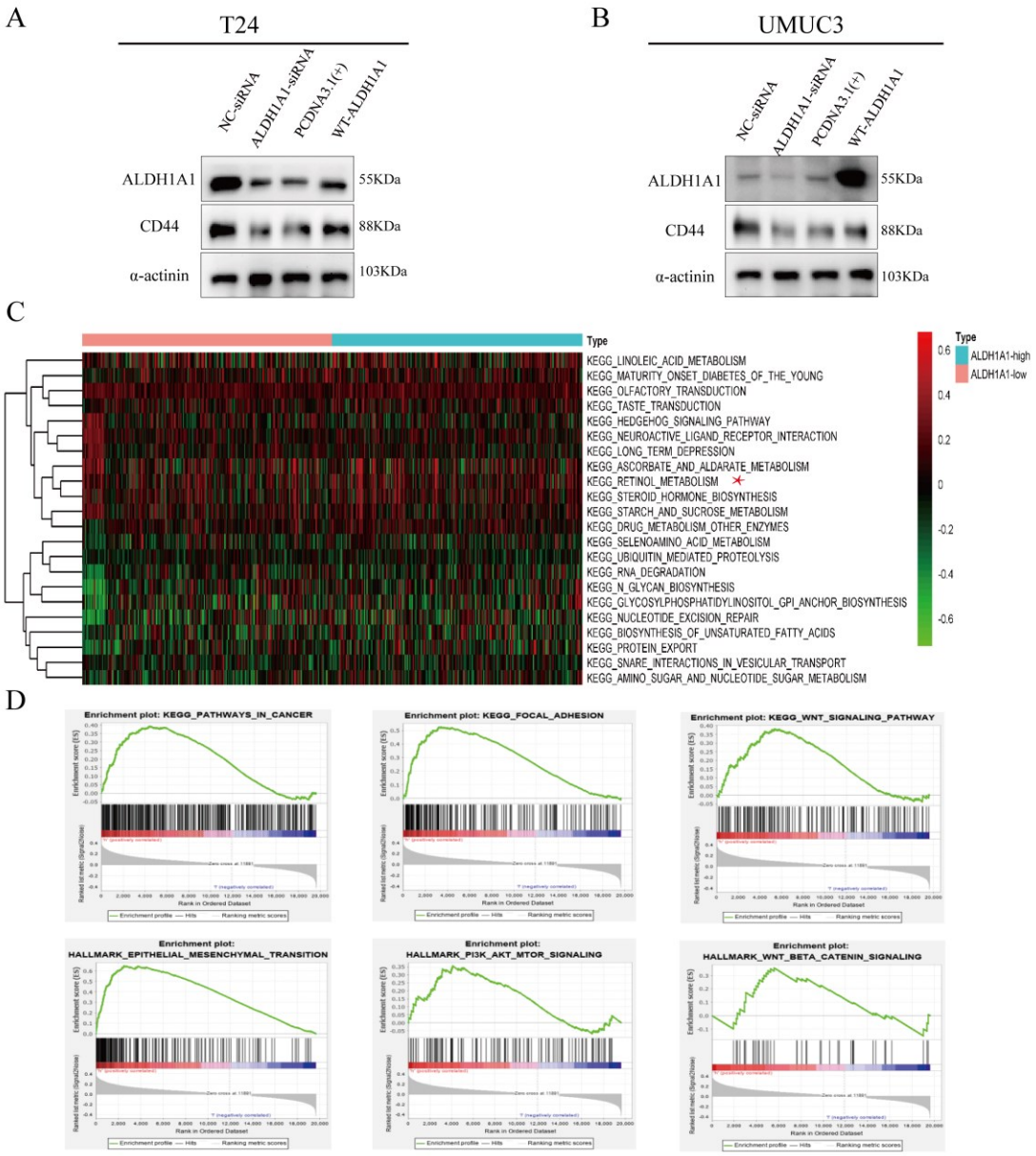

Figure S1. The signaling pathways of ALDH1A1 in TCGA-BLCA database, related with Figure 1 and Figure 2. **(A,B)** The expression of CD44 was analyzed by the different treatments of ALDH1A1 in T24 and UMUC3 cells. **(C)** GSVA enrichment with different ALDH1A1 expression. **(D)** GSEA analysis to identify the relevant signal pathways with ALDH1A1. For cell experiments, each experiment was performed at least three times.

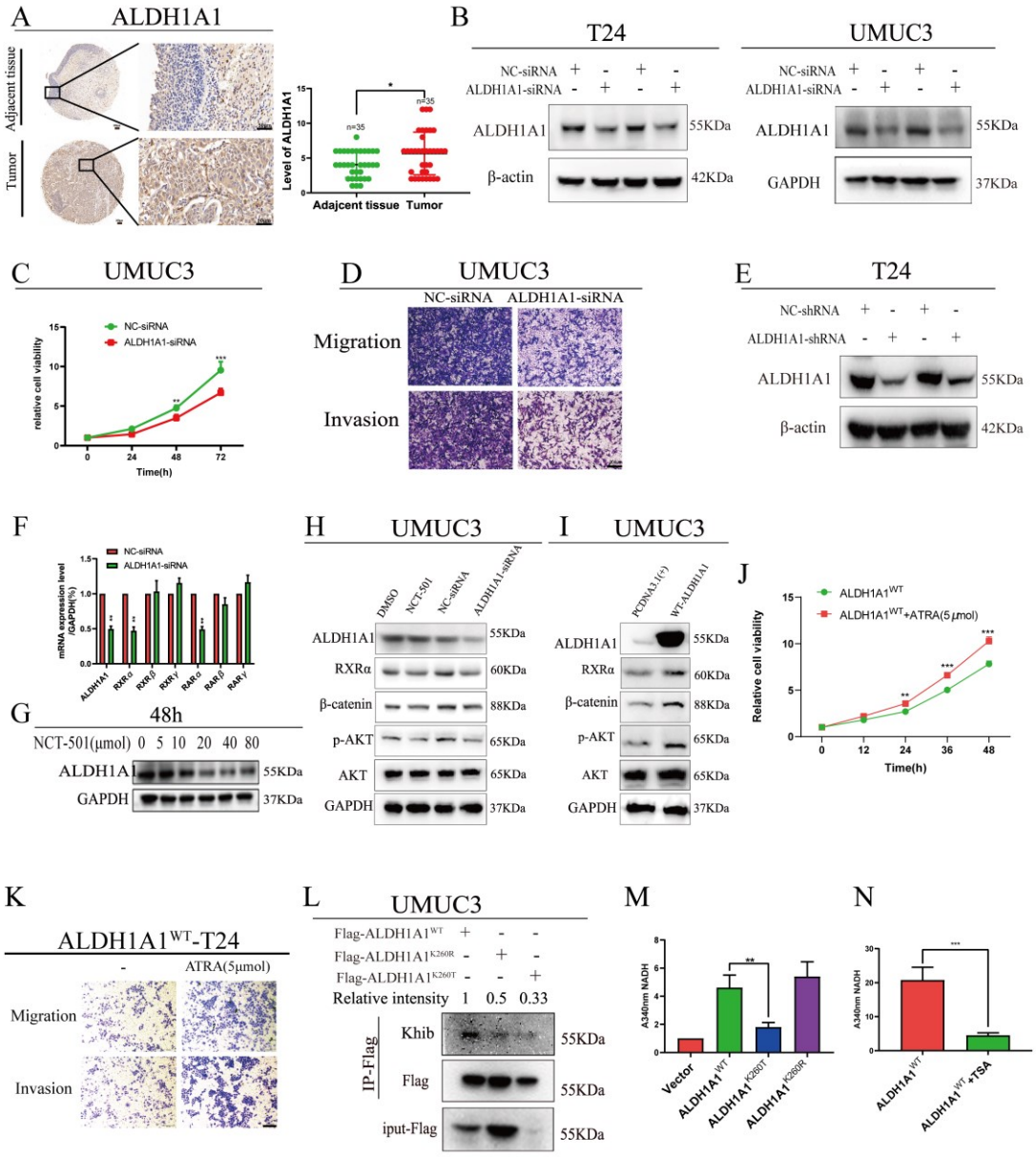

Figure S2. ALDH1A1 promotes tumor progression through RA pathway in bladder cancer, related with Figure 2 and Figure 3. (A) The expression levels of ALDH1A1 in bladder cancer tissue and adjacent tissue was evaluated by IHC analysis (n=35 per group). Left: scale bars 200μm. Right: scale bars 50μm. **(B)** The protein expression of ALDH1A1 was efficiently knocked down by ALDH1A1-siRNA in T24 and UMU3 cell lines. **(C)** The cell proliferation and growth measured by the method of MTT in UMUC3 cells. **(D)** The migration and cell invasive ability was reduced in UMUC3 cell line by knocking down ALDH1A1. Scale bars 200μm. **(E)** T24 cells were infected with NC-shRNA and ALDH1A1-shRNA lentivirus and then were subjected to Western blot analysis of ALDH1A1. **(F)** Identification of genes downregulated by ALDH1A1 knockdown in T24 cells. **(G)** T24 cells were treated with indicated different concentration of NCT-501 and then were subjected to western blot analysis of ALDH1A1. **(H,I)** Western blotting analysis of ALDH1A1,RXRα, p-AKT and β-catenin in UMUC3 cell lines with different treatments. **(J)** The cell proliferation and growth measured by the method of MTT in ALDH1A1^{WT} cells. **(K)** The migration and cell invasive ability of 3 × 10^4 cells/200 μL ALDH1A1^{WT} cells was increased after

treatment with ARTA (5μmol). Scale bars 200μm.**(L)** K260 mutation decreased the 2-hydroxyisobutyrylation levels of ALDH1A1 in UMUC3 cell line. **(M)** Flag-tagged ALDH1A1 ^{WT}, ALDH1A1^{K260T} and ALDH1A1^{K260R} mutants were individually transfected into the 293T cells, and the dehydrogenase activity assay of ALDH1A1 was conducted with the NADH as the substrate, and changes of the absorbance at 340 nm were measured to show the decrease of NADH. **(N)** Flag-tagged ALDH1A1^{WT} was transfected into HEK293T cells, which were treated with or without 4 μM TSA for 6 h, and the activity of ALDH1A1 was measured. For cell experiments, each experiment was performed at least three times. Data are represented as mean +/- SEM.* $p<0.05$, **$p < 0.01$, ***$p < 0.001$ by two-sided Student's t-test or one-way ANOVA.

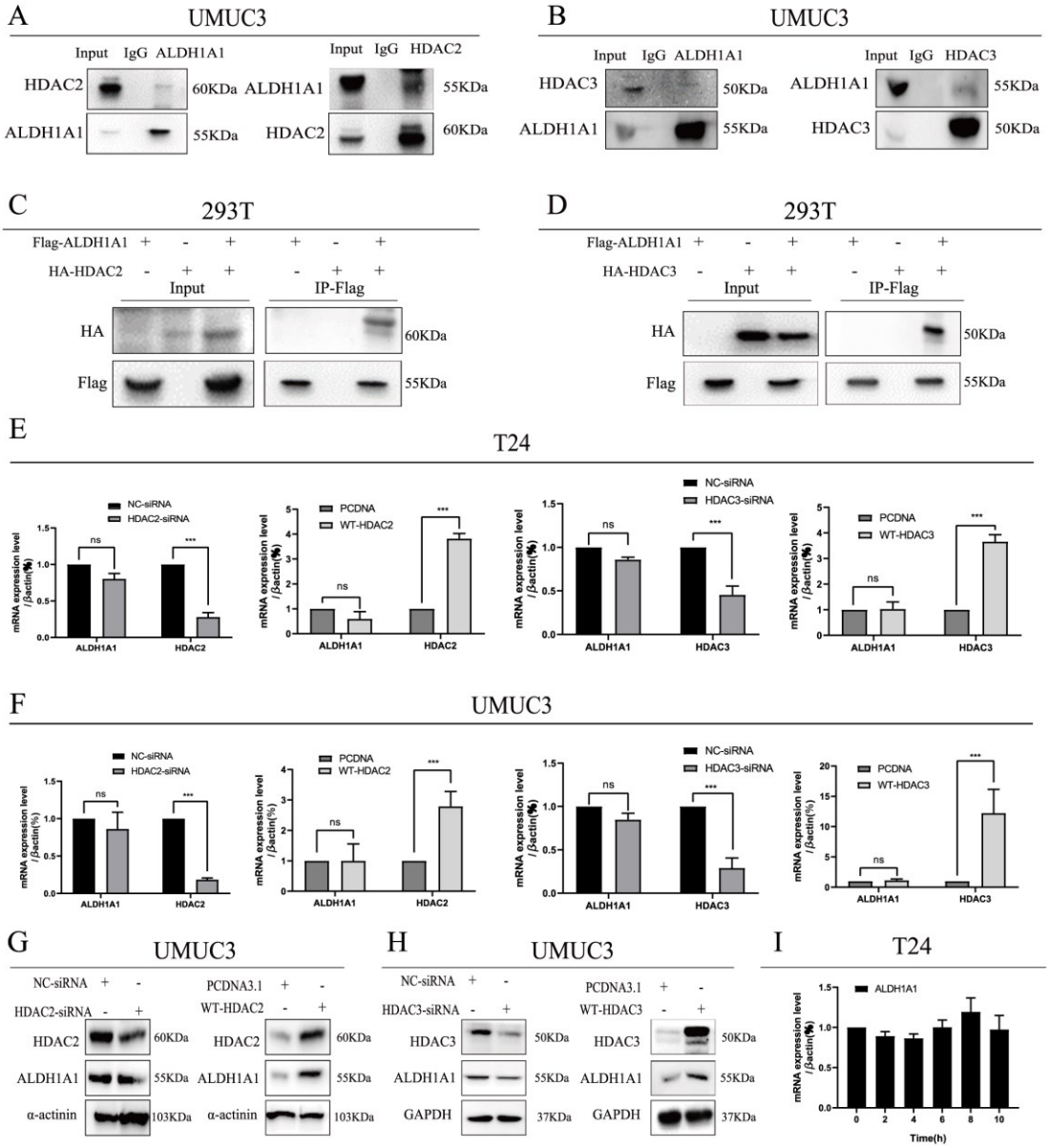

Figure S3. HDAC2/3 could regulate the expression of ALDH1A1, related with Figure 4. (A,B) Endogenous ALDH1A1 was IPed from UMUC3 cells. Rabbit IgG was used as a control. **(C,D)** Flag-tagged ALDH1A1 and HA-tagged HDAC2/3 were transfected individually or together into HEK293T cells followed by immunoprecipitation. **(E,F)** RT-qPCR was performed to analyze the expression of ALDH1A1 in T24 and UMUC3 cells by knocking down and overexpressing HDAC2/3 respectively. **(G,H)** Western blot analysis of HDAC2/3 and ALDH1A1 in HDAC2/3 overexpressed and knocking down in UMUC3 cells. **(I)** The mRNA expression of ALDH1A1 was measured by RT-qPCR in T24 cells after treatment with TSA at indicated time. Independent experiments were performed in triplicate. For cell experiments, each experiment was performed at least three times. Data are represented as mean +/- SEM.***$p < 0.001$ by two-sided Student's t-test or one-way ANOVA, ns: not significant.

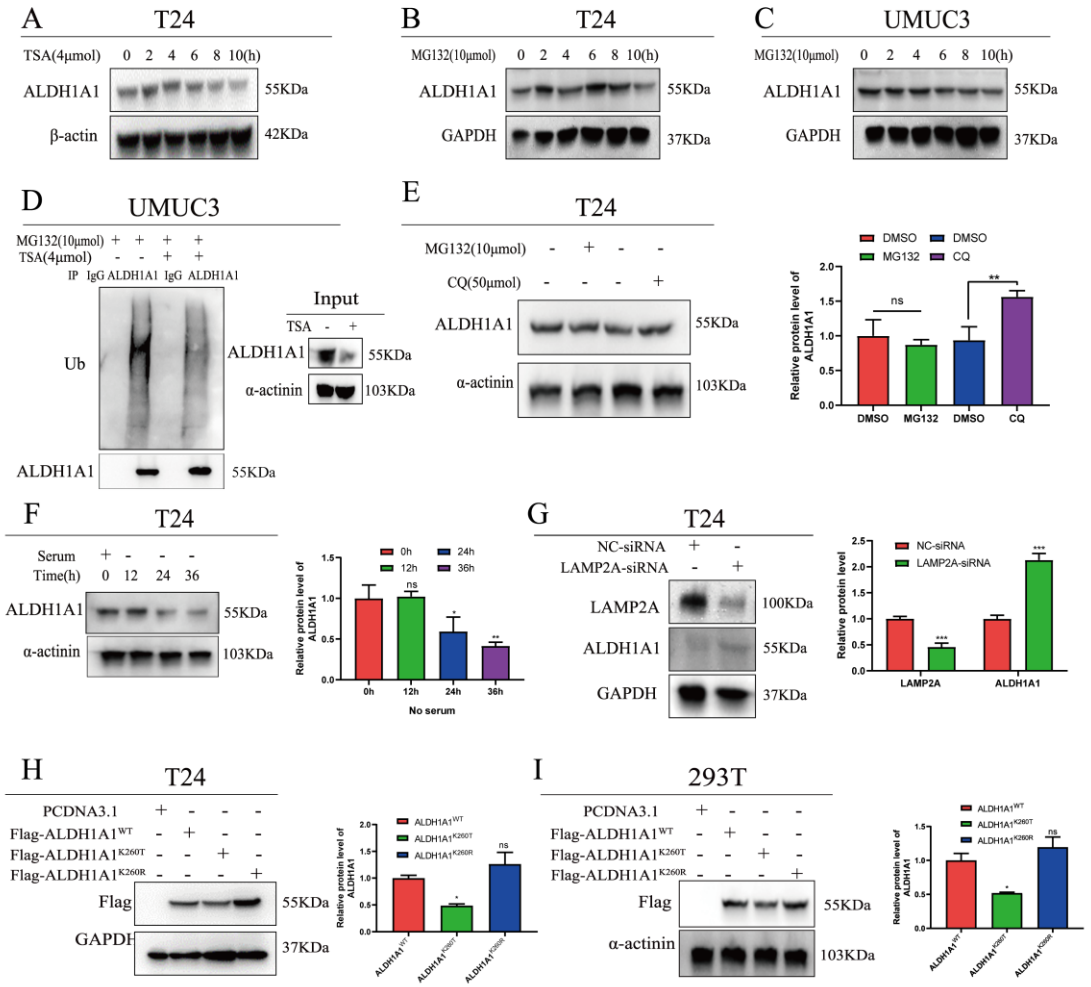

Figure S4. K260hib could promote ALDH1A1 protein degradation, related with Figure 4 and Figure 5. (A) T24 cells was treated with TSA for different lengths of time as indicated. The protein levels of ALDH1A1 were determined by western blotting and normalized against β-actin. **(B,C)** Western blot analysis of ALDH1A1 in T24 and UMUC3 cells after treatment with MG132. **(D)** MG132 was added into the culture medium with or without TSA in UMUC3 cells as indicated to determine the ubiquitination of ALDH1A1. **(E)** The protein expression of ALDH1A1 was measured by Western blotting in T24 cells after treatment with MG132 or CQ. **(F)** ALDH1A1 level was determined by western blotting after serum starvation for different lengths of time in T24 cells. **(G)**ALDH1A1 protein level was examined by western blotting in T24 cells with LAMP2A knocking down efficaciously. **(H)** Flag-tagged ALDH1A1^{WT}, ALDH1A1^{K260T} and ALDH1A1^{K260R} were overexpressed the in the T24 cell line, and the protein level of ALDH1A1 was measured by immunoblotting. **(I)** Flag-tagged ALDH1A1^{WT}, ALDH1A1^{K260T} and ALDH1A1^{K260R} were overexpressed the in the 293T cell line, and the protein level of ALDH1A1 was measured by immunoblotting. For cell experiments, each experiment was performed at least three times. Data are represented as mean +/- SEM. * $p<0.05$, **$p < 0.01$, ***$p < 0.001$ by two-sided Student's t-test or one-way ANOVA. ns: not significant.

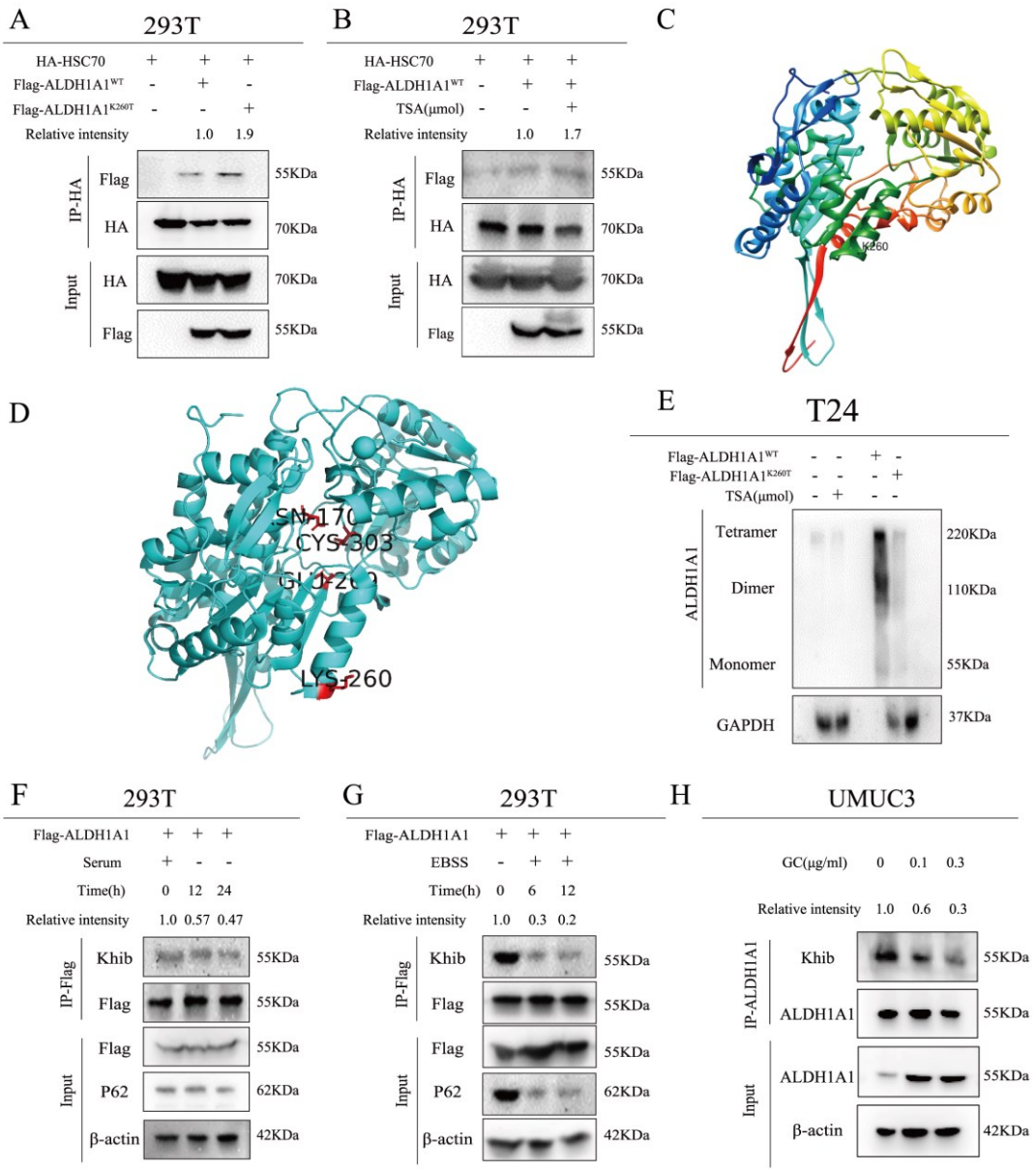

Figure S5. K260hib affects the crystal structure and function of ALDH1A1, related with Figure 5 and Figure 6. (A) HA-tagged HSC70 was co-transfected with vector, flag-tagged ALDH1A1^{WT} or flag-tagged ALDH1A1^{K260T} into 293T cells, ALDH1A1-HSC70 binding was determined by immunoprecipitation-western analysis. **(B)** HA-tagged HSC70 was co-transfected with vector or flag-tagged ALDH1A1^{WT} into the 293T cells followed by the treatment with TSA to observe the ALDH1A1-HSC70 binding by western blot. **(C)** Crystal structure of ALDH1A1. **(D)** The catalytic site of ALDH1A1enzyme demonstrated by crystal structure. **(E)** The cell lysates were collected with ALDH1A1-WT, K260T or K260R overexpressed in T24 cells followed treatment with 1% glutaraldehyde, then the polymerizations of ALDH1A1 were examined by immunoblotting. **(F,G)** Flag-tagged ALDH1A1 was transfected into 293T cells, and cells were treated with serum starvation and EBSS stresses for the indicated time to observe the level of 2-hydroxyisobutyrylation. **(H)** UMUC3 cells were treated with gemcitabine and cisplatin (GC) for the indicated time to observe the level of 2-hydroxyisobutyrylation. The final concentration of gemcitabine and cisplatin were 0,

0.1μg/ml and 0.3μg/ml respectively. For cell experiments, each experiment was performed at least three times.

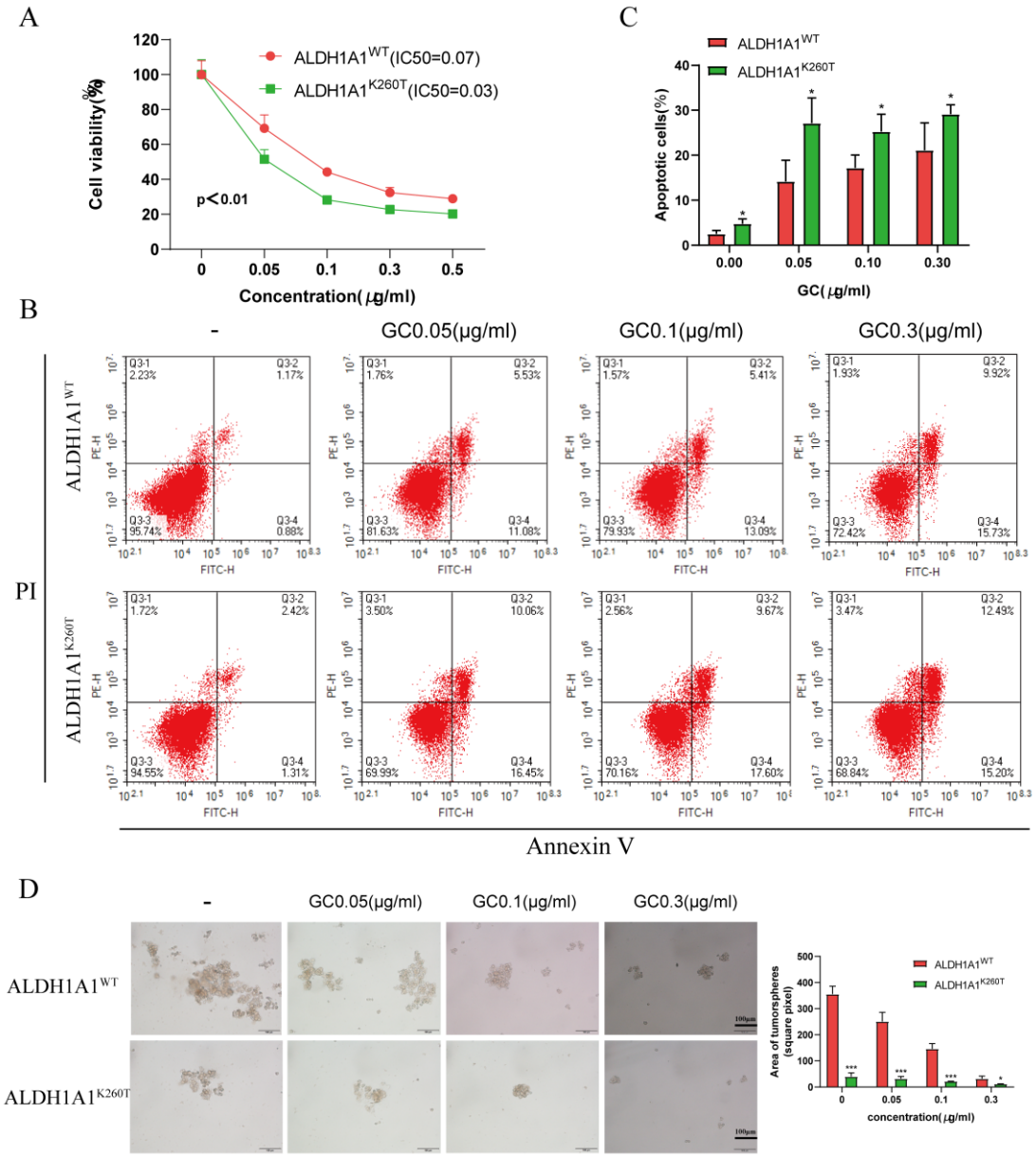

Figure S6. K260hib modification could sensitize the bladder cancer cells to gemcitabine and cisplatin treatment, related with Figure 6. (A) The T24 ALDH1A1^{WT} or ALDH1A1^{K260T} rescued cells were treated with an increasing concentrations of gemcitabine and cisplatin. The final concentration of gemcitabine and cisplatin were 0, 0.05μg/ml, 0.1μg/ml, 0.3μg/ml and 0.5μg/ml respectively. After 48 h, the viable cells were determined by MTT assay. **(B,C)** The apoptotic cells were determined by flow cytometry using FITC-labeled Annexin V staining. **(D)** ALDH1A1^{WT} and ALDH1A1^{K260T} were analyzed by sphere-forming assay with different concentration of gemcitabine and cisplatin (GC). The final concentration of gemcitabine and cisplatin were 0, 0.05μg/ml, 0.1μg/ml and 0.3μg/ml respectively. Scale bars 100μm. For cell experiments, each experiment was performed at least three times. Data are represented as mean +/- SEM. *p < 0.05, ***p < 0.001 by two-sided Student's t-test.

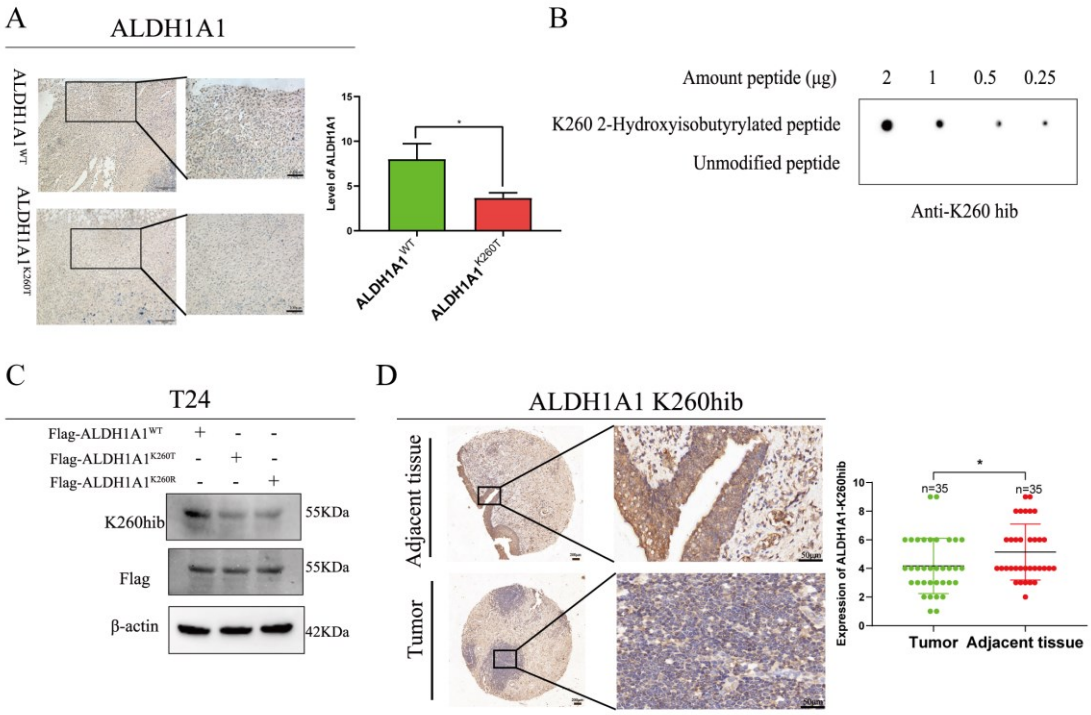

Figure S7. The ALDH1A1 K260hib expression between normal bladder tissues and the corresponding bladder cancer tissues with a specific K260hib antibody, related with Figure 6. **(A)** Immunohistochemical staining of ALDH1A1 protein in ALDH1A1^{WT} and ALDH1A1^{K260T} tumors from BABL/c nude mice (n=6 per group). Left: scale bars 200μm. Right: scale bars 100μm. **(B)**The dot blot assay was performed to confirm the specificity of the antibody to K260 hib-ALDH1A1 (K260 hib-ALDH1A1 Ab). K260 2-hydroxyisobutylated peptide and the unmodified peptide with different concentrations were dotted on Nitrocellulose membrane and were detected by K260 hib-ALDH1A1 Ab. (**C**) Flag-ALDH1A1^{WT}, Flag-ALDH1A1^{K260T} and Flag-ALDH1A1^{K260R} mutant were transfected into the T24 cells respectively, then the cell lysis was followed by western blot analysis using K260 hib-ALDH1A1 antibody. **(D)** The expression levels of ALDH1A1-K260hib in bladder cancer tissue and adjacent tissue was evaluated by IHC analysis (n=35 per group). Left: scale bars 200μm. Right: scale bars 50μm. For cell experiments, each experiment was performed at least three times. For animal experiments, six per group were used for in vivo studies. Data are represented as mean +/- SEM. * $p<0.05$ by two-sided Student's t-test.

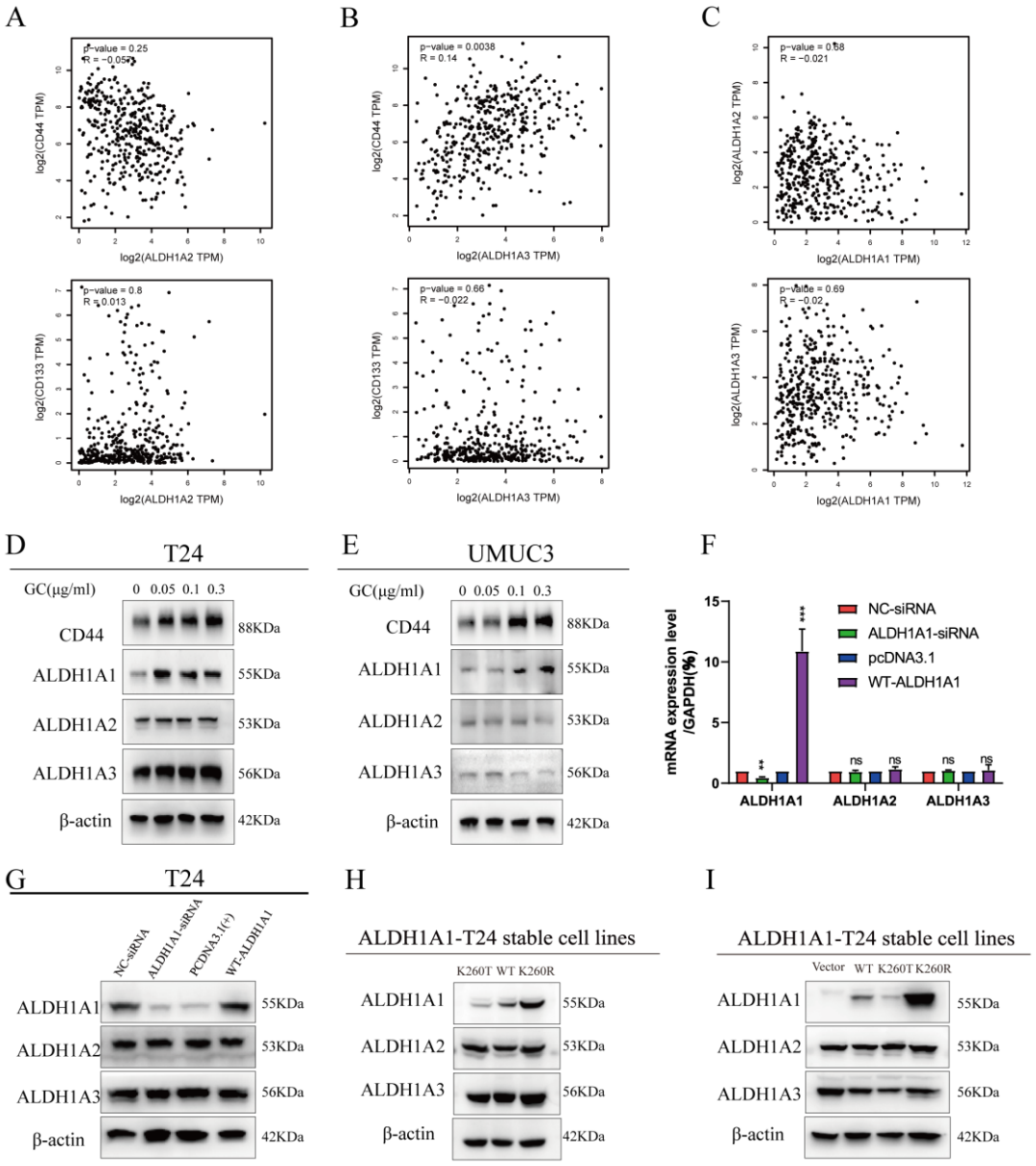

Figure S8. The relationship among ALDH1A2, ALDH1A3, CD133 and CD44 in bladder cancer, related with Figure 1. (**A,B**) The relationship among ALDH1A2, ALDH1A3, CD133 and CD44 in the GEPIA-TCGA-BLCA database. (**C**) The relationship among ALDH1A2, ALDH1A3 and ALDH1A1in the GEPIA-TCGA-BLCA database. (**D,E**) Western blot analysis of CD44, ALDH1A1, ALDH1A2 and ALDH1A3 after different concentration of gemcitabine and cisplatin in T24 and UMUC3 cells. The final concentration of gemcitabine and cisplatin were 0, 0.05μg/ml, 0.1μg/ml and 0.3μg/ml respectively. **(F)** The mRNA expression level of ALDH1A2 and ALDH1A3 evaluated by RT-qPCR after knockdown and over expression of ALDH1A1. **(G)** Analysis of ALDH1A2 and ALDH1A3 by western blotting after knockdown and over expression of ALDH1A1. **(H,I)** The protein expression of ALDH1A1, ALDH1A2 and ALDH1A3 were evaluated by western blot in the ALDH1A1-T24 rescued stable cell lines. For cell experiments, each experiment was performed at least three times. Data are represented as mean +/- SEM. **p < 0.01, ***p < 0.001 by two-sided Student's t-test. ns: not significant.

Table. S1 Name of primer, related with Figure 2 and Figure 4.

Name of primer	Sequence of primer
ALDH1A1-F	TCCTGGTTATGGGCCTACAG
ALDH1A1-R	CAAGTCGGCATCAGCTAACA
HDAC2-F	GAGCTGTGAAGTTAAACCGACA
HDAC2-R	ACCGTCATTACACGATCTGTTG
HDAC3-F	CCTGGCATTGACCCATAGCC
HDAC3-R	CTCTTGGTGAAGCCTTGCATA
RXRα-F	GACGGAGCTTGTGTCCAAGAT
RXRα-R	AGTCAGGGTTAAAGAGGACGAT
RXRβ-F	ACGGCTATGTGCAATCTGC
RXRβ-R	CGGATGGTGCGTTTGAAGAA
RXRγ-F	CCGGATCTCTGGTTAAACACATC
RXRγ-R	GTCCTTCCTTATCGTCCTCTTGA
RARα-F	GGGCAAATACACTACGAACAACA
RARα-R	CTCCACAGTCTTAATGATGCACT
RARβ-F	CCCCAGAACAAGACACCATGA
RARβ-R	TTTTGTCGGTTCCTCAAGGTC
RARγ-F	ATGCTGCGTATCTGCACAAG
RARγ-R	AGGCAAAGACAAGGTCTGTGA
GAPDH-F	TCATGGGTGTGAACCATGAGAA
GAPDH-R	GGCATGGACTGTGGTCATGAG
β-actin-F	GGCATCGTCACCAACTGGGAC
β-actin-R	CGATTTCCCGCTCGGCCGTGG