Supplementary Data

CSN6 mediates nucleotide metabolism to promote tumor development and chemoresistance in colorectal cancer

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Supplementary Materials and Methods

Patient-derived organoid (PDO)

Fresh human CRC tumor tissues were collected and cut into pieces, then washed with ice-cold PBS, digested with EDTA. Matrigel polymerization for 10 min at 37°C. Then, organoids were cultured with advanced DMEM/F12 supplemented with 2mM GlutaMAX, penicillin/streptomycin, 1 × B27, 10 mM HEPES, 10 nM gastrin I (Biogems), 1 × N2 (Life Technologies), 1mM N-acetylcysteine (Sigma), 500 nM A83-01 (Biogems), 50 ng/mL recombinant EGF, 100 ng/ mL recombinant Noggin (Peprotech), 10mM nicotinamide (Sigma), 500ng/mL R-spondin-1(Peprotech), 10 μM Y-27632 (Abmole) and 10 μM SB202190 (Sigma). After seeding, organoids were treated with shRNA virus or control for 12h. Removed the virus and added the new culture medium with nucleosides supplementation. Organoid diameters were calculated by Image J (RRID: SCR_003070).

Immunoblot and Immunoprecipitation

Total cells lysates for immunoblotting or immunoprecipitation were lysed with buffer (0.1%Triton-100, 50 mM Tris-Cl, pH 7.5, 0.1% NP-40, 150 mM NaCl, 0.1 M EDTA) supplemented with a cocktail of phosphate and proteinase inhibitors for 30 minutes at 4℃. For immunoblot, samples were separated by SDS–PAGE. Antibodies specific for CSN6 (Enzo Life Sciences Cat# BML-PW8295, RRID: AB_10539117), DDX5 (Cell Signaling Technology, #9877), β-Trcp (Cell Signaling Technology, #4394S), Flag-Tag (Sigma, F1804), HA-Tag (Cell Signaling Technology, #3724S),

Myc-Tag (Cell Signaling Technology, #2276S), PHGDH (Sigma, HPA021241), and GAPDH (Proteintech, 10494-1-AP) were purchased from the indicated companies. For immunoprecipitation, cell lysates were prepared as before and rotated 1mg proteins with antibody at 4℃ overnight, then immunoprecipitated by Protein A/G beads; or incubated cell lysates with anti-Flag or anti-myc beads at 4℃ overnight. The immunoprecipitates and input were subjected to western blot analysis.

Tissue microarray assay

For tissue microarray, we performed immunohistochemistry (IHC) on 267 paraffin-embedded samples obtained from the First Affiliated Hospital of Sun Yat-sen University. Then the original immunohistochemistry slides were scanned and captured images by Aperio Versa (Leica Biosystems). Subsequently, H-score was calculated by the Aperio Versa base on the staining intensity and percentage of positive stained tumor cells. The cut-off point was defined based on the receiver operating characteristic curve. Spearman correlation analysis or Chi-square test was used to estimate the association between CSN6, DDX5 and PHGDH staining intensities.

Immunohistochemistry

Immunohistochemical staining was performed on 5-μm paraffin sections prepared from tumor tissues. Briefly, sections were deparaffinized in xylene, hydrated in graded ethanol, then immersed in 3% hydrogen peroxide to quench for endogenous peroxidase and probed with primary antibody against CSN6 (1:500, Enzo Life Sciences Cat# BML-PW8295, RRID: AB_10539117), DDX5 (1:4000, Abcam, ab21696) or PHGDH

(1:1000, Sigma, HPA021241), Ki67 (1:500, Cell Signaling Technology, #9449) at 4°C overnight. Subsequently, immunostaining was visualized with diaminobenzidine and slices were counterstained with hematoxylin.

Cell proliferation assay

The incucyte live cell analysis system (Essen Bioscience) is performed for detecting cell proliferation. Cells were seeded in 12-well plates and put inside incubators, then machine would automatically obtain and analyze images during the progress of an experiment while cells remain unperturbed. The cell counting kit-8 (CCK8) assay was also used to examine cell viability. Cells (3000/well) were seeded in 96-well plates and were incubated for 24, 48, or 72 hours. Subsequently, 10μL of CCK8 (APExBIO) solution was added to each well and incubated for 3 h, then the absorbance value (OD) was measured at 450 nm. Data represent the means ± SD. of three independent experiments.

Colony formation assay

Cells were seeded into 6-well plates (800 cells per well) and then cultured for 10-14 days. Culture mediums were refreshed every 3 days. After visible colonies formed, cells were fixed with 4% paraformaldehyde (PFA) and stained with crystal violet. Finally, the number of colonies were counted by image J.

DESI-MS imaging

Imaging experiments were carried out on a 2D DESI system (Prosolia, Indianapolis, USA) mounted on a Xevo-G2-XS quadrupole-time of flight (Q-TOF) (Waters Corporation, Wilmslow, UK). The solvent spray consisted of 95% methanol and 5% water. Spray conditions used were a flow rate of 2 μl/minutes (using a syringe pump from Harvard Apparatus, Inc., Holliston, MA), with a nebulising gas of nitrogen at 4 bar pressure. Typical positions of the sprayer were used (sprayer 1.5mm above surface, 6mm sprayer to capillary distance, 75˚ sprayer impact angle, 5˚ collection capillary angle). The source temperature was 100 ˚C and the capillary voltage was set between 4.82 and 5kV. Images were acquired using a scan rate of 1-10 scans/second, and a mass range of 50-1200 Da. The spatial resolution varied from 40-200 μm. Images were processed and normalised to total ion current (TIC) using the High-Definition Imaging (HDI) v1.4 (Waters, Wilmslow, UK). MS/MS experiments were carried out on a Xevo-G2-XS Q-TOF mass spectrometer using argon as the collision gas and a collision energy range of 20 V - 40 V. Spray conditions were the same as DESI imaging.

Real Time qPCR

Total RNA was isolated with TRIZOL reagent (Invitrogen) and reverse transcription was performed by ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO), according to the manufacturer's protocol. Then the gene expression was quantified dependent on the LightCycler480 PCR system (Roche) by using 2×SYBR Green qPCR Master Mix (biotool, #B21203). All the target genes were

normalized to GAPDH. Primer sequences were listed as follows: *CSN6* F: TCATCGAGAGCCCCCTCTTT; *CSN6* R: CCAATGCGTTCCGCTTCCT; *DDX5* F: GCCGGGACCGAGGGTTTGGT; *DDX5* R: CTTGTGCTGTGCGCCTAGCCA; *PHGDH* F: CTGCGGAAAGTGCTCATCAGT; *PHGDH* R: TGGCAGAGCGAACAATAAGGC; *GAPDH* F: GGAGCGAGATCCCTCCAAAAT; *GAPDH* R: GGCTGTTGTCATACTTCTCATGG.

Exogenous RNA pull-down assays

For exogenous RNA pull-down assay, RNAs were *in vitro* transcribed using Thermo Scientific Transcript Aid T7 High Yield Transcription Kit (Thermo, #K0441) according to the manufacturer's instructions. Next, the Thermo Scientific Pierce RNA 3' End Desthiobiotinylation Kit uses T4 RNA ligase to attach a single biotinylated nucleotide to the 3′ terminuses of an RNA strand (Thermo, #20163). Cell lysates were prepared using standard lysis buffer, binding of Labeled RNA to Streptavidin Magnetic Beads, then binding RNA-Binding Proteins, incubate 30-60 minutes at 4°C with agitation or rotation. Washing and elution of RNA-Binding Protein complexes, finally RNA-interactive proteins were detected by WB analysis.

mRNA stability analysis

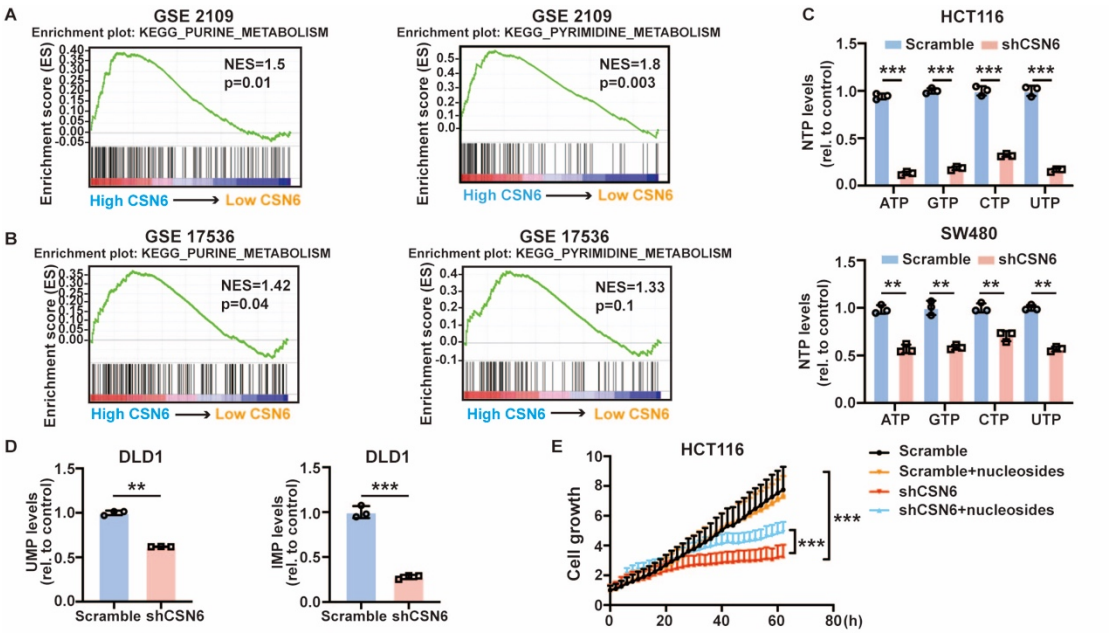
DLD1 cells were treated with DDX5 shRNA or scramble, then incubated with Actinomycin D (10mg/mL) for 0, 1, 2 or 4h. Following RNA isolation with Trizol and quantification with qPCR.

Turnover assay

Cells were infected with virus carried shRNA or transfected with indicated plasmids, then cycloheximide was added into the media at a final concentration of 100 μg/ml. Harvested cells at the indicated time points after cycloheximide treatment, and analyzed protein levels by immunoblotting.

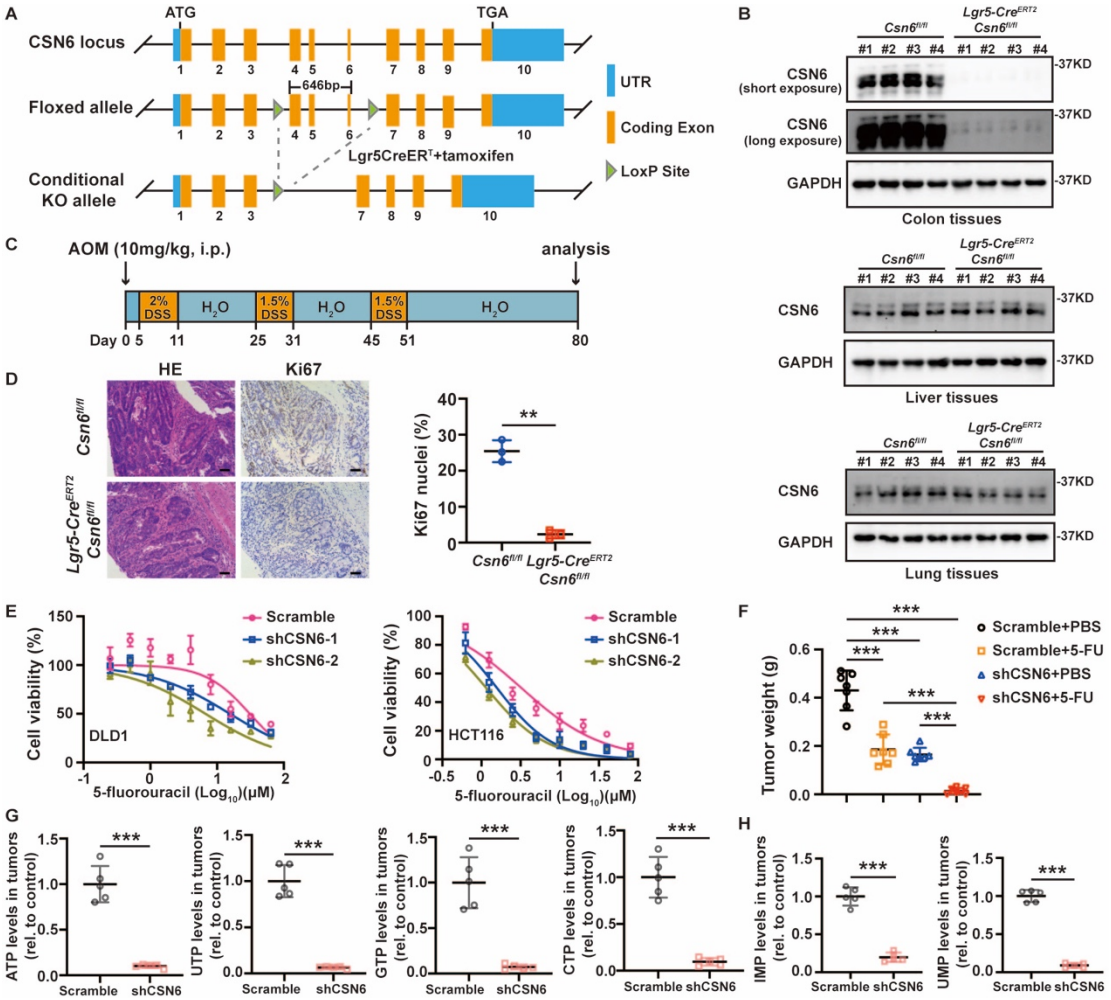
Ubiquitination assay

HCT116 and HEK293T cells were co-transfected with the indicated plasmids for 48 h and treated with MG132 for an additional 6 h. The cell lysates were incubated with Ni-NTA agarose beads to pull down proteins conjugated to His-ubiquitin, then washed, and analyzed by western blotting with indicated antibody.



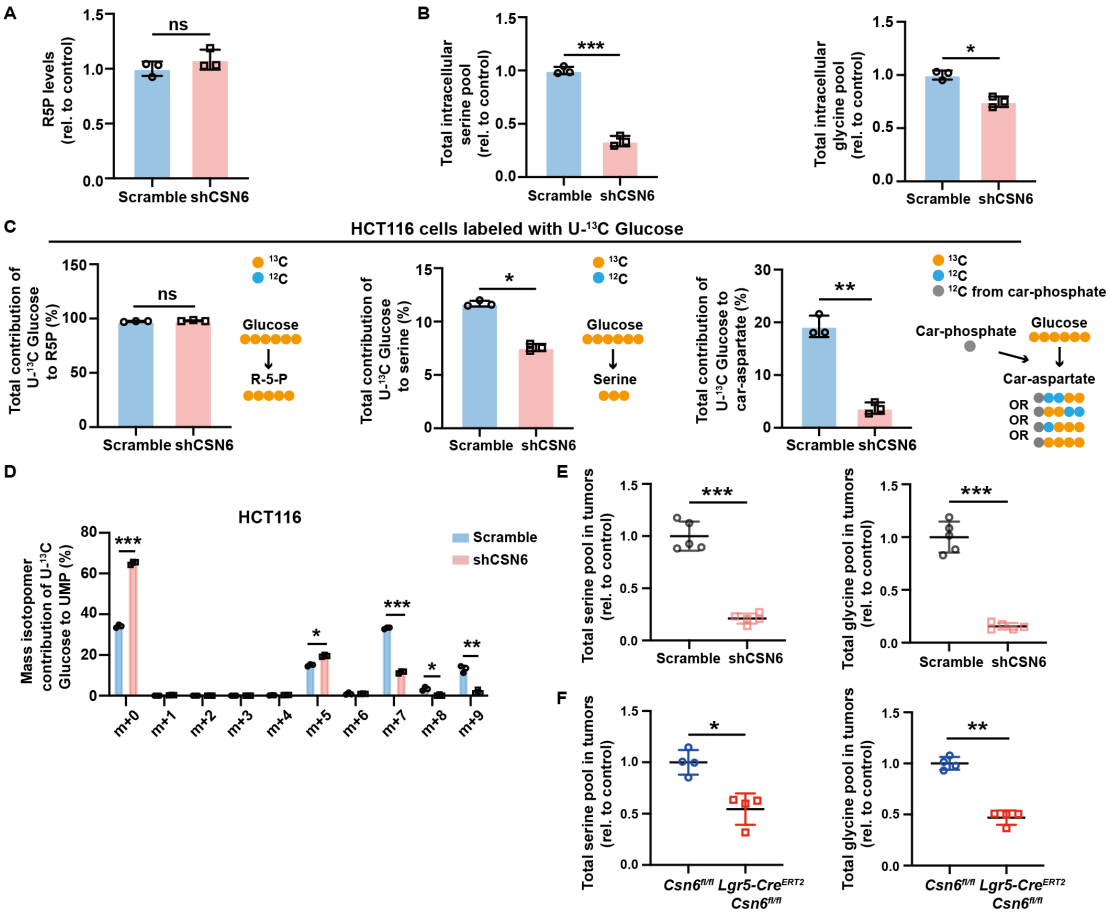
Supplementary Fig.S1

Depletion of CSN6 decreased nucleotide synthesis. **A, B,** An enrichment analysis of gene sets available from the CRC database GSE2109 and GSE17536 revealed that CSN6 expression is positively correlated with pyrimidine and purine metabolism. **C,** NTPs (ATP, GTP, UTP, CTP) levels in HCT116 and SW480 cells with or without CSN6 shRNA treatment. The data are presented as the means ± SD. **p<0.01, ***p<0.001, t test. **D,** UMP and IMP levels in DLD1 cells stably expressing CSN6 shRNA (n=3). The data are presented as the means ± SD. **p<0.01, ***p<0.001, t test. **E,** The effects of nucleosides repletion on rescuing shCSN6 inhibiting cell growth. ***p<0.001, by the two-way ANOVA.



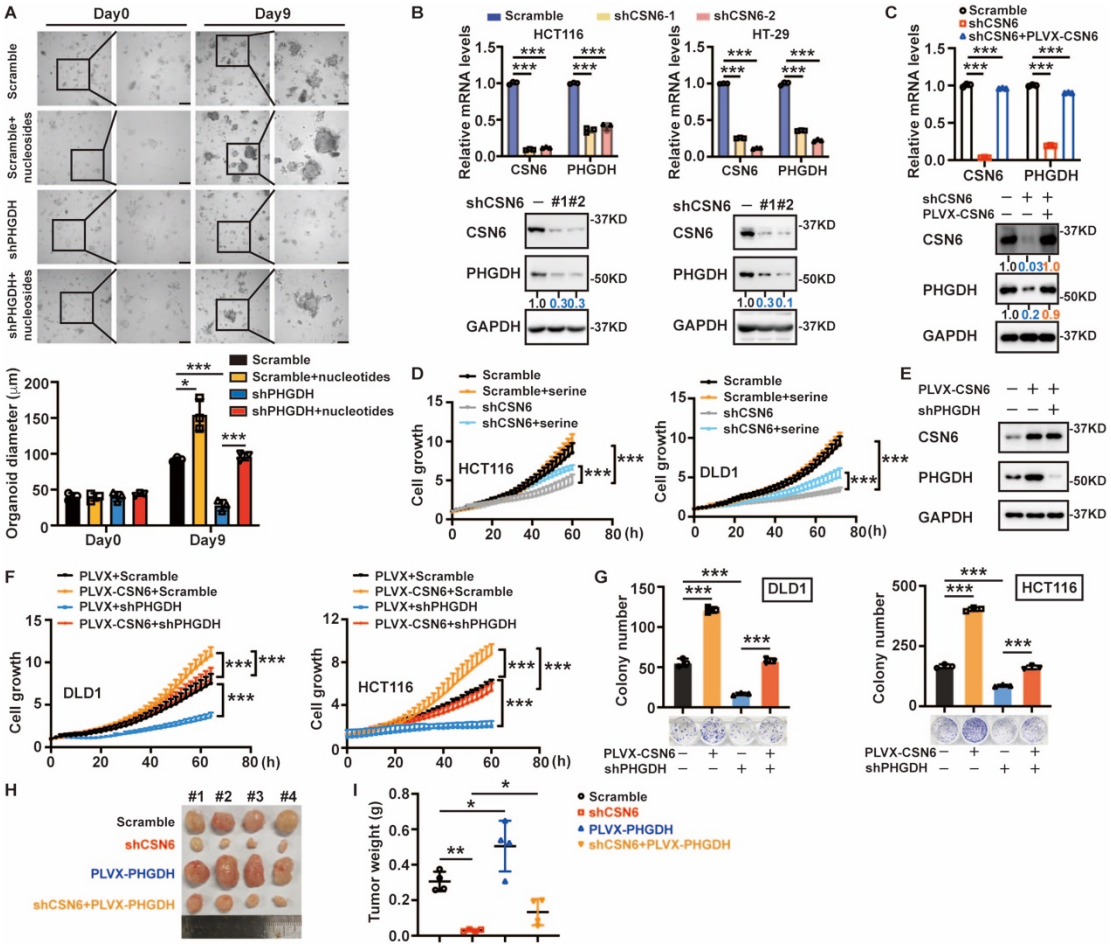
Supplementary Fig.S2

CSN6 promotes CRC progression and drug resistance. **A,** Overview of the CSN6 conditional knockout (*Csn6*^{CKO}) mice targeting strategy. **B,** CSN6 expression in colon, liver and lung tissues from *Lgr5-Cre*^{ERT2}*;Csn6*$^{fl/fl}$ *and Csn6*$^{fl/fl}$ mice were determined by western blot. **C,** Scheme of AOM/DSS model. **D,** H&E (hematoxylin and eosin) staining and Ki67 immunohistochemistry of colon tumor sections from *Lgr5-Cre*^{ERT2}*;Csn6*$^{fl/fl}$ *and Csn6*$^{fl/fl}$ mice. Scale bar, 100μm. The data are presented as the means ± SD. **p<0.01. **E,** DLD1 and HCT116 cells were treated with 5-FU at different concentrations, and the cell viability was then evaluated using the CCK8 assay. The data are presented as the means ± SD. **F,** Tumor weight of different nude mice groups (scramble; scamble+5-FU; shCSN6; shCSN6+5-FU, n=7 for each group). The data are presented as the means ± SD. ***p<0.001 by One-Way ANOVA. **G,** NTPs (ATP, CTP, UTP, GTP) levels in control tumors (scramble) compared to CSN6 knockdown (shCSN6) DLD1 xenograft tumors (n=5 per group). The data are presented as the means ± SD. ***p<0.001, by non-parametric test. **H,** IMP and UMP levels in the control tumors (scramble) compared to CSN6 knockdown (shCSN6) DLD1 xenograft tumors (n=5 per group). The data are presented as the means ± SD. ***p<0.001, by non-parametric test.



Supplementary Fig.S3

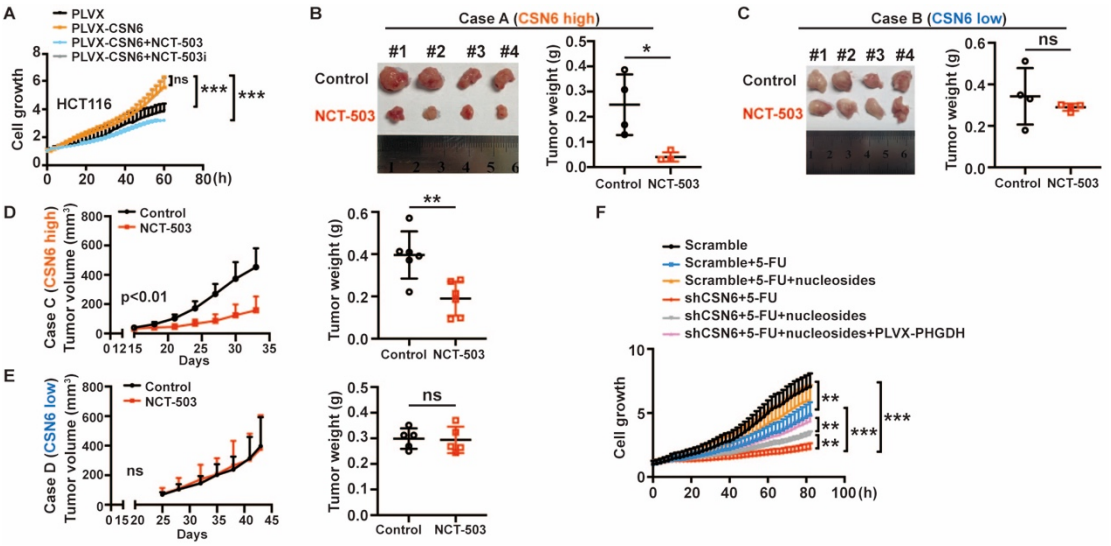
Silencing of CSN6 impaired *de novo* nucleotide synthesis. **A,** Ribose-5-phosphate levels in the control and shCSN6 expressing DLD1 cells. The data are presented as the means ± SD. ns: no significance, t-test. **B,** Intracellular serine and glycine levels in the control and shCSN6 expressing DLD1 cells. The data are presented as the means ± SD. *p<0.05, ***p<0.001 by t-test. **C,** Incorporation of carbon atoms from [U-^{13}C] glucose into R5P, serine and car-aspartate in the control and shCSN6 expressing HCT116 cells. The data are presented as the means ± SD. *p<0.05, **p<0.01, ns: no significance by t-test. **D,** Fractional contribution of carbon atoms from [U-^{13}C] glucose into the UMP isotopomers (m+0 to m+9) in the control and shCSN6 expressing HCT116 cells. The data are presented as the means ± SD. *p<0.05, **p<0.01, ***p<0.001 by t-test. **E,** Serine and glycine levels in the control and shCSN6 expressing DLD1 xenograft tumor tissues (n = 5 per group). The data are presented as the means ± SD. ***p<0.001 by non-parametric test. **F,** Serine and glycine levels in the colonic tumors from *Lgr5-Cre^{ERT2};Csn6$^{fl/fl}$ and Csn6$^{fl/fl}$* mice (n = 4 per group). The data are presented as the means ± SD. *p<0.05, **p<0.01, by non-parametric test.



Supplementary Fig.S4

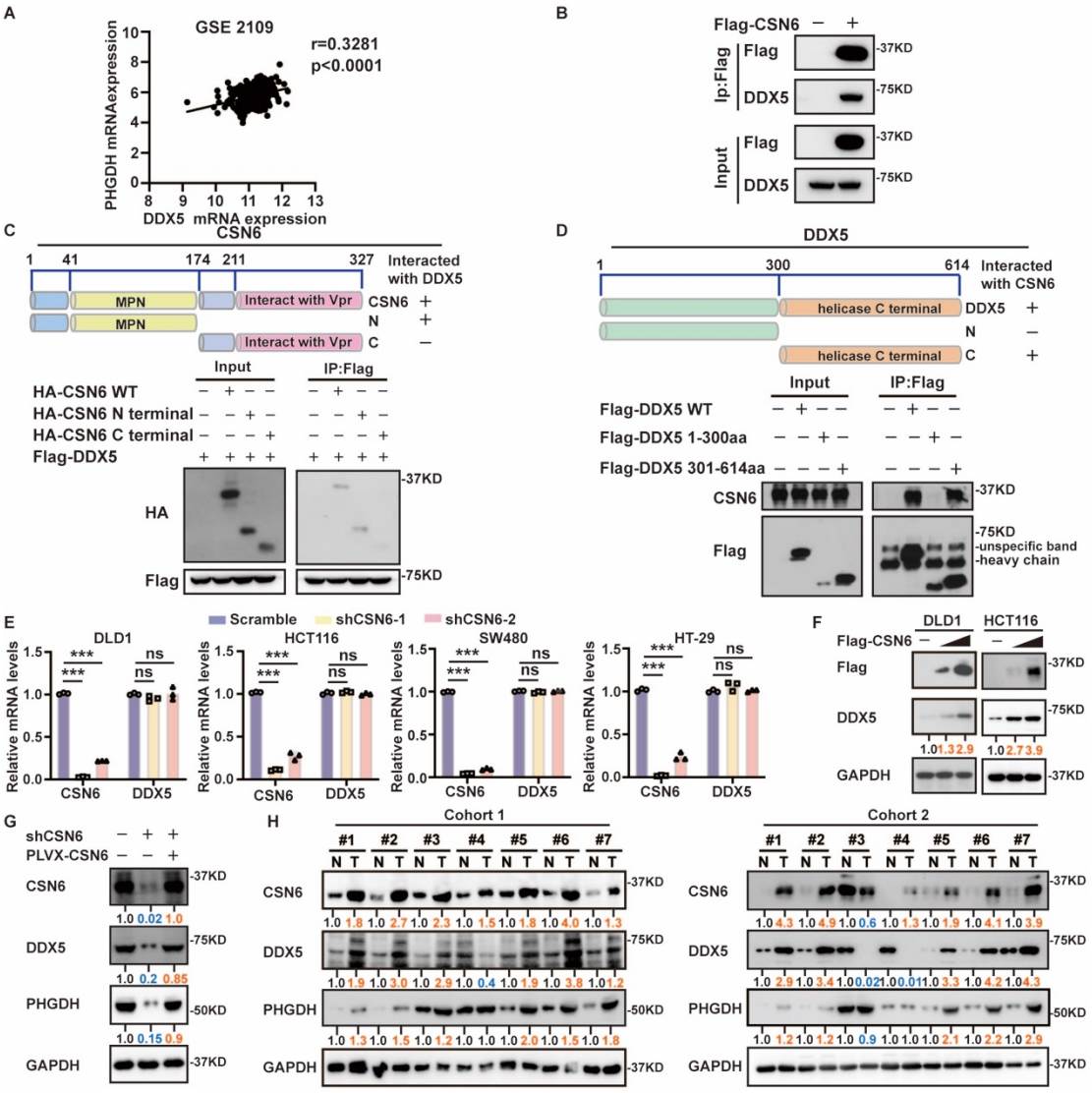
CSN6 promotes CRC progression in PHGDH-dependent manner. **A,** Nucleosides repletion (adenine, guanine, cytosine, uracil; 100 μM) could rescue patient-derived tumor organoid (PDO) growth upon PHGDH depletion. The morphology of the organoids is shown. Scale bar: 100 μm. Quantifications of organoid diameter are presented as the means ± SD. *p<0.05, ***p<0.001. **B,** qRT-PCR and western blot analysis of PHGDH expression in HCT116 and HT-29 cells stably expressing CSN6 shRNA or scramble. The data are presented as the means ± SD. ***p<0.001 by One-Way ANOVA. **C,** DLD1 cells were infected with indicated virus. qRT-PCR and western blot were used to detected PHGDH expression. The data are presented as the means ± SD. ***p<0.001 by the two-way ANOVA. **D,** Supplementation of serine rescued shCSN6 inhibited HCT116 and DLD1 cell growth. The data are presented as the means ± SD. ***p<0.001 by the two-way ANOVA. **E,** Detection of CSN6 and PHGDH expression in control and PLVX-CSN6 DLD1 cells treated with shPHGDH. **F,** Proliferation assay of DLD1 and HCT116 cells with or without PLVX-CSN6 or shPHGDH treatment. The data are presented as the means ± SD. ***p<0.001 by the two-way ANOVA. **G,** Effects of PHGDH depletion on the clonogenic ability of PLVX or PLVX-CSN6 DLD1 and HCT116 cells. Colony numbers were quantified. The data are presented as the means ± SD. ***p<0.001. **H, I,** DLD1 cells with or without shCSN6 or PLVX-PHGDH infection were subcutaneously injected into nude mice

(n=4). The tumors were isolated at the end of the experiments. The tumor pictures (H), and tumor weight (I) were shown.



Supplementary Fig.S5

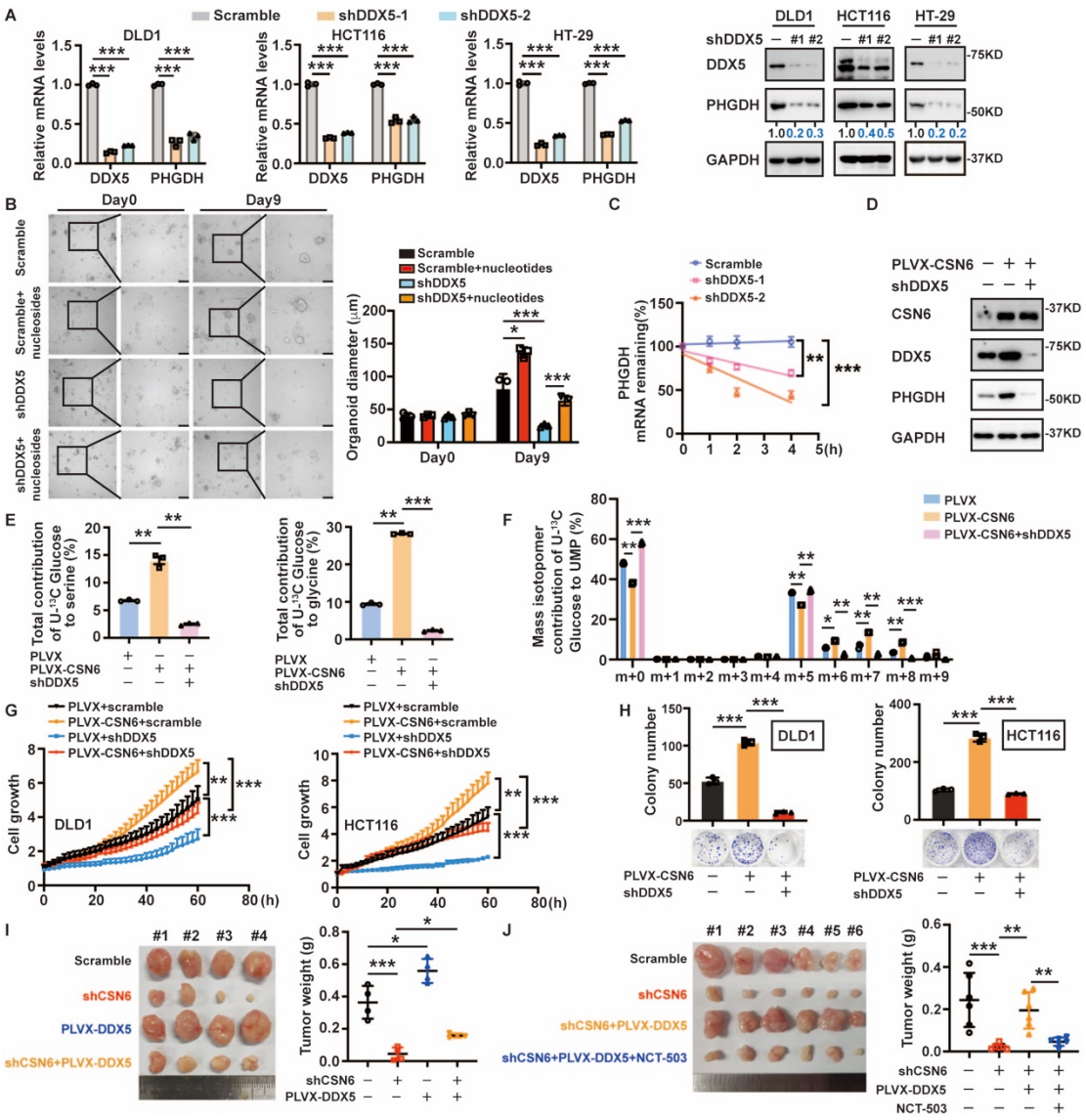
CSN6 promotes CRC tumorigenesis and chemoresistance through PHGDH. **A,** Proliferation assay of control and PLVX-CSN6 HCT116 cells treated with NCT-503 or NCT-503i. The data are presented as the means ± SD. ***p<0.001 by the two-way ANOVA. **B,** Impact of NCT-503 on tumor growth and weight in mice bearing CSN6-high expressed PDXs (n=4 per group). The data are presented as the means ± SD. *p<0.05 by non-parametric test. **C,** Impact of NCT-503 on tumor growth and weight in mice bearing CSN6-low expressed PDXs (n=4 per group). The data are presented as the means ± SD. ns: no significance, by non-parametric test. **D,** Impact of NCT-503 on tumor volume and weight in mice bearing CSN6-high expressed PDXs (Case C). The data are presented as the means ± SD. **p<0.01 by non-parametric test. **E,** Impact of NCT-503 on tumor volume and weight in mice bearing CSN6-low expressed PDXs (Case D). The data are presented as the means ± SD. ns: no significance, by non-parametric test. **F,** Incucyte machine was used to evaluate the effects of nucleosides repletion (adenine, guanine, cytosine, uracil; 100 μM) on cancer cells proliferation upon CSN6 depletion, PHGDH overexpression and 5-FU treatment. The data are presented as the means ± SD. **p<0.01; ***p<0.001 by the two-way ANOVA.



Supplementary Fig.S6

CSN6 interacts with DDX5 and regulates DDX5 protein expression. **A,** Pearson correlation analysis of DDX5 and PHGDH in GEO: GSE2109. **B,** DLD1 cells were transfected with Flag-CSN6 and vector. Cell lysates were then immunoprecipitated with anti-Flag beads and immunoblotted with the indicated antibodies. **C,** Mapping of CSN6-binding domains on DDX5. HEK293T cells were co-transfected with HA-CSN6 (aa 1-327), the N terminal of CSN6 (aa 1-174) or the C terminal of CSN6 (aa 175-327) and Flag-DDX5. Cell lysates were immunoprecipitated with anti-Flag beads and immunoblotted with anti-HA antibody. **D,** Mapping of DDX5-binding domains on CSN6. HEK293T cells were transfected with Flag-DDX5 (aa 1-614), the N terminus of DDX5 (aa 1-300) or the C terminus of DDX5 (aa 301-614). Cell lysates were immunoprecipitated with anti-Flag beads and immunoblotted with anti-CSN6 antibody. **E,** qRT-PCR analysis of DDX5 expression in control and shCSN6 expressing CRC cells. The data are presented as the means ± SD. ***p<0.001, ns: no significance, by One-Way ANOVA. **F,** Western blot analysis of DDX5 expression in vector and CSN6 overexpressing CRC cells. **G,** DLD1 cells were infected with indicated virus. Western blot was used to detected CSN6, DDX5 and PHGDH expression. **H,** Immunoblotting

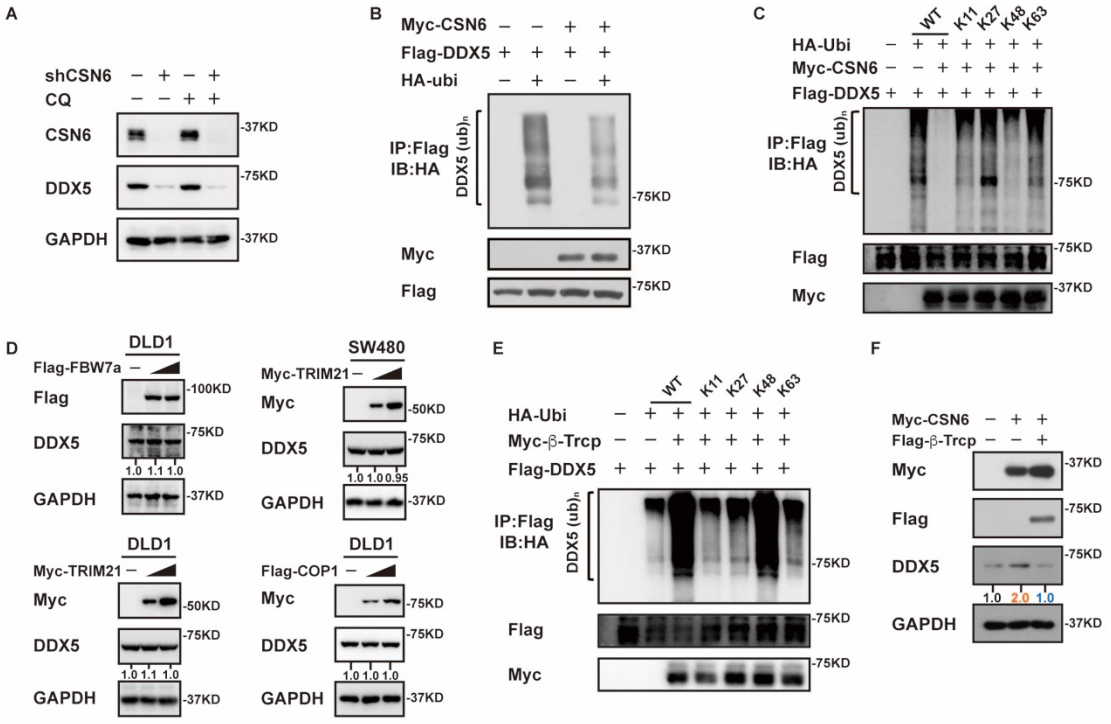
analysis of CSN6, DDX5 and PHGDH protein levels in 14 pairs of CRC tumors (T) and corresponding adjacent normal tissues (N).



Supplementary Fig.S7

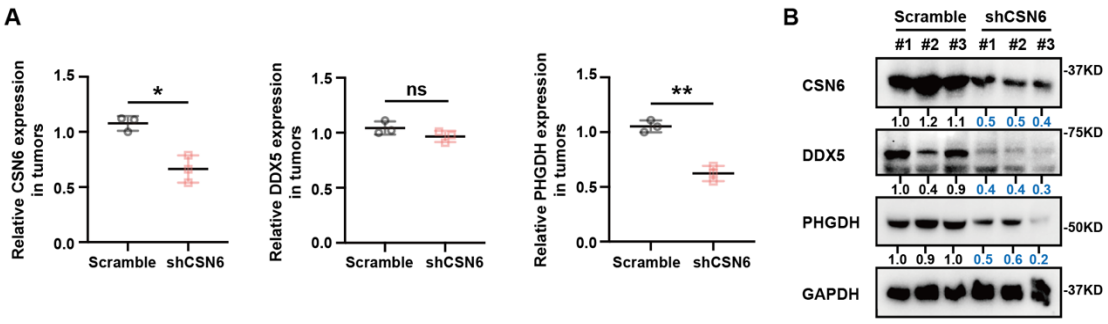
CSN6-DDX5-PHGDH axis promotes nucleotide biosynthesis and CRC progression. **A,** qRT-PCR and western blot analysis of PHGDH expression in control and shDDX5. expressing CRC cells. For the qRT-PCR, the data are presented as the means ± SD. ***p<0.001 by One-Way ANOVA. **B,** Nucleosides repletion could rescue patient-derived tumor organoid (PDO) growth upon DDX5 depletion. The morphology of the organoids is shown. Scale bar: 100 μm. Quantifications of organoid diameter are presented as the means ± SD. *p<0.05, ***p<0.001. **C,** Quantification of the half-life of PHGDH (% mRNA remaining) over time after actinomycin D (10 μg/ml) addition in DLD1 cells. Graph represents means ±SD. **p<0.01; ***p<0.001 by the two-way ANOVA test. **D,** Detection of CSN6, DDX5 and PHGDH expression in control and PLVX-CSN6 DLD1 cells treated with shDDX5. **E,** Incorporation of carbon atoms from [U-^{13}C] glucose into serine, glycine in the cells with or without PLVX-CSN6 or shDDX5 treatment. The data are presented as the means ± SD. **p<0.01, ***p<0.001 by One-Way ANOVA. **F,** Fractional contribution of carbon atoms from [U-^{13}C] glucose into the UMP in the cells with or without PLVX-CSN6 or shDDX5 treatment.

The data are presented as the means ± SD. *p<0.05, **p<0.01, ***p<0.001 by One-Way ANOVA. **G,** Cell proliferation of PLVX and PLVX-CSN6 DLD1 and HCT116 cells infected with scramble or shDDX5. Graph represents means ±SD. **p<0.01; ***p<0.001 by the two-way ANOVA test. **H,** Effects of DDX5 depletion on the clonogenic ability of PLVX-CSN6 DLD1 and HCT116 cells. Colony numbers were quantified. The data are presented as the means ± SD. ***p<0.001. **I,** DLD1 cells with or without shCSN6 or PLVX-DDX5 infection were subcutaneously injected into nude mice (n=4). The tumors were isolated at the end of the experiments. The tumor pictures and tumor weight were shown. **J,** CSN6 knockdown through the injection of shRNA lentivirus strongly inhibits the subcutaneous tumor growth of nude mice, which could be rescued by stable overexpression of PLVX-DDX5. Furthermore, NCT-503 treatment could abrogate these effects. The tumor pictures and tumor weight were shown.



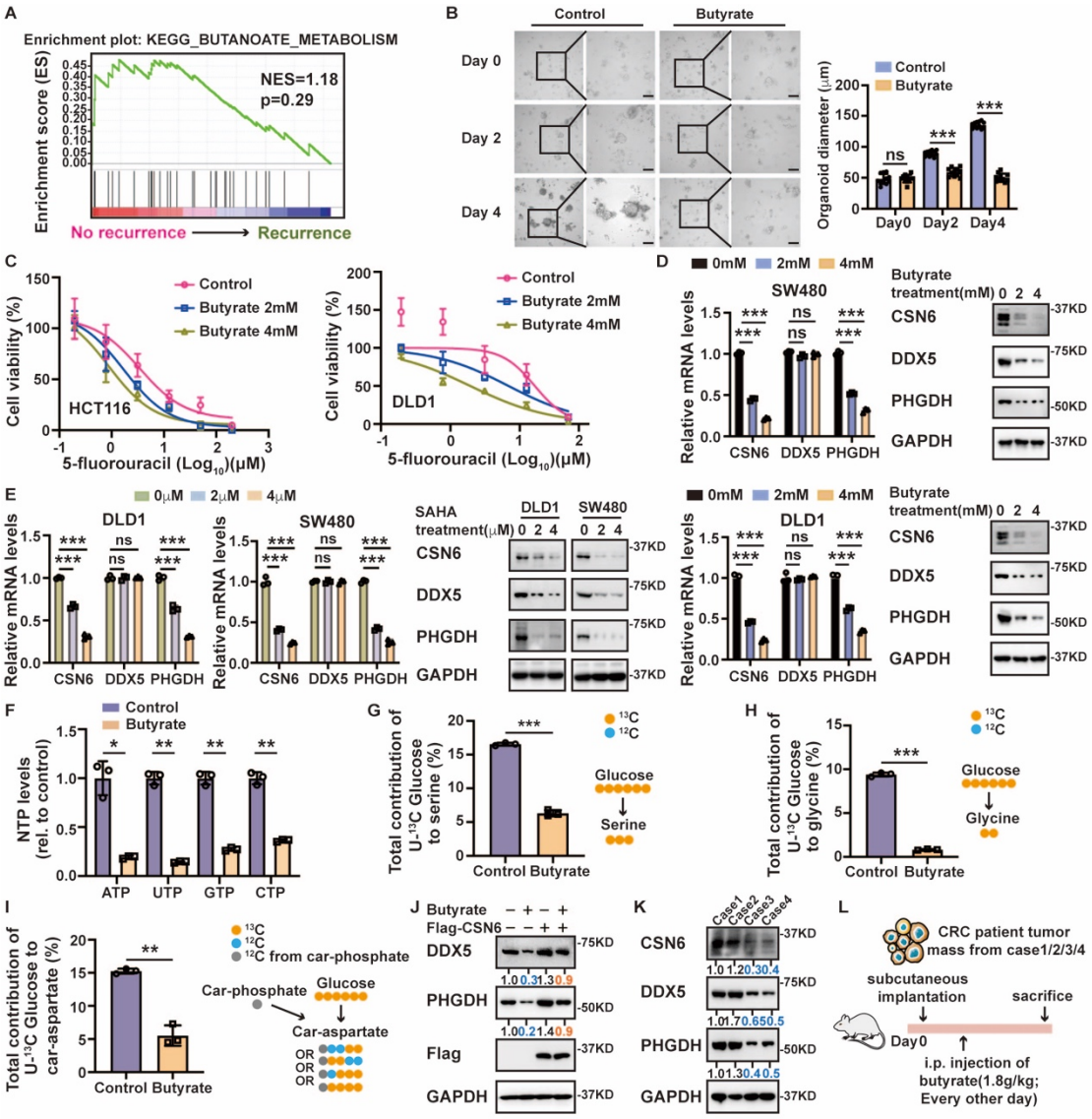
Supplementary Fig.S8

CSN6 stabilizes DDX5 protein by decreasing β-Trcp-mediated ubiquitination. **A,** Cell lysates were prepared from DLD1 cells expressing CSN6 shRNA or scrambled shRNA that had been treated with or without chloroquine (CQ) and then immunoblotted with the indicated antibodies. **B,** HCT116 cells were transfected with the indicated plasmids. MG132 was added to the cells 6 h prior to harvesting. The ubiquitinated DDX5 proteins were pulled down with anti-Flag beads and immunoblotted with the indicated antibodies. **C,** 293T cells were transfected with the indicated plasmids. MG132 was added to the cells 6 h prior to harvesting. The cell lysates were immunoprecipitated with anti-Flag beads and then immunoblotted with indicated antibody. **D,** Cells were transfected with the indicated plasmids and immunoblotted with the indicated antibodies. **E,** HEK293T cells were co-transfected with the indicated plasmids. Cells were then treated with MG132 6 h prior to harvesting. Polyubiquitinated DDX5 was immunoprecipitated with nickel beads and immunoblotted with indicated antibody. **F,** HCT116 cells were transfected with the indicated plasmids and immunoblotted with the indicated antibodies.



Supplementary Fig.S9

The expression of CSN6-DDX5-PHGDH axis in xenograft models. **A,** qPCR analysis of the indicated genes expressions in mouse DLD1 xenograft tissue samples (n = 3 per group). The data are presented as the means ± SD. *p<0.05, **p<0.01, ns: no significance, by non-parametric test. **B,** Western blot analysis of the indicated proteins in mouse DLD1 xenograft tissue samples (n = 3 per group).



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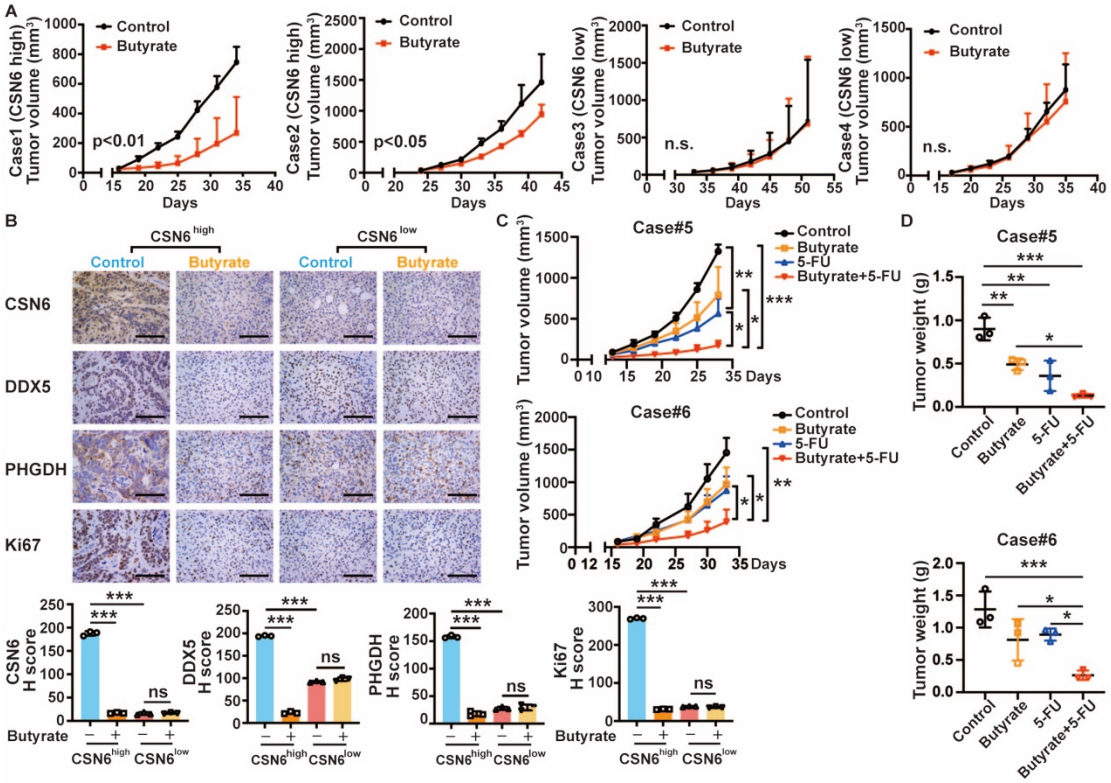
422 **Supplementary Fig.S10**

423 Butyrate may act as potential CSN6 antagonist. **A,** Gene set enrichment score and
424 distribution of butanoate metabolism between CRC patient samples with or without
425 recurrence. **B,** Images of organoids after 0, 2, 4 days of butyrate treatment. Scale bar:
426 100 μm. Quantifications of organoid diameter are presented as the means ± SD.
427 ***p<0.001, ns=no significance. **C,** HCT116 and DLD1 cells were treated with 5-FU
428 at different concentrations combined with butyrate (2 mM or 4 mM) for 48 h, and the
429 cell viability was then evaluated using the CCK8 assay. The data are presented as the
430 means ± SD. **D,** qRT-PCR and western blot analysis of the indicated protein levels after
431 treatment with different concentrations of butyrate in SW480 and DLD1 cells. The data
432 are presented as the means ± SD. ***p<0.001, ns: no significance, by One-Way
433 ANOVA. **E,** qRT-PCR and western blot analysis of the indicated protein levels after
434 treatment with different concentrations of SAHA in DLD1 and SW480 cells. The data
435 are presented as the means ± SD. ***p<0.001, ns: no significance, by One-Way
436 ANOVA. **F,** NTPs (ATP, GTP, UTP, CTP) levels in butyrate-treated DLD1 cells. The
437 data are presented as the means ± SD. *p<0.05, **p<0.01 by t-test. **G-I,** Incorporation

of carbon atoms from [U-^{13}C] glucose into serine, glycine and car-aspartate in control and butyrate-treated DLD1 cells. The data are presented as the means ± SD.**p<0.01, ***p<0.001, t test. **J,** HCT116 cells were transient transfected with Flag-CSN6 or control with or without butyrate treatment, and then immunoblotted with the indicated antibodies. **K**, Expression level of CSN6 in indicated patient-derived xenografts (PDXs). **L,** Treatment schedule of butyrate is indicated.



Supplementary Fig.S11

Butyrate is a CSN6 antagonist and improves the antitumor therapy efficacy. **A,** Impact of butyrate on tumor growth in mice bearing indicated PDXs. The data are presented as the means ± SD. ns: no significance, *p<0.05, **p<0.01 by the two-way ANOVA test. **B,** Representative IHC images and quantification of indicated proteins in CSN6 high (Case#1) or low (Case#3) PDX tumors after butyrate treatment. Scale bar: 100 μm. For the quantification, data are presented as the means ± SD. ***p<0.001, ns: no significance, by One-Way ANOVA. **C, D,** Impact of butyrate and 5-FU combination on tumor growth (C) and weight (D) in mice bearing indicated PDXs. Graph represents means ±SD. *p<0.05, **p<0.01; ***p<0.001 by the two-way ANOVA test.