Supplementary methods

Reagents and inhibitors

N-acetylcysteine (NAC) (Cat. #A0737), formate (Cat. #67253), crystal violet (Cat. #V5265) and H_2O_2 (Cat. #88597) and were obtained from Sigma-Aldrich (St. Louis, MO, USA). CM-H_2DCFDA (Cat. #C6827) and CM-H_2XRos (Cat. #M7513) probes were purchased from Invitrogen Life Technologies (Carlsbad, USA). GSH/GSSG-Glo Assay kit (Cat. #V6612) and a NADPH/NADP-Glo Assay kit (Cat. #S1008) were purchased from Promega (Madison, MI, USA). Growth factor-reduced Matrigel (Cat. #354230) and Annexin-V/PI assay kit (Cat. #556547) were purchased from BD Biosciences (Bedford, MA, USA). The Akt inhibitor LY294002 (Cat. #S1005), ERK1/2 inhibitor AZD-6244 (Cat. #S1008) and NF-κB inhibitor BAY11-7082 (Cat. #S2913) was purchased from Selleck Chemicals (Houston, TX, USA) and dissolved in DMSO. The MTHFD2 inhibitor LY345899 was synthesized by School of Pharmaceutical Sciences (Sun Yat-sen University, China).

RNA isolation and qPCR analysis

Total RNA was isoltated with TRIzol regent (Cat. # 15596-08, Life Technologies, Carlsbad, USA) and then reverse transcribed with an iScript cDNA Synthesis Kit (Cat. #170-8896, Bio-Rad, Hercules, USA). The resulting complementary DNA was analyzed by qPCR performed with SYBR reagent using the IQ5 PCR system (Bio-Rad, Hercules, USA). β-Actin was used as the internal control gene and data were analyzed using the $2^{-\Delta\Delta ct}$ method. The sequences of used primers were described as following: G6PD (forward: 5'-cgaggccgtcaccaagaac-3', reverse: 5'-gtagtggtcgatgcggtaga-3.); ME1 (forward: 5'-

gagtgctgacatctgacattga-3', reverse: 5'-ttggcttccgaaacaccaaac-3.); ME2 (forward: 5'-gtgcaggaatacggcctgata-3', reverse: 5'-aatgggtcttttaagagtgcgat-3.); IDH1 (forward: 5'-tgtggtagagatgcaaggaga-3', reverse: 5'-ttggtgacttggtcgttggtg-3.); IDH2 (forward: 5'-aaccgtgaccagactgatgac-3', reverse: 5'-atggtggcacacttgacagc-3.); MTHFD1 (forward: 5'-gttgaaggagcaagtacctgg-3', reverse: 5'-ggtagctgcactaagaaccca-3.); MTHFD2 (forward: 5'-gatcctggttggcgagaatcc-3', reverse: 5'-tctggaagaggcaactgaaca-3.); NNT (forward: 5'-tggtcaagcagggttttaatgt-3', reverse: 5'-tcctttgccccttggatttgg-3.); Kras (forward: 5'-acagagagtggaggatgcttt-3', reverse: 5'-tttcacacagccaggagtctt-3'.); c-Myc (forward: 5'-ggctcctggcaaaaggtca-3', reverse: 5'-ctgcgtagttgtgctgatgt-3'.); β-Actin (forward: 5'-catgtacgttgctatccaggc-3', reverse: 5'-ctccttaatgtcacgcacgat-3.) .

Immunoblotting and IHC analysis

Immunoblotting and IHC analysis were conducted with standard procedures as previously described [1]. Blotting membranes were stripped and re-probed with anti-β-Actin antibody as a loading control. The degree of immunostaining of formalin-fixed, paraffin-embedded sections was reviewed and scored independently by two pathologists, based on both the proportion of positively stained tumor cells (1, <25%; 2, 25%-50%; 3, 50%-75%; 4, 75%-100%) and the intensity of staining (0,1,2,3). The expression levels of MTHFD2 was considered high (⩾4) or low (<4) based on the final scores generated by multiplying the staining proportion scores with staining extent scores. Sections were also stained with hematoxylin and eosin (HE) according to standard procedures. The following antibodies were used for immunoblotting or IHC analysis: MTHFD2 (1:200, sc-100750), Kras (1:200, sc-30) (Santa Cruz, CA,

USA); c-Myc (1:1000, #ab32072); p-Akt (1:2000, #4060), AKt (1:1000, #4691), p-ERK1/2 (1:2000, #4370), ERK1/2 (1:1000,#4695), c-PARP (1:1000, #9185), N-cadherin (1:1000, #13116), E-cadherin (1:1000, #14472), β-Actin (1:1000, #3700) (Cell Signaling, Beverly, USA).

RNAi assay and lentiviral transduction

The siRNA targeting against the following genes were purchased from RiboBio (Guangzou, China), including MTHFD2 (#1: gcgagaatcctgcaagtca; #2: gcctcttccagagcatatt); Kras (#1: cgagtggttgtacgatgcattggtt; #2: gggtggtggtgtgccaagacattaa); c-Myc (#1: cgagctaaaacggagcttt; #2: cgagctaaaacggagcttt). Cells (~50% confluent) were transfected with 20 nM siRNA or NC using Lipofectamin3000 reagent according to manufacturer's instructions (Life Technologies). For rescue experiments, siRNA#2 resistant MTHFD2 with same-sense mutation (MTHFD2-R#2; mutant sequence: CCA CTG CCT GAA CAC ATC) was synthesized by GeneCopoeia (Rockville, USA). Short hairpin RNA (shRNA) directed against MTHFD2 were ligated into the pLV12 vector (GenePharma, Shanghai, China). Lentiviruses were generated by transfecting lentiviral vector pLV12 together with packaging vector psPAX2 and envelope plasmid pMD2.G into 293T cells, and the infected cells were selected with puromycin.

Sphere formation assay

Sphere-forming assay was preformed according to published protocol [2]. Briefly, single-cell suspensions were plated in six-well ultralow attachment plates (Corning Inc. Corning, USA) at a density of $2.0x10^3$ cells/well. Cells were cultured in serum-free Dulbecco's Modified Eagle Medium with nutrient mixture

F-12 (DMEM/F12, Mediatech Inc; Manassas, V) medium supplemented with 20 ng/ml epidermal growth factor (Miltenyi Biotec; Auburn, CA), 20 ng/ml of basic fibroblast growth factor (Miltenyi Biotec; Auburn, CA), and 2 mM L-glutamine (Mediatech Inc; Manassas, VA). The number and size of tumor spheres formed were evaluated by light microscopy after 7 days.

Cell apoptosis analysis

For cell apoptosis analysis, CRC cells were harvested, washed twice and resuspended in 500 µl of PBS plus Annexin V-FITC and propidium iodide (PI) (Cat. #556547, BD Biosciences, Bedford, USA). The degree of apoptosis was determined as the percentage of cells positive for Annexin V/PI evaluated by flow cytometry (FACSCalibur, Becton Dickinson) as described previously [3].

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using a Chromatin Immunoprecipitation Assay Kit (Cat. #17-2, Millipore, Bedford, MA, USA) according to the manufacturer's instructions as previously described [1]. CRC cells were grown to 80% confluence, and crosslinking was performed with 1% formaldehyde for 10 min. The cell lysates were sonicated to shear DNA to sizes of 300 to 1,000 bp. Equal aliquots of chromatin supernatants were incubated with anti-c-Myc or anti-IgG antibody (Millipore, Bedford, MA, USA) overnight at 4°C with rotation. After reverse cross-link of protein/DNA complexes to free DNA, RT-PCR was carried out using the specific primer (forward: 5’-accgtttttaggagactcattggt-3’, reverse: 5’-acttctagcctccaggaagga-3) detecting the c-Myc binding site on MTHFD2 promoter region.

Luciferase promoter assay

293T cells (~50% confluent) were split into a 48-well plate to achieve 50% confluence on the day of transfection. A dual luciferase reporter assay system (Promega, Madison, USA) was used per the manufacturer's protocol. Briefly, a mixture containing Lipofectamine LTX reagent (Invitrogen, Carlsbad, USA), 50 ng of luciferase pGL3-MTHFD2 promoter (~2000 bp upstream of the start site) plasmid DNA, and 50 ng of either vector control or fully sequenced c-Myc cDNA (GE Dharmacon, Lafayette, USA) was added to each well. All transfections were performed in quintuplicate. Luciferase and renilla signals were measured 24 h after transfection according to the recommended protocol.

References

1. Ju HQ, Ying H, Tian T*, et al.* Mutant Kras- and p16-regulated NOX4 activation overcomes metabolic checkpoints in development of pancreatic ductal adenocarcinoma. Nat Commun 2017;8:14437.
2. Ju HQ, Lu YX, Chen DL*, et al.* Redox Regulation of Stem-like Cells Though the CD44v-xCT Axis in Colorectal Cancer: Mechanisms and Therapeutic Implications. Theranostics 2016;6(8):1160-75.
3. Ju HQ, Gocho T, Aguilar M*, et al.* Mechanisms of Overcoming Intrinsic Resistance to Gemcitabine in Pancreatic Ductal Adenocarcinoma through the Redox Modulation. Mol Cancer Ther 2015;14(3):788-98.

Supplementary Tables

Table S1. Correlation between MTHFD2 expression and clinicopathological features in 462 primary CRC.

Parameters	Low expression N=279	High N=183	*P**
Age			
< 57	148 (53.0%)	99 (54.1%)	
≥57	131 (47.0%)	84 (45.9%)	.84
Gender			
Male	163 (58.4%)	110 (60.1%)	
Female	116 (41.6%)	73 (39.9%)	.77
Differentiation status			
Well/Moderate	61 (21.9%)	43 (23.5%)	
Poor and others	218 (78.1%)	140 (76.5%)	.73
Tumor depth (T stage)			
m/sm/mp	22 (7.9%)	13 (7.1%)	
ss/se/si	257 (92.1%)	170 (92.9%)	.85
Lymph node invasion (N stage)			
Absent	105 (37.6%)	82 (44.8%)	
Present	174 (62.4%)	101 (55.2%)	.14
Vascular invasion			
Absent	214 (76.7%)	121 (66.4%)	
Present	65 (23.3%)	62 (33.9%)	.01
Perineural invasion			
Absent	138 (49.5%)	92 (50.3%)	
Present	141 (50.5%)	91 (49.7%)	.92
Clinical stage			
I, II	94 (33.7%)	73 (39.9%)	
III, IV	185 (66.3%)	110 (60.1%)	.19

**P* values determined by Chi-square test using SPSS 20.0. All statistical tests were two-sided. CRC=colorectal cancer; m=tumor invasion of mucosa; p=muscularis propria; sm=submocosa; ss=subserosa; se=serosa penetration; si=invasion to adjacent structures.

Table S2. Effect of factors on overall survival in CRC patients in the univariate and multivariate cox regression model.

	Univariate *		Multivariate *, †, ‡	
Factors	HR (95% CI)	*P*	HR (95% CI) *	*P*
Age (<60/≥60)	1.28 (0.90-1.84)	.17	—	—
Gender (male/female)	1.58 (1.08-2.33)	.02	1.40 (0.94-2.08)	.09
Differentiation (poor/well, moderate)	1.64 (1.11-2.42)	.01	1.22 (0.82-1.83)	.33
Clinical Stage (III-IV/I-II)	4.49 (2.65-7.62)	<.001	1.07 (0.40-2.88)	.88
Tumor depth (m, sm, mp/ss, se, si)	2.83 (1.04-7.69)	.04	1.13 (0.40-3.17)	.81
Lymph node invasion (present/absent)	3.38 (2.14-5.33)	<.001	2.47 (1.10-5.55)	.02
Vascular invasion (abnormal/normal)	4.45 (3.09-6.41)	<.001	1.99 (1.28-3.07)	.002
Perineural invasion (abnormal/normal)	3.40 (2.26-5.10)	<.001	1.71 (1.08-2.72)	.02
Recrudesce (present/absent)	4.69 (3.26-6.76)	<.001	3.21 (2.19-4.72)	<.001
MTHFD2 (high/low)	1.60 (1.11-2.29)	.01	1.55 (1.07-2.23)	.02

* Hazard ratios and *P* values were obtained from Cox proportional hazards regression. All statistical test were two-sided. † For the multivariate model, HR and *P* values were shown for the final set of stepwise selected variables only. ‡ The parameters with *P* value less than .05 in the univariate were included in the multivariate Cox analysis using SPSS 20.0. CRC=colorectal cancer; m= tumor invasion of mucosa; sm=submocosa; mp=muscularis propria; ss=subserosa; se=serosa penetration; si=invasion to adjacent structures.

Supplementary Figure 1

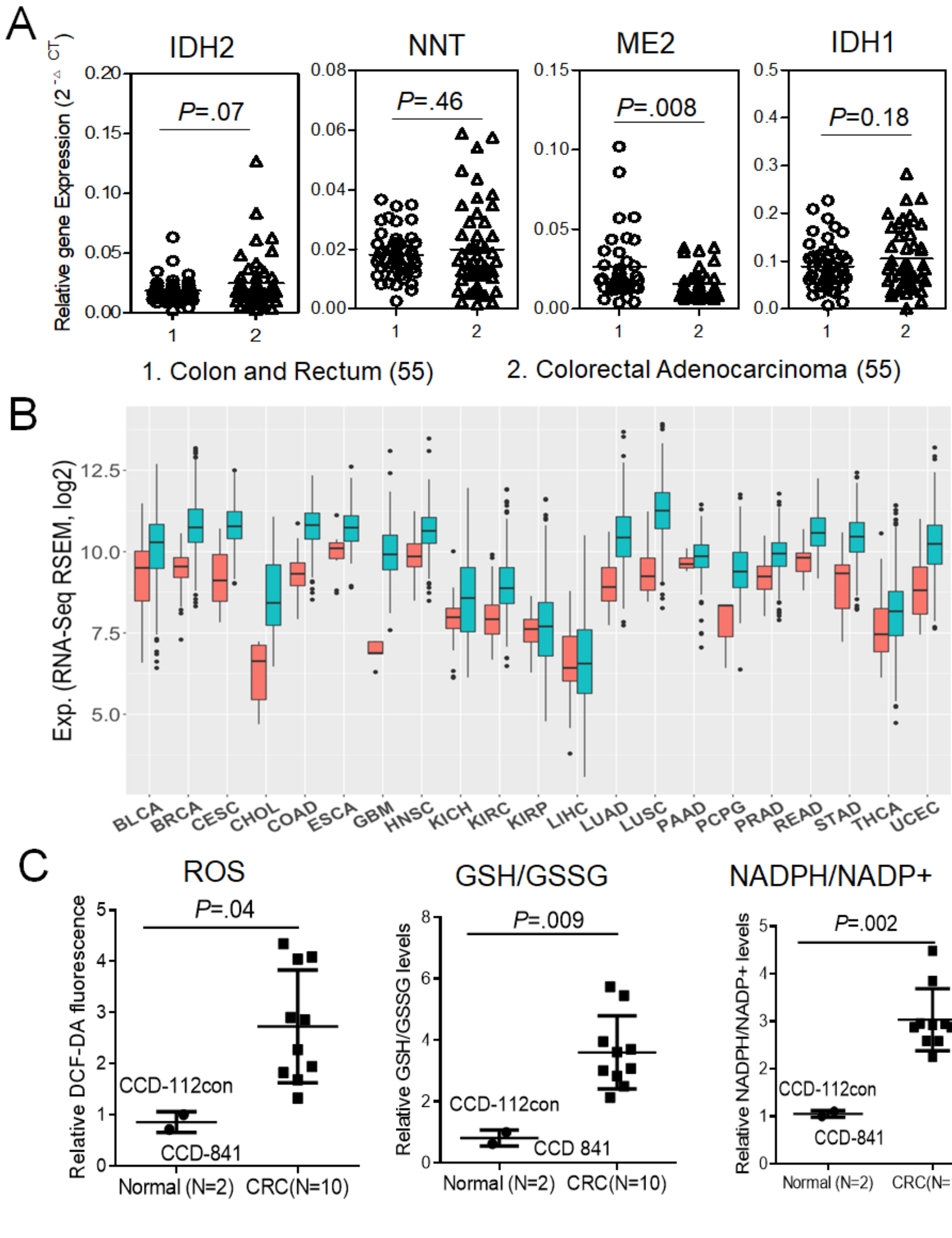


Figure S1. Redox status in CRC cells and MTHFD2 levels in human tumors. **A)** qPCR analysis of expression of these enzymes (including ME2, IDH1, IDH2, NNT) in 55 paired CRC tissues and tumor-adjacent tissues. The horizontal line indicates the median and the analyses were performed using Student's paired *t*-test. **B)** MTHFD2 is overexpressed in multiple human tumors (TCGA database). **C)** Cellular ROS (H_2O_2, CM-H2DCF-DA as a fluorescent probe), NADPH/NADP+ and GSH/GSSG levels were measured in CRC (N=10) and normal cell lines (N=2). Data are presented as the mean ± SD, and statistical analyses were performed using Student's unpaired t-test. All statistical tests were two-sided. CRC=colorectal cancer; ME2=malic enzyme 2; IDH1=isocitrate dehydrogenase 1; IDH2=isocitrate dehydrogenase 2; NNT=transhydrogenase; MTHFD2=methylene tetrahydrofolate dehydrogenase 2; ROS=reactive oxygen species; NADP=nicotinamide adenine dinucleotide phosphate; GSH=reduced glutathione; GSSG=oxidized glutathione; TCGA=The Cancer Genome Atlas.

Supplementary Figure 2

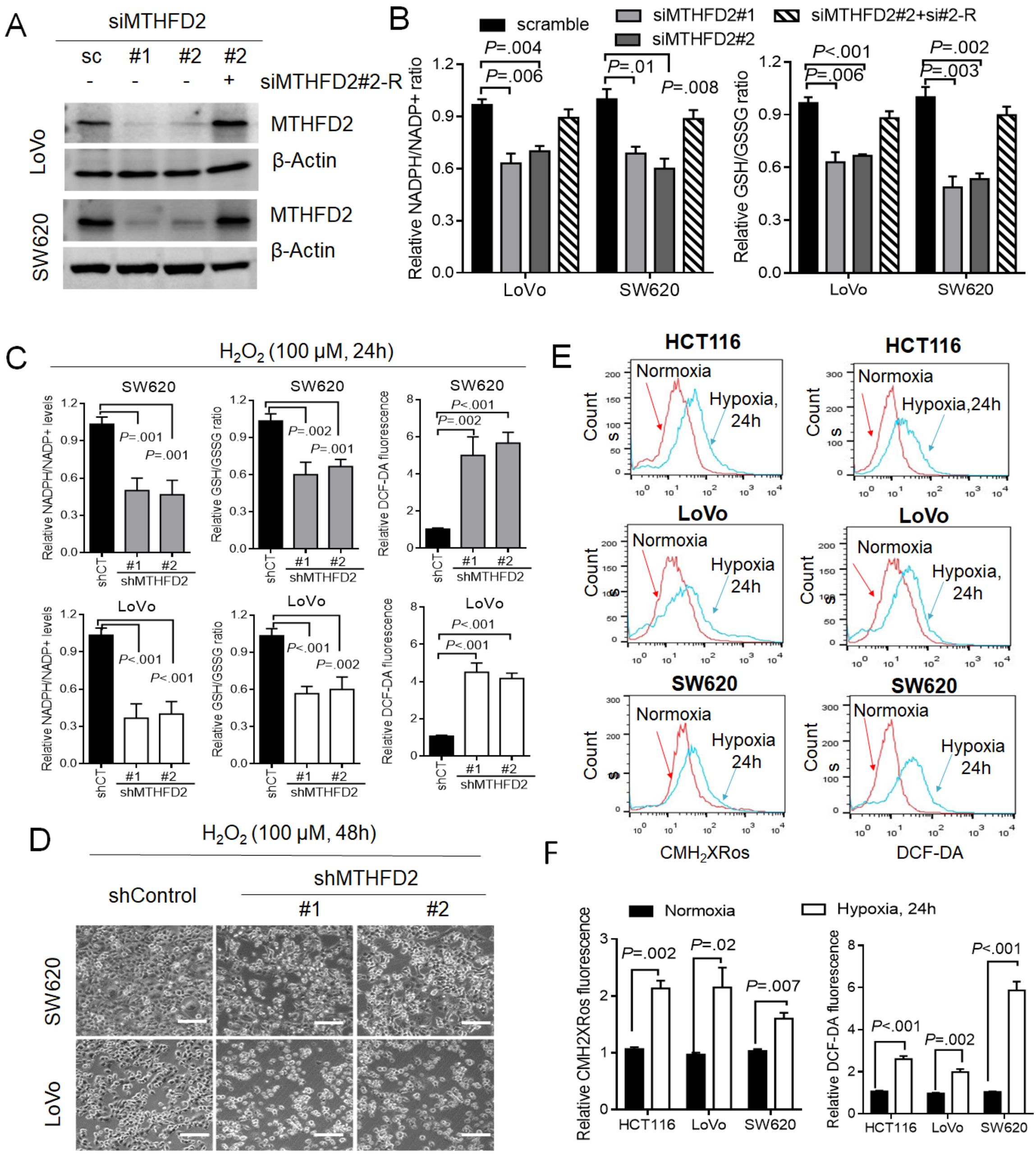
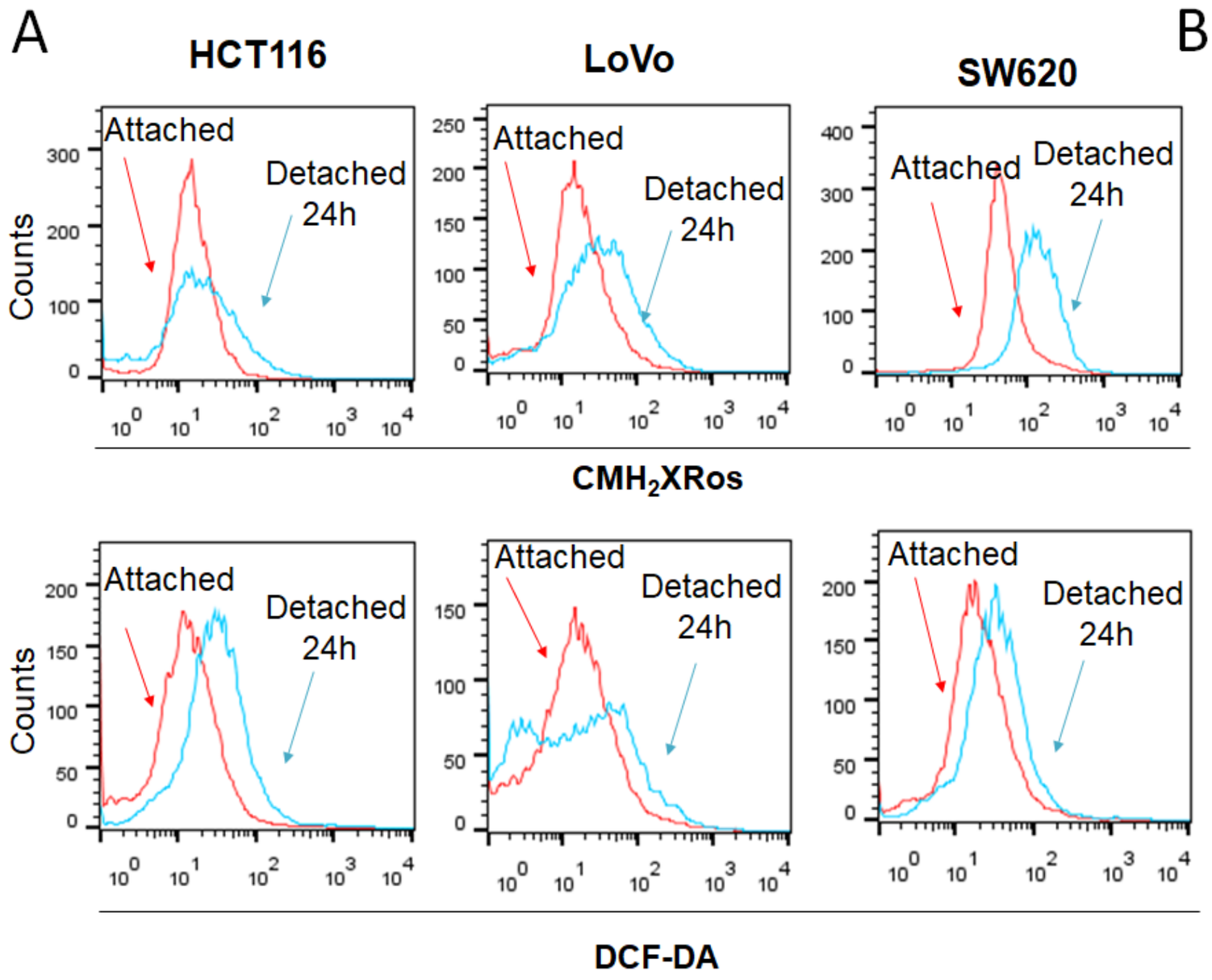
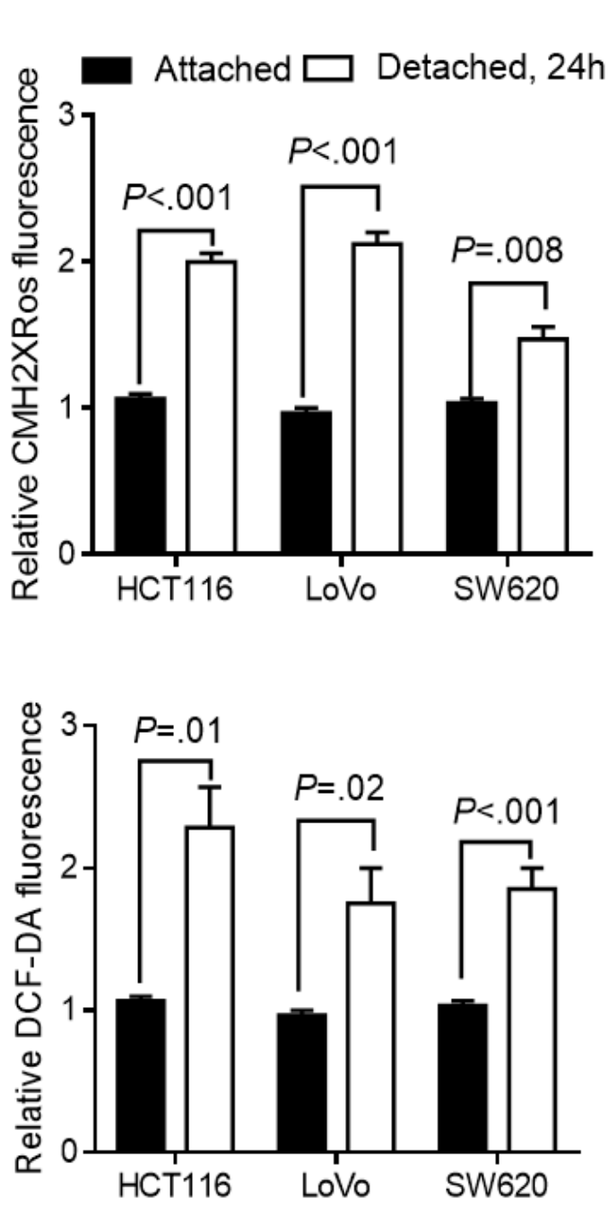
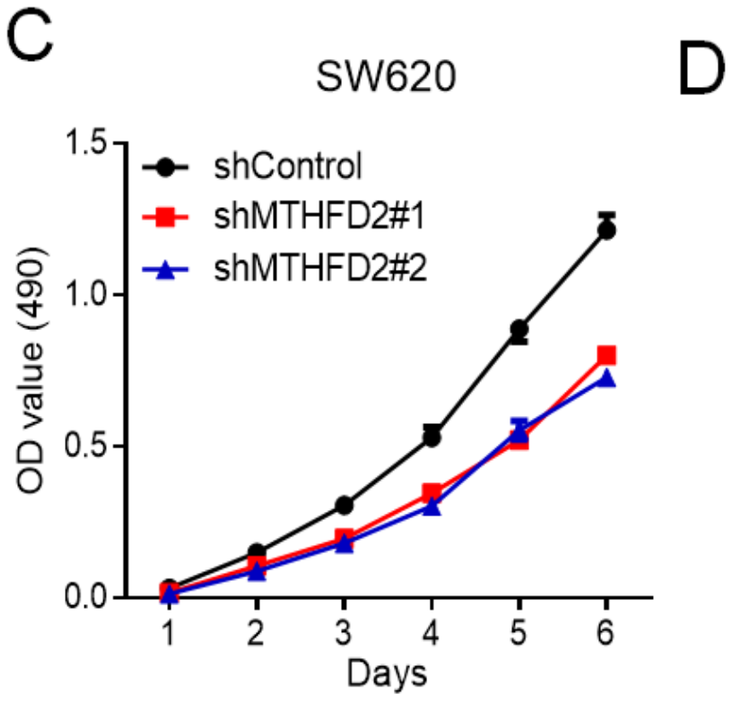


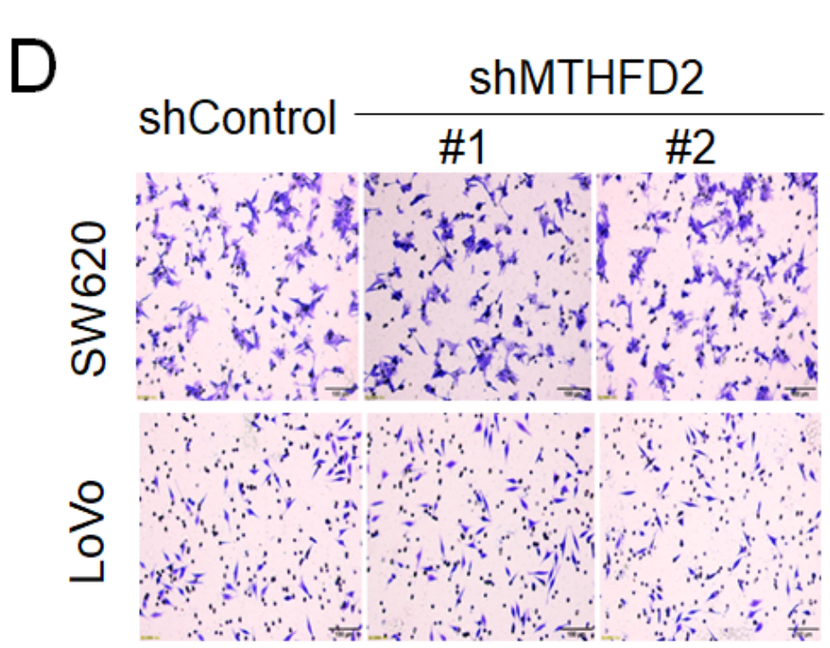
Figure S2. Effects of MTHFD2 suppression on NADPH homeostasis and cell survival in CRC. **A)** Immunoblotting analysis of MTHFD2 expression in SW620 and LoVo cells after siRNAs (#1 and #2) depletion of MTHFD2 or overexpressing siRNA#2 resistant MTHFD2 (siMTHFD2#2-R) in indicated CRC cells. **B)** NADPH/NADP+ and GSH/GSSG levels were measured in indicated control or MTHFD2-knockdown CRC cells. **C**) Relative NADPH/NADP+, GSH/GSSG and ROS levels were measured in the indicated cells treated with H_2O_2 (100 μM) for 24 h. **D)** Bright field images of indicated CRC cells treated with H_2O_2 (100 μM) for 48 h. **E-F)** Representative histogram and quantification of mitochondrial and cellular ROS levels was measured in the indicated cells under normoxia or hypoxia (1% O_2) for 24 h, as detected by flow cytometry using CMH_2XRos and CM-H2DCF-DA as fluorescent probes. Data in B, C, E are presented as the mean ± SD (n=3). All statistical analyses were performed using Student's unpaired *t*-test. All statistical tests were two-sided. NADP=nicotinamide adenine dinucleotide phosphate; GSH=reduced glutathione; GSSG=oxidized glutathione; H_2O_2=hydrogen peroxide; ROS= reactive oxygen species;

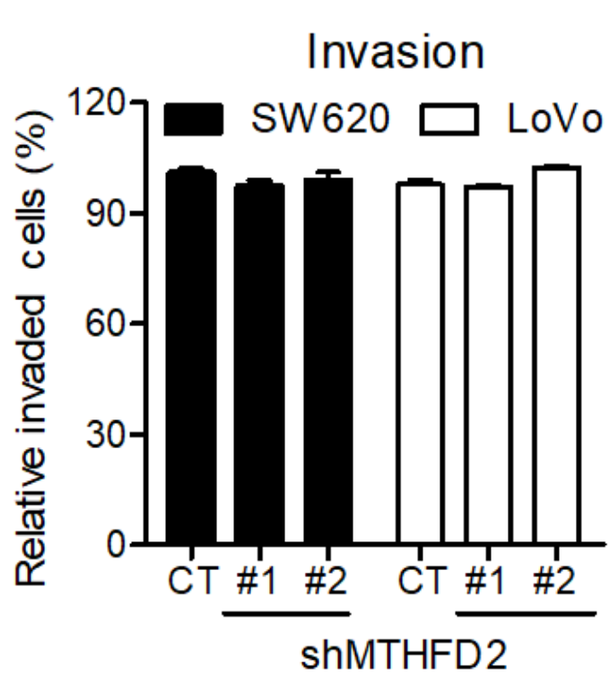
Supplementary Figure 3











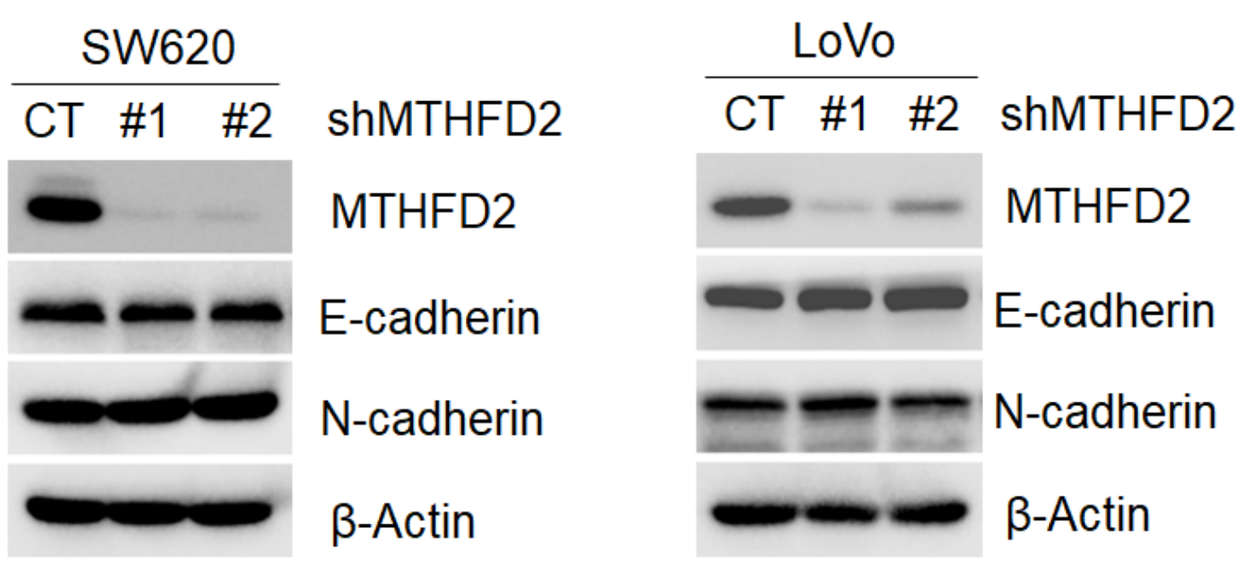
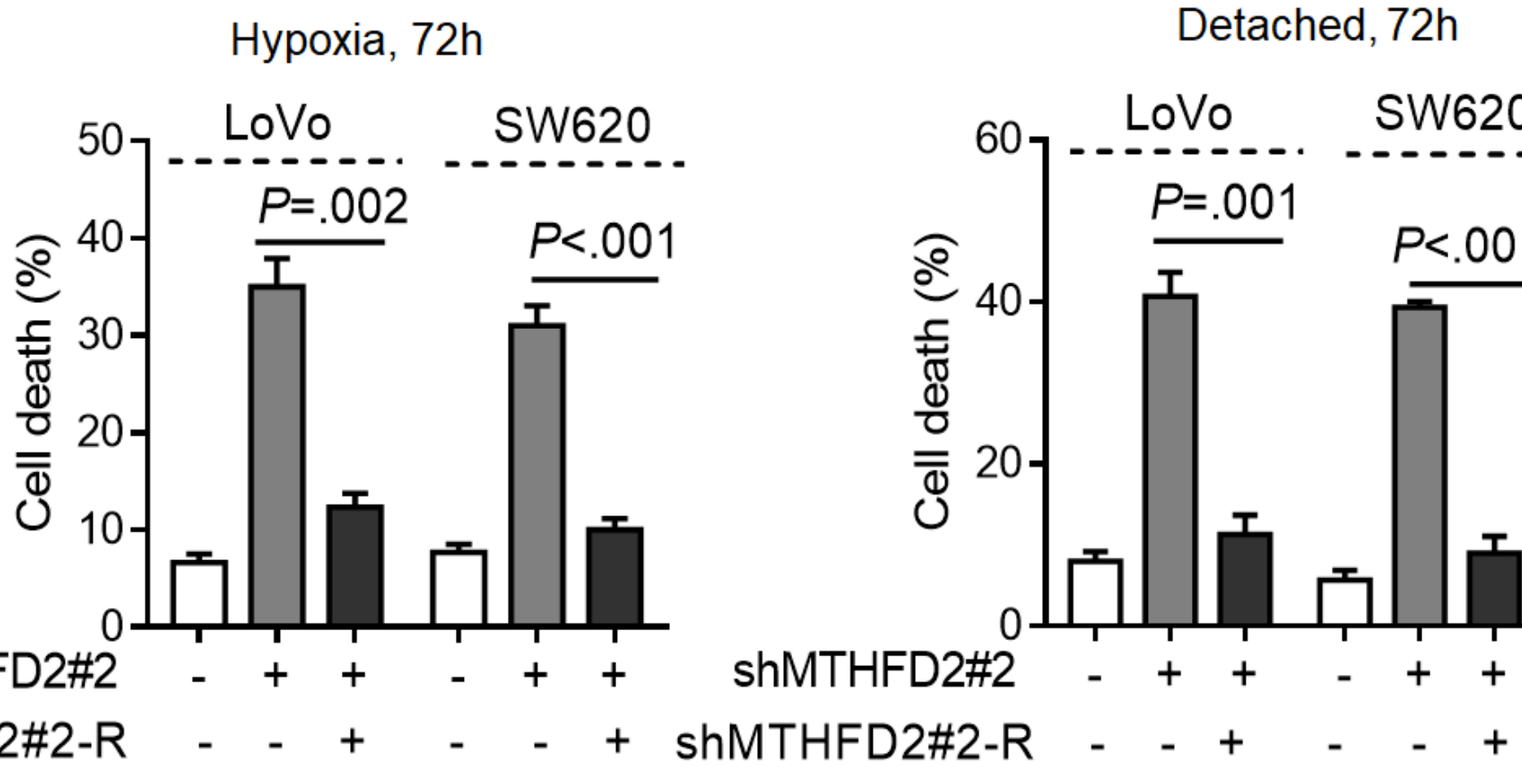


Figure S3. Effects of MTHFD2 suppression on CRC cell growth and metastasis. **A-B)** Representative histogram and quantification of mitochondrial and cellular ROS levels was measured in the indicated cells for 24 h after plating in adherent (Attached) or nonadherent (poly-HEMA-coated) plates (Detached). **C)** Growth curves of the indicated MTHFD2-knockdown and control CRC cells were measured by MTS assay. **D)** Images and quantification of invaded CRC cells with knockdown of MTHFD2 using two independent siRNAs (scale bar: 100 μm). **E)** Immunoblotting analysis of MTHFD2, E-cadherin and N-cadherin expression in the indicated CRC cells with knockdown of MTHFD2 using two independent siRNAs. Data in B, D are presented as the mean ± SD (n=3). All statistical analyses were performed using Student's unpaired *t*-test. All statistical tests were two-sided. CRC=colorectal cancer; ROS=reactive oxygen species; CT=control.

Supplementary Figure 4

A



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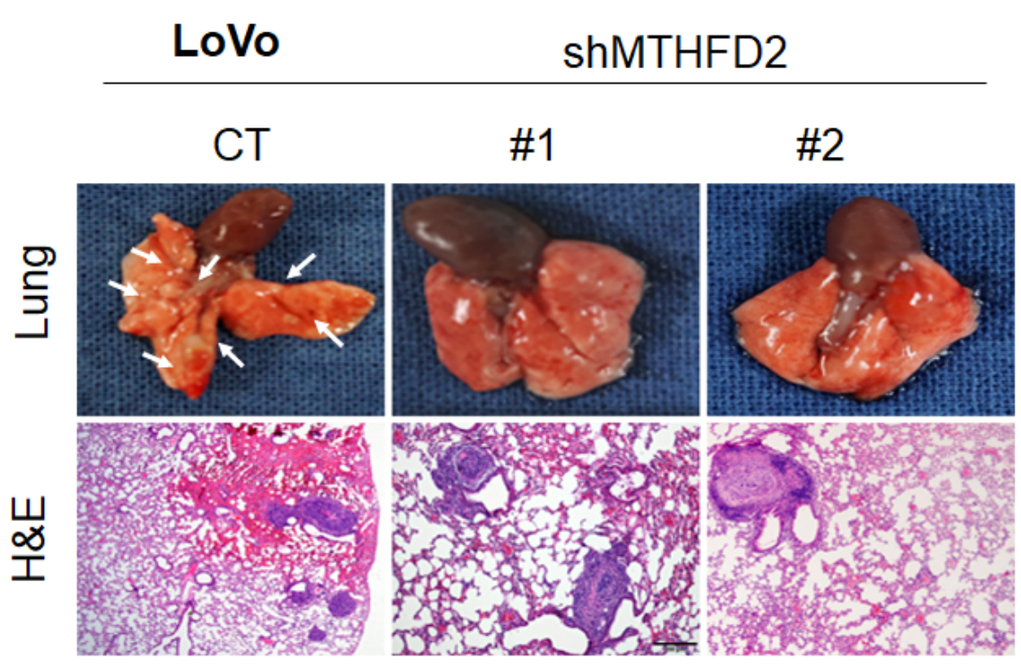

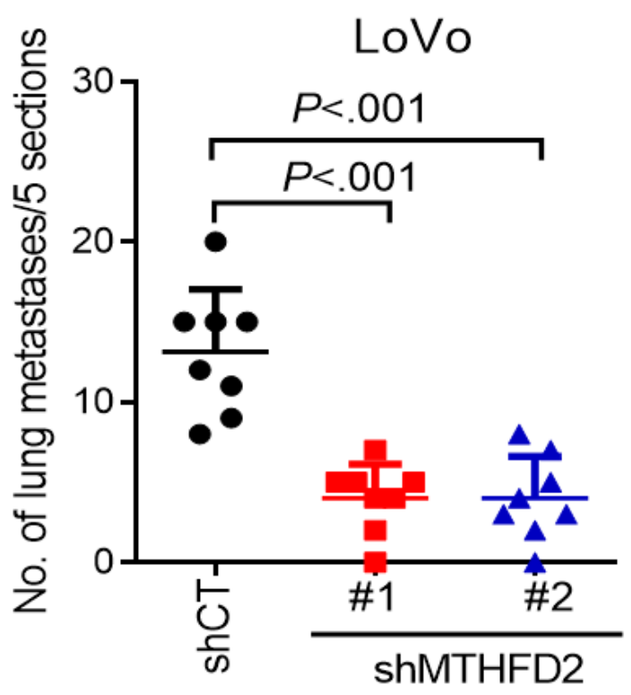

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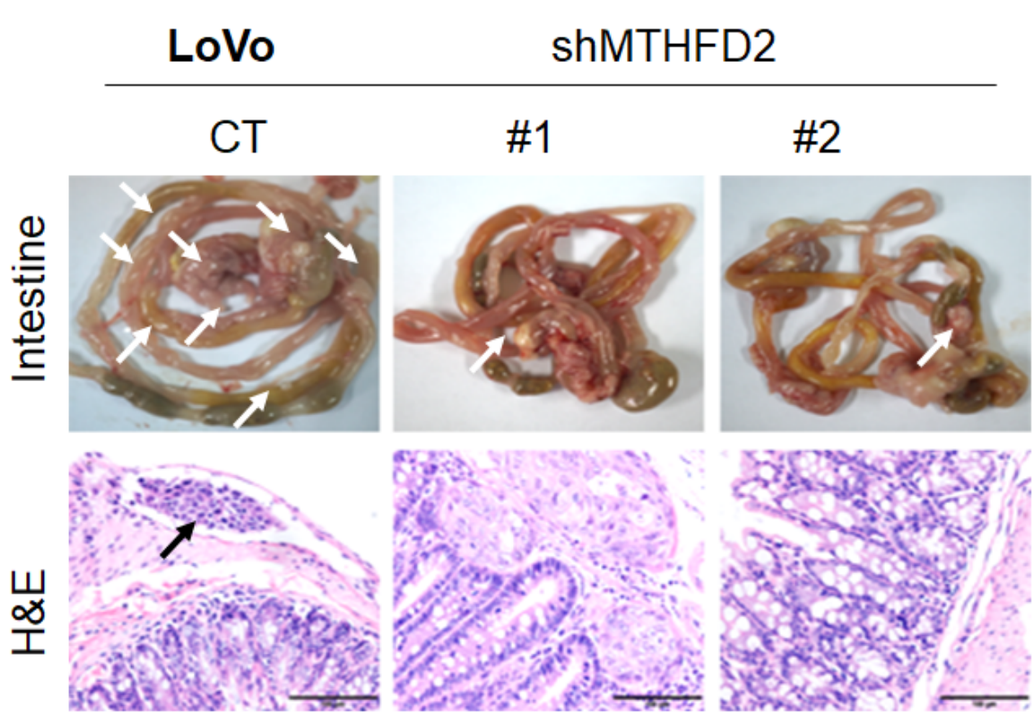


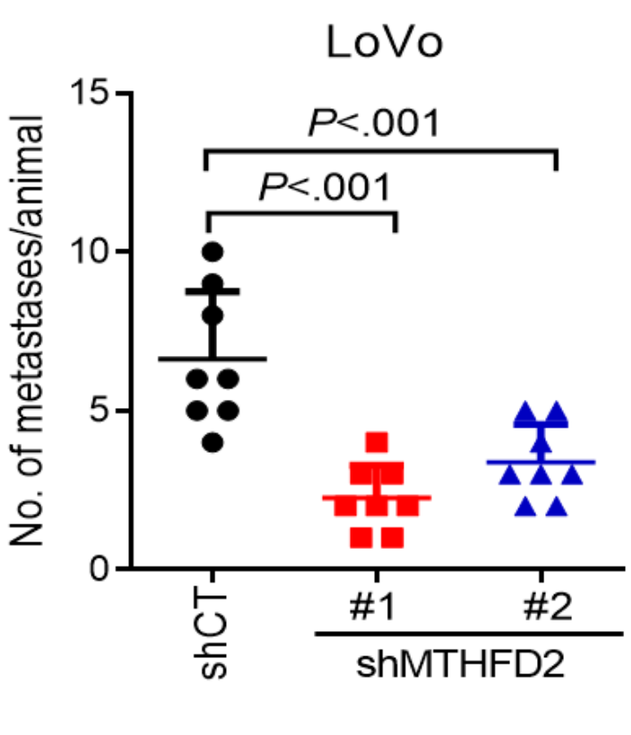
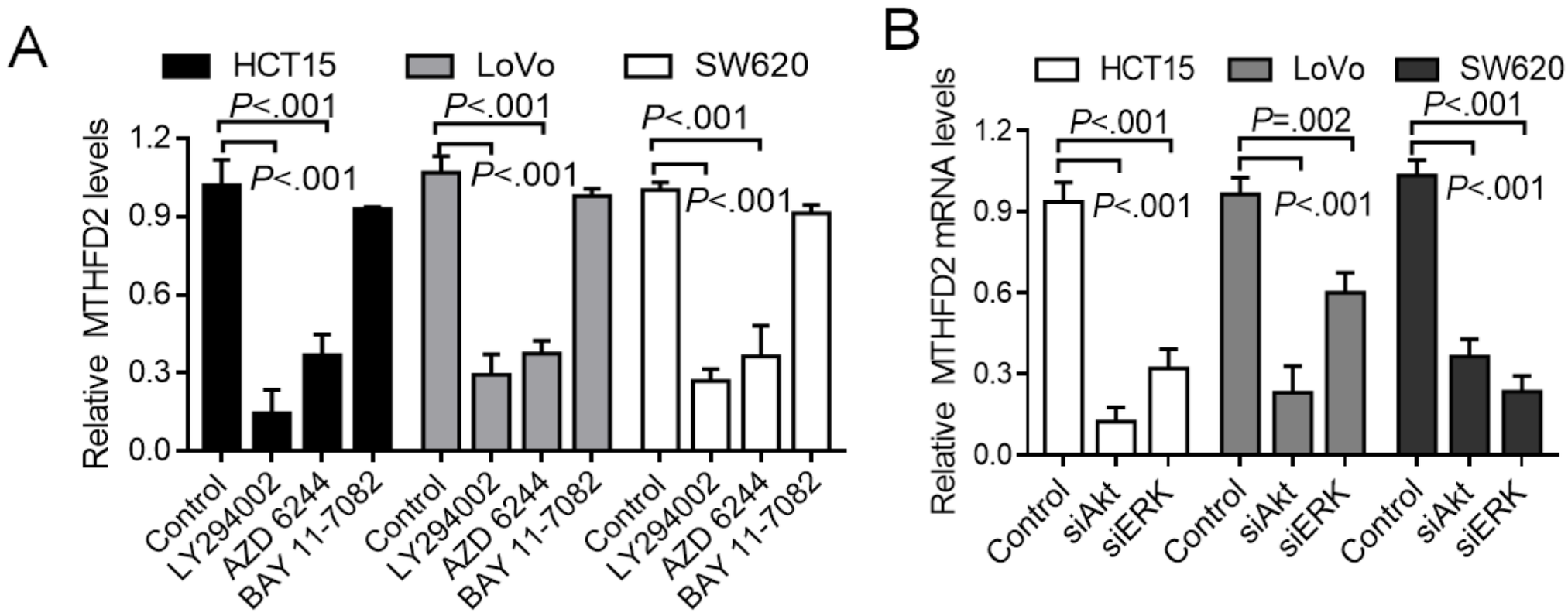


Figure S4. Effects of MTHFD2 inhibition on CRC tumorigenesis and metastasis. **A)** Cell death was measured by Annexin-V/PI assays in the indicated MTHFD2 knockdown CRC cells overexpressing siRNA#2 resistant MTHFD2 (siMTHFD2#2-R) under hypoxia or detachment for 72h. **B)** Representative H&E staining and statistical results of metastatic lung nodules from mice injected via the tail vein with MTHFD2 knockdown and control LoVo cells for 60 days. (5 sections evaluated per lung). **C)** Images (upper) and statistical results of scattered tumors in the excised intestines of mice (N=8) orthotopically implanted with the indicated cells. Representative H&E staining (lower) of tumors formed by the indicated cells. Arrows indicate the metastatic foci. (scale bar: 100 μm. Data are presented as the mean ± SD (n=3). All statistical analyses were performed using Student's unpaired *t*-test. All statistical tests were two-sided. CRC=colorectal cancer; CT=control; H&E=haematoxylin and eosin staining.

Supplementary Figure 5



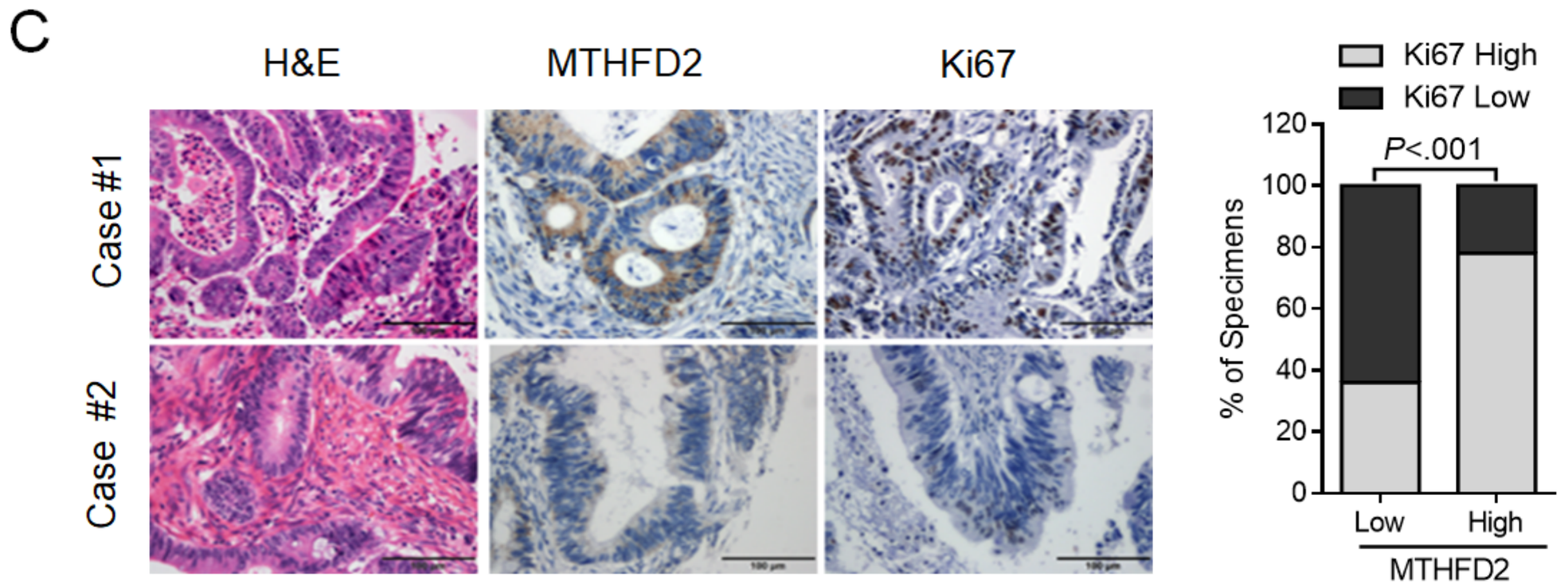
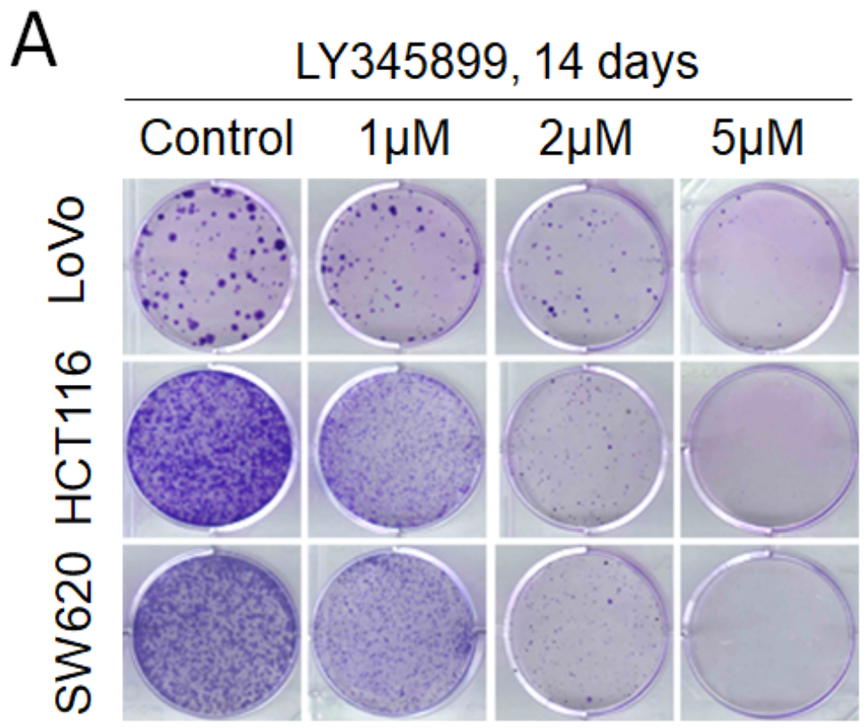
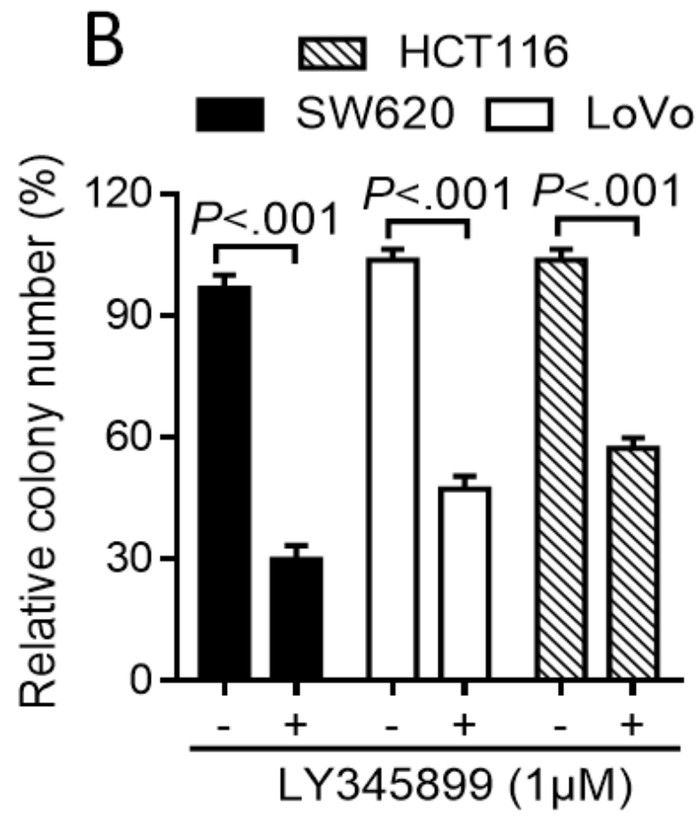
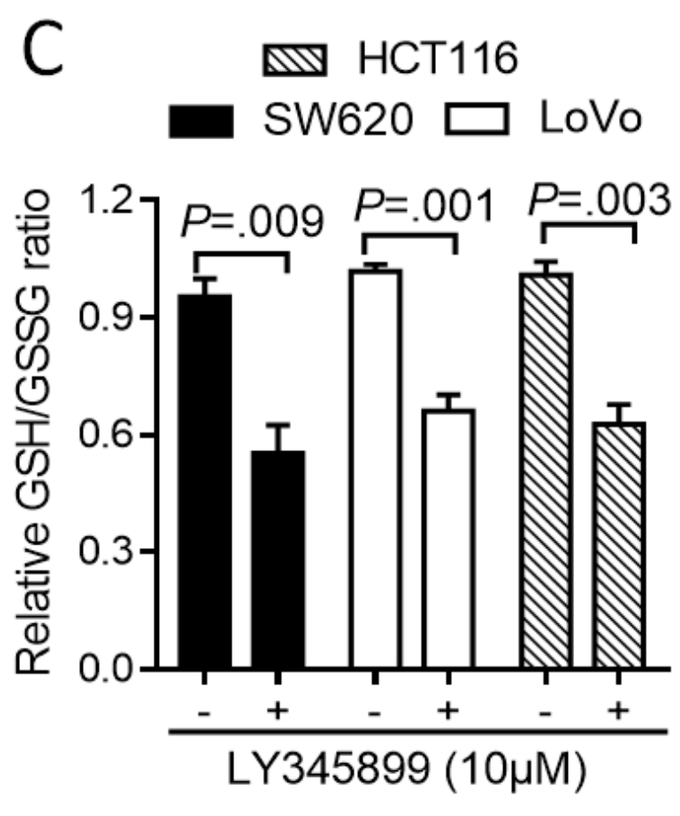


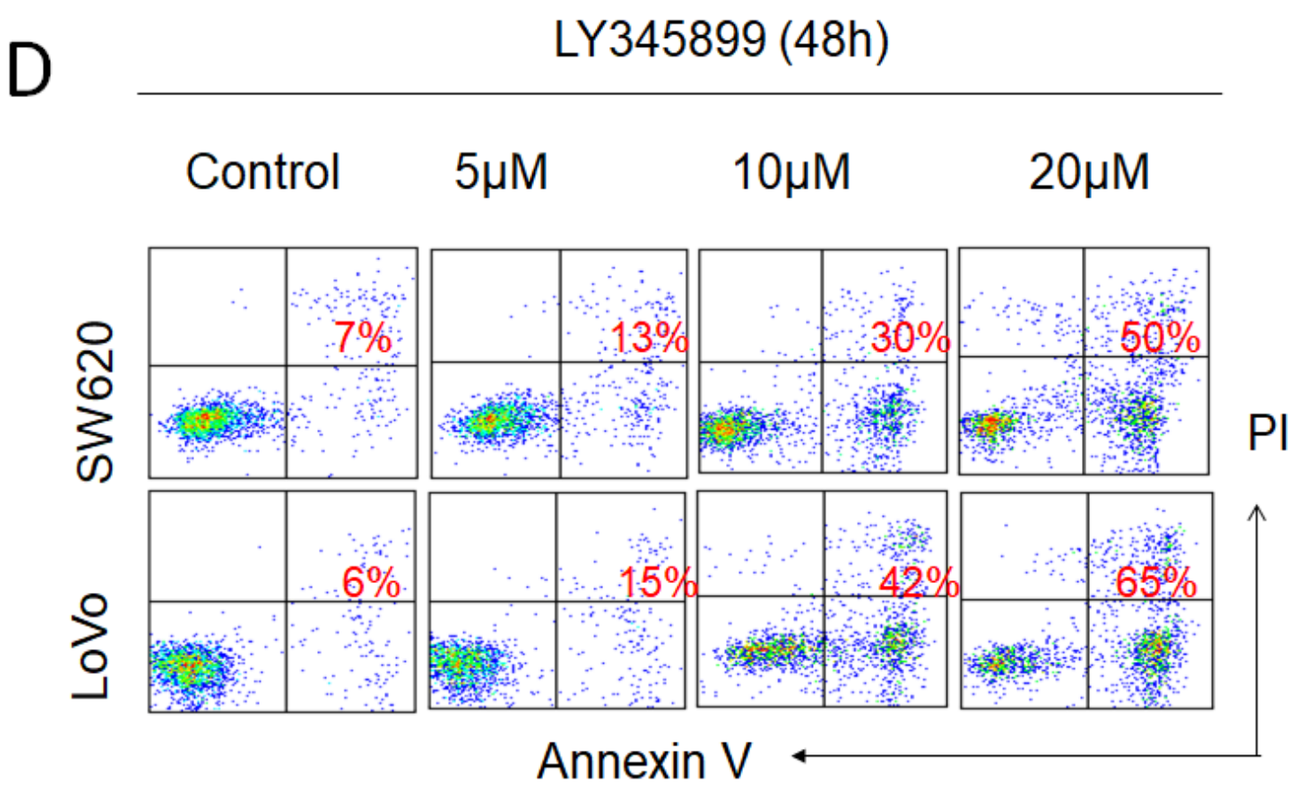
Figure S5. Signaling pathway involved in MTHFD2 expression in CRC. **A)** qPCR analysis of MTHFD2 expression in the indicated cells treated with the Akt inhibitor LY294002 (10 μM), the ERK1/2 inhibitor AZD-6244 (2 μM) and the NF-κB inhibitor BAY11-7082 (10 μM) for 48 h. **B)** qPCR analysis of the MTHFD2 mRNA expression in the indicated CRC cells transfected with siRNA targeting the Akt or ERK pathway. **C)** MTHFD2 expression was inversely associated with Ki67 expression in 462 clinical CRC specimens. Shown are the visualizations of two representative cases (Scale bars: 100 μm) and percentages of samples showing low or high MTHFD2 expression relative to Ki67. Data in A, B are presented as the mean ± SD (n=3), and statistical analyses were performed using Student's unpaired *t*-test. Chi-square test was used to study the association between MTHFD2 and Ki67 expression. All statistical tests were two-sided. CRC=colorectal cancer; H&E=haematoxylin and eosin staining.

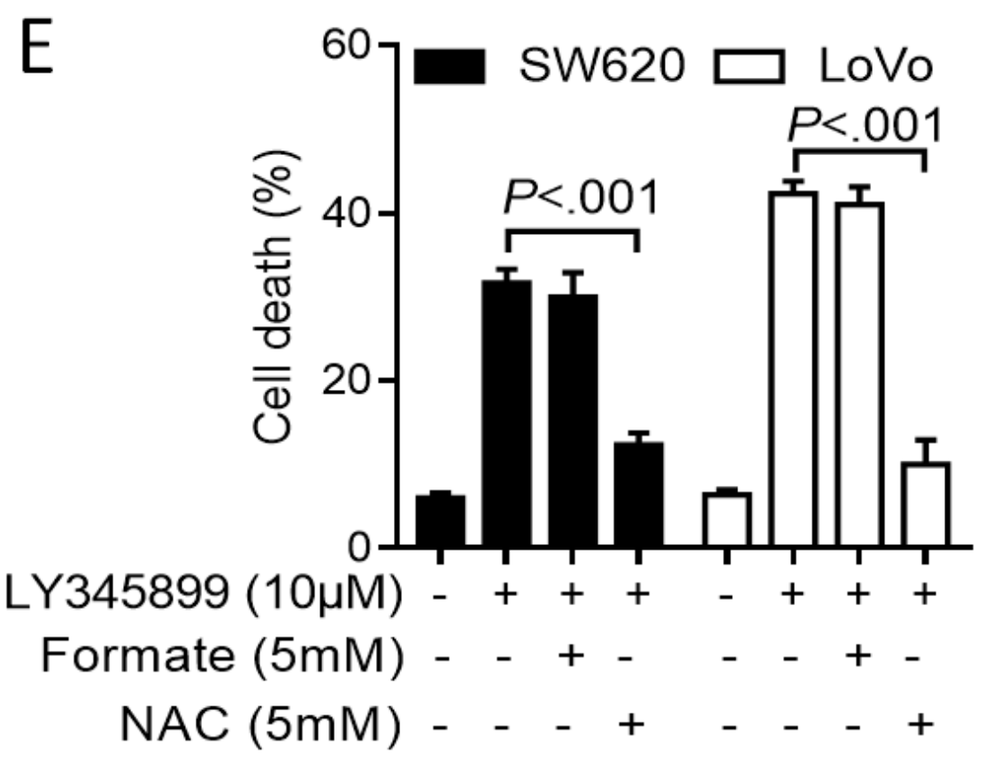
Supplementary Figure 6

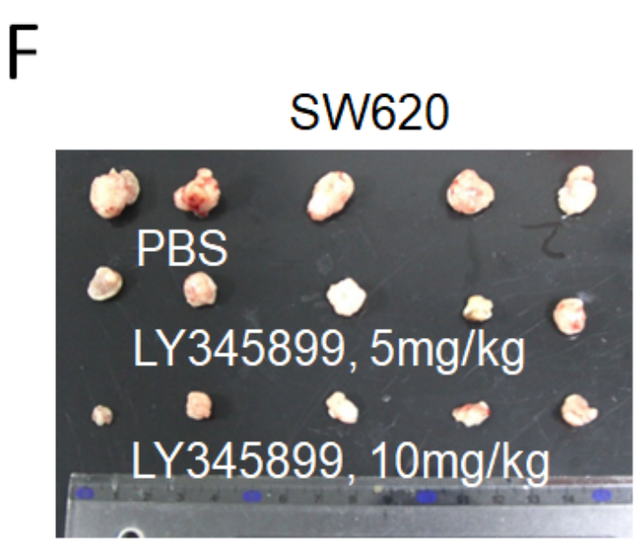


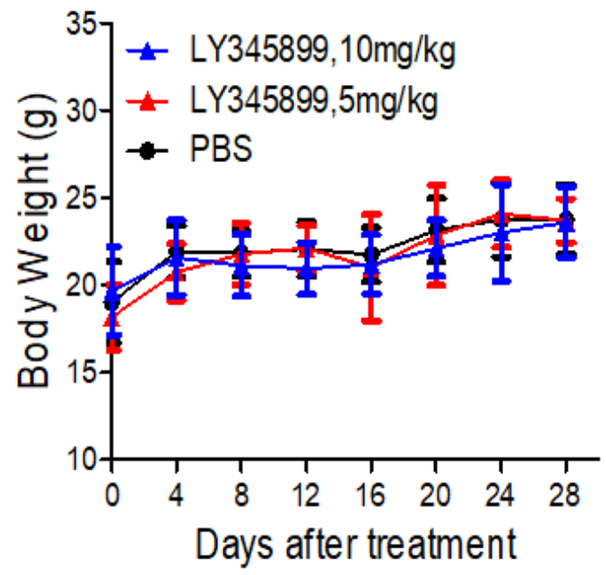


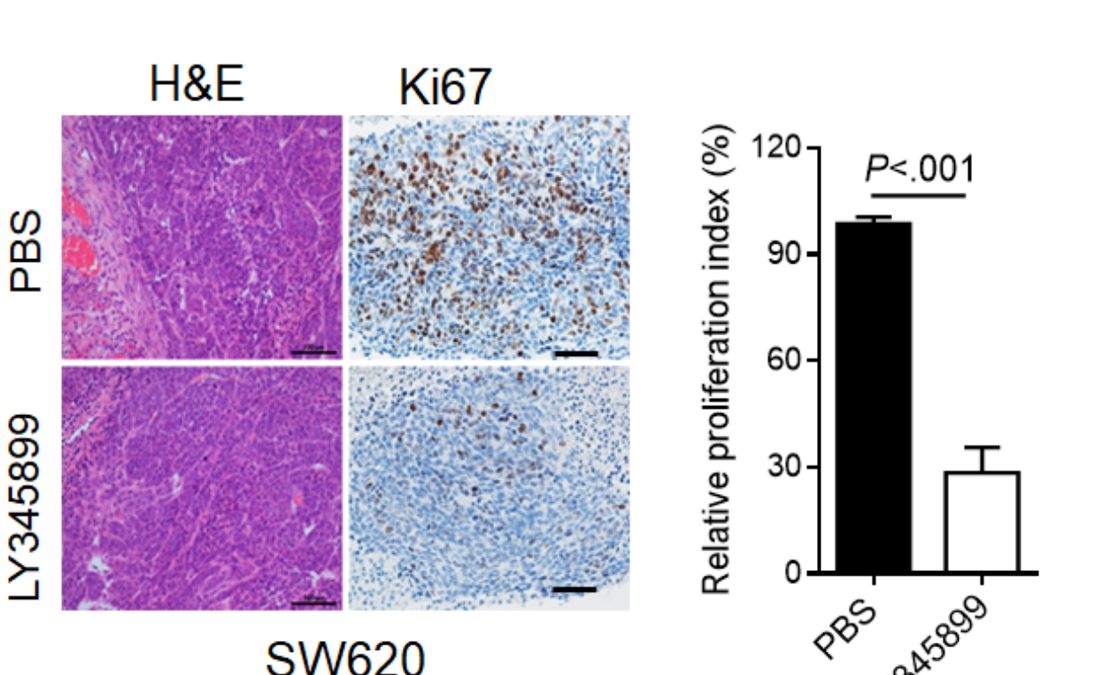


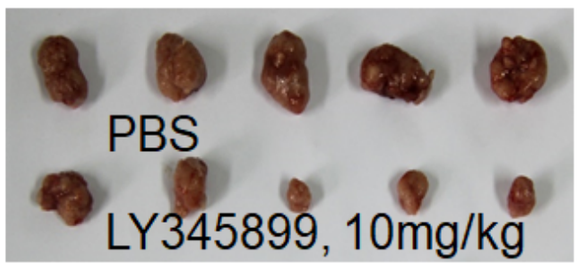












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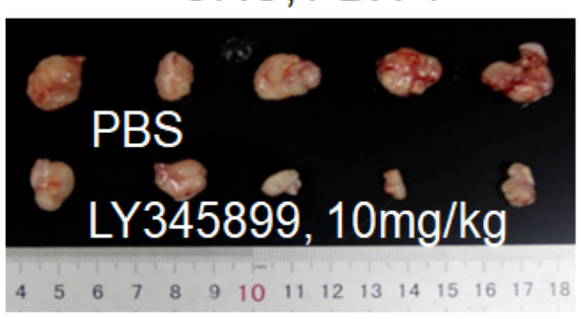

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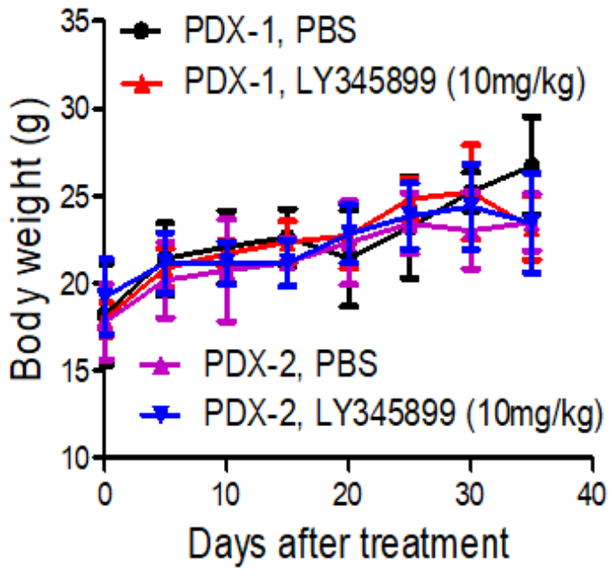

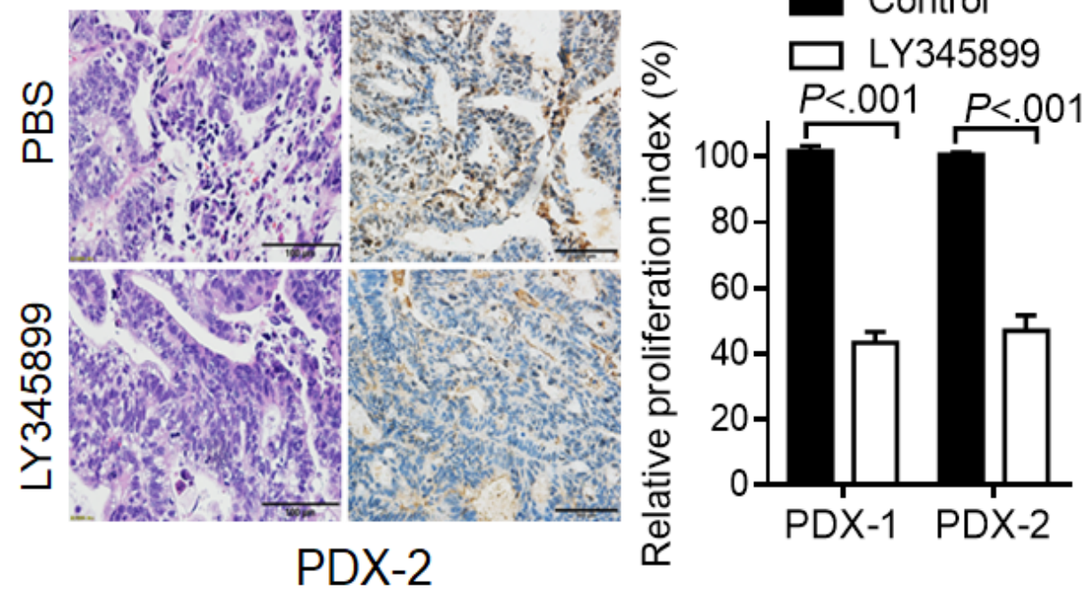

Figure S6. Antitumor activity of MTHFD2 inhibitor LY345899 in CRC. **A-B)** Images and quantification of cell colonies in the indicated CRC cells incubated with LY345899 for 2 weeks. **C)** The relative GSH/GSSG levels were measured in the indicated CRC cells treated with LY345899 for 24h (10μM). **D)** Representative and statistical results of Annexin V/PI staining in the indicated CRC cells treated with LY345899 for 48h. **E)** Statistical results of cell death in the indicated CRC cells treated with LY345899 for 48h with and without formate (5 mM) or NAC (5mM) supplementation in media. **F)** Photograph of excised tumor size, and the mice body weights were measured and recorded for SW620 or PDX (#1, #2)-based xenograft mice treated with LY345899 (N=5, 5mg/kg or 10mg/kg). **G)** Representative and quantification of Ki67 staining for proliferation in the indicated groups (scale bar: 100 μm). All statistical analyses were performed using Student's unpaired *t*-test. All statistical tests were two-sided. GSH=reduced glutathione; GSSG=oxidized glutathione; CRC=colorectal cancer; NAC=*N*-acetyl-*L*-cysteine PDX=patient derived xenograft; PBS=phosphate buffered saline; H&E=haematoxylin and eosin staining.