The YAP1/SIX2 axis is required for DDX3-mediated tumor aggressiveness and cetuximab resistance in *KRAS*-wild-type colorectal cancer

Wu et al.,

Supplementary Materials and methods

MiR-29c mimics and inhibitor transfection

Cells were grown to confluence in 6-well plates. The miR-29c mimics (40 nM) (Ambion, Foster city, CA), miR-29c inhibitors (80 nM per well) (Ambion, Foster city, CA, USA) and negative control (Ambion, , Foster city, CA) cells were transfected using Lipofectamine 3000 transfection reagent (Invitrogen, Foster city, CA) according to the manufacturer's protocol. Transfection efficiency was evaluated by the real-time polymerase chain reaction (PCR).

Real-time RT-PCR analysis of miR-29c mRNA expression levels

DNase I-treated total RNA (10 ng) was subjected to microRNA polymerase chain reaction (PCR) analysis with the TaqMan® miRNA Reverse Transcription Kit (Life technologies, Foster city, CA), miRNA Assays (Life technologies, Foster city, CA), and a Real-Time Thermocycler 7500 (Life technologies, Foster city, CA). RNU6B was used as the small RNA reference housekeeping gene.

Colony formation assay

Cells were transfected with indicated plasmids for 24 h. The cells were plated in 6-well plates in complement media for another 10 days. Before the pictures of these colonies were taken, cells were stained with 0.01% crystal violet for 1 h at room temperature.

Supplementary Figures

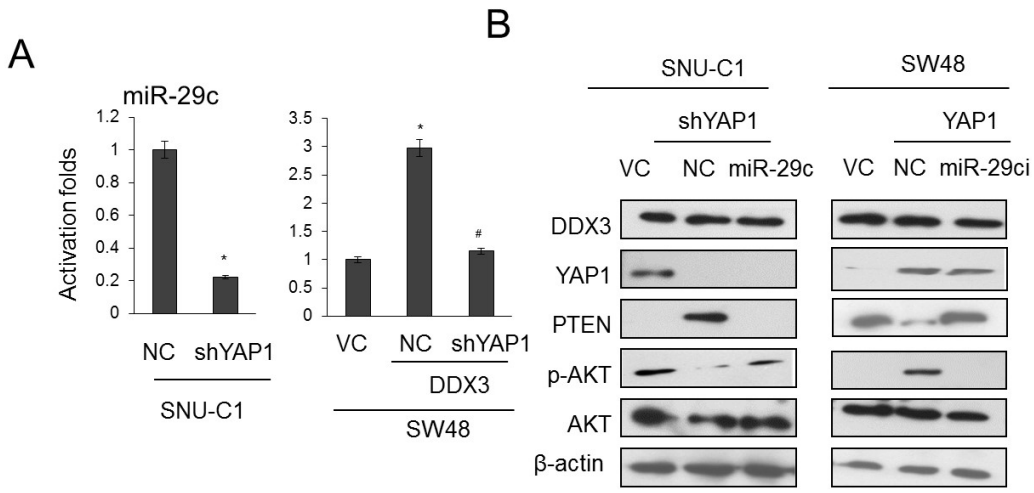

Figure S1. DDX3-induced YAP1 expression elevated miR-29c expression, and then PTEN targeted by miR-29c to activate PI3K/AKT signaling. (A) In the SNU-C1 and DDX3-overexpressing SW48 cells, YAP1 was knocked down by YAP1 shRNA. The miR-29c expression was determined by real-time PCR. (B) SNU-C1 cells were transfected with the indicated combination of YAP1 shRNA and miR-29c precursor for 48 h. SW48 cells were transfected with the indicated combination of YAP1 expression vector and miR-29c inhibitor for 48 h. The expression of DDX3, YAP1, PTEN, p-AKT, AKT and β-actin was determined by western blotting. P value was calculated by the Student's *t*-test. The significant differences in experimental groups were compared to VC or NC (*$P < 0.05$). The significant differences in experimental groups were compared to the group of DDX3-overexpression alone (#$P < 0.05$).

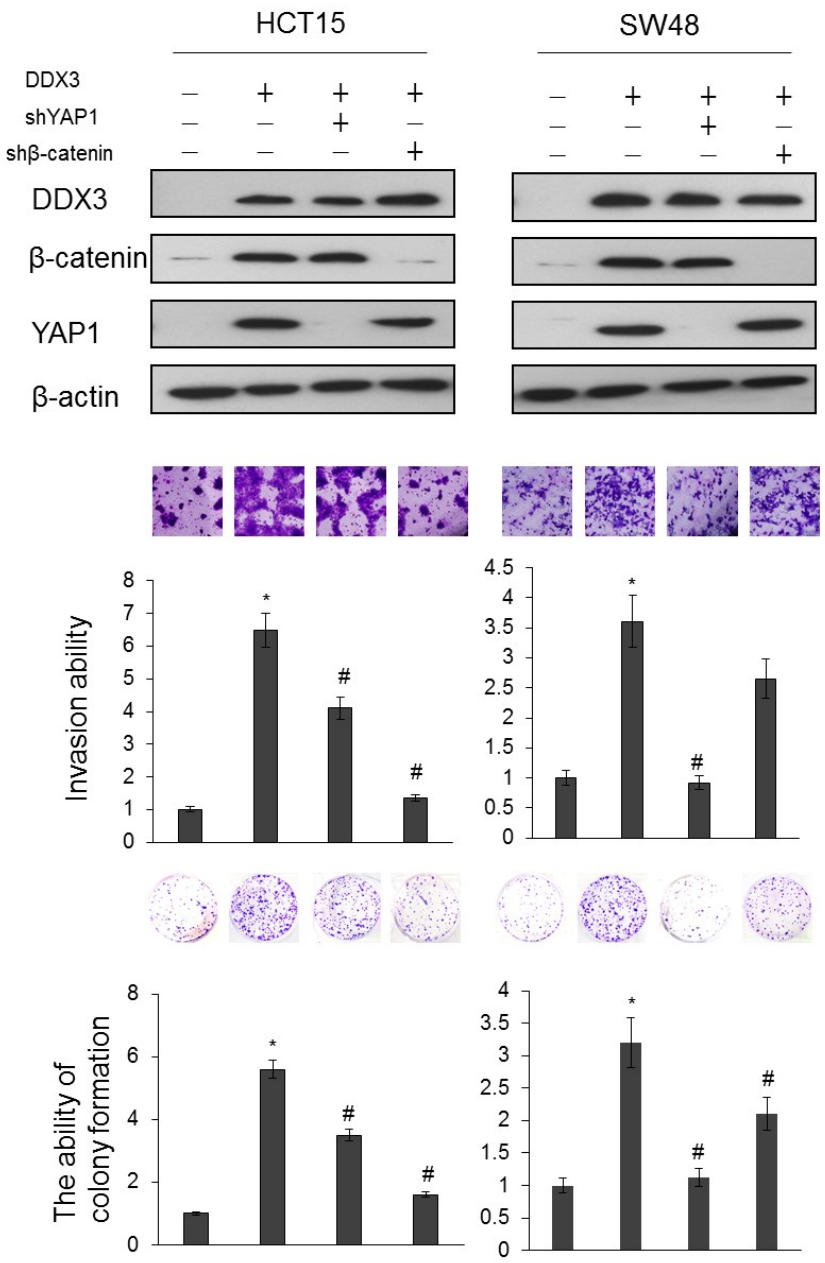

Figure S2. Different pathways for DDX3-mediated cell invasion and colony formation occurred in *KRAS*-mutated and *KRAS*-WT colon cancer cells**.** HCT15 and SW48 cells were transfected with the indicated combination of DDX3 expression vector, YAP1 shRNA and β-catenin shRNA for 24 h. The invasion ability was evaluated by a Boyden chamber assay. The colony formation ability was evaluated by the colony

formation assay. The expression of DDX3, YAP1, and β-catenin was determined by western blotting. P value was calculated by the Student's *t*-test. The significant differences in experimental groups were compared to VC or NC (*P < 0.05). The significant differences in experimental groups were compared to the group of DDX3-overexpression alone (#P < 0.05).

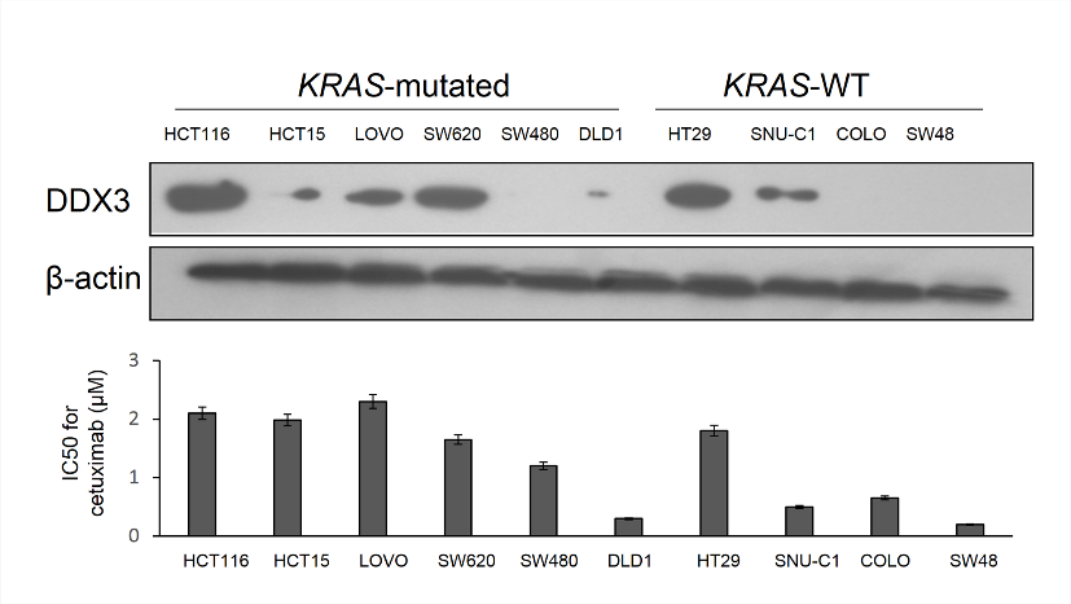

Figure S3. A positive association of DDX3 expression with CTX resistance in six *KRAS*-mutated and four *KRAS*-WT colon cancer cell lines. Six *KRAS*-mutated and four *KRAS*-WT colon cancer cell lines were collected to treat with four concentrations of CTX. After 72 h, the IC50 value of each cell type was calculated by dose-response curves which are determined by the MTT assay. DDX3 expression of these cell types were evaluated by western blotting.

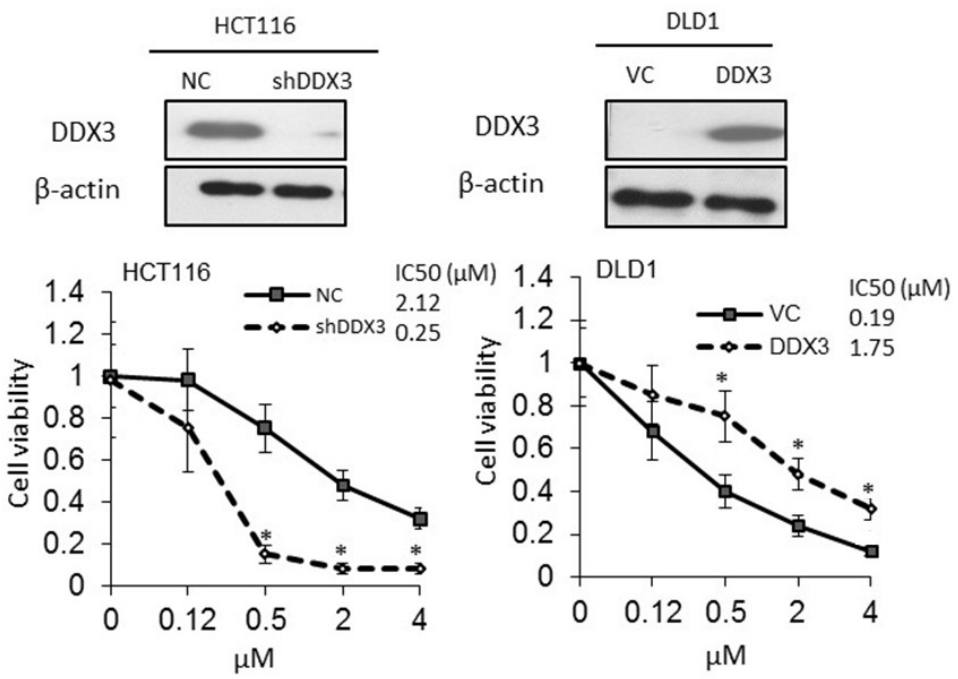

Figure S4. DDX3 confers CTX resistance in *KRAS*-mutated colon cancer cells. High-DDX3-expressing HCT116 and low-DDX3-expressing DLD1 colon cancer cells were transfected with DDX3 shRNA and DDX3 expression vector to determine the IC50 value for CTX using the MTT assay. P value was calculated by the Student's *t*-test. The significant differences in experimental groups were compared to VC or NC (*$P < 0.05$).

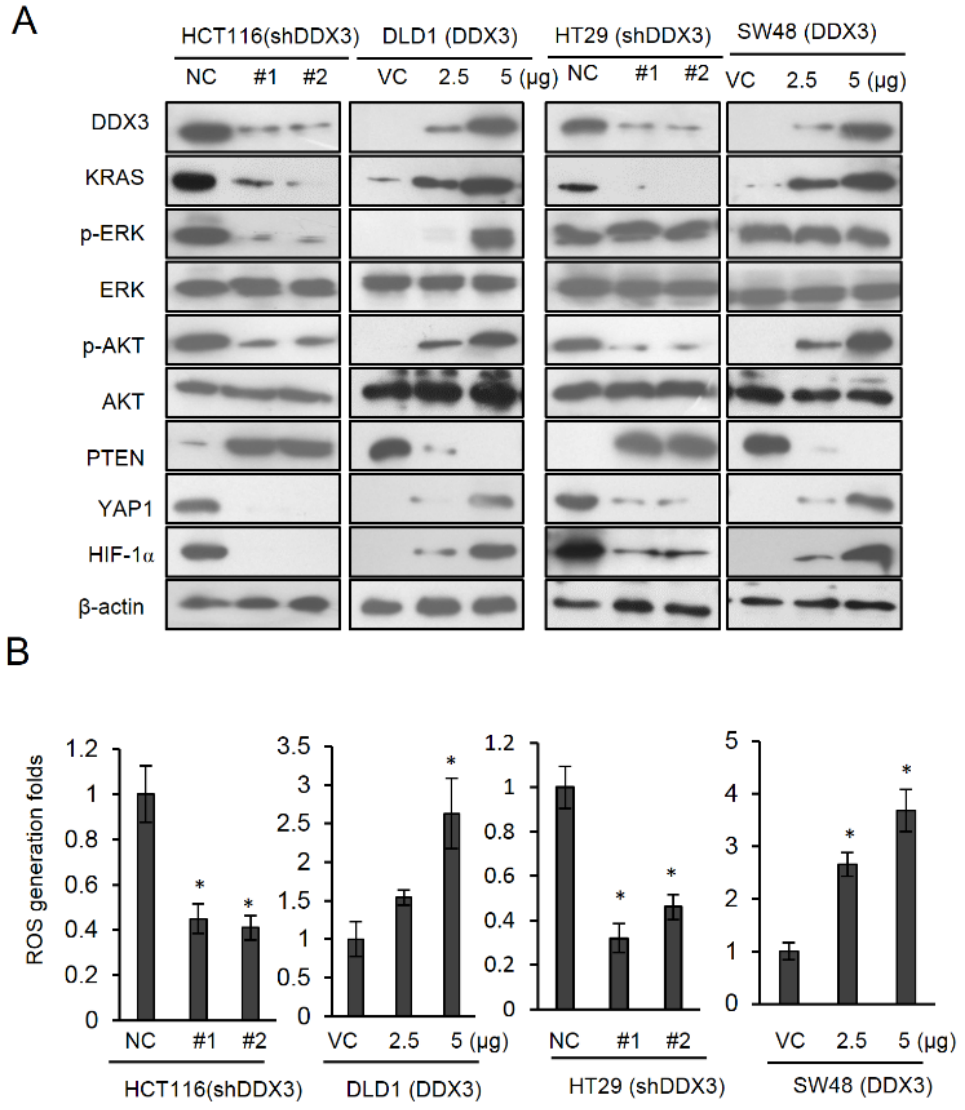

Figure S5. Different gene expression profile modulated by DDX3 manipulation in *KRAS*-mutated and *KRAS*-WT cells, but the ROS generation depended on DDX3 expression regardless of KRAS mutational status. (A) The expression of KRAS, p-ERK, p-AKT, ERK, AKT, PTEN, YAP1 and HIF-1α in DDX3-knockdown HCT116 and HT29 cells and DDX3-overexpression DLD1 and SW48 cells were evaluated by western blotting using their specific antibodies. (B) The ROS level in DDX3-

knockdown HCT116 and HT29 cells and DDX3-overexpression DLD1 and SW48 cells were evaluated by a flow cytometry analysis. P value was calculated by the Student's t-test. P value was calculated by the Student's *t*-test. The significant differences in experimental groups were compared to VC or NC (*$P < 0.05$).

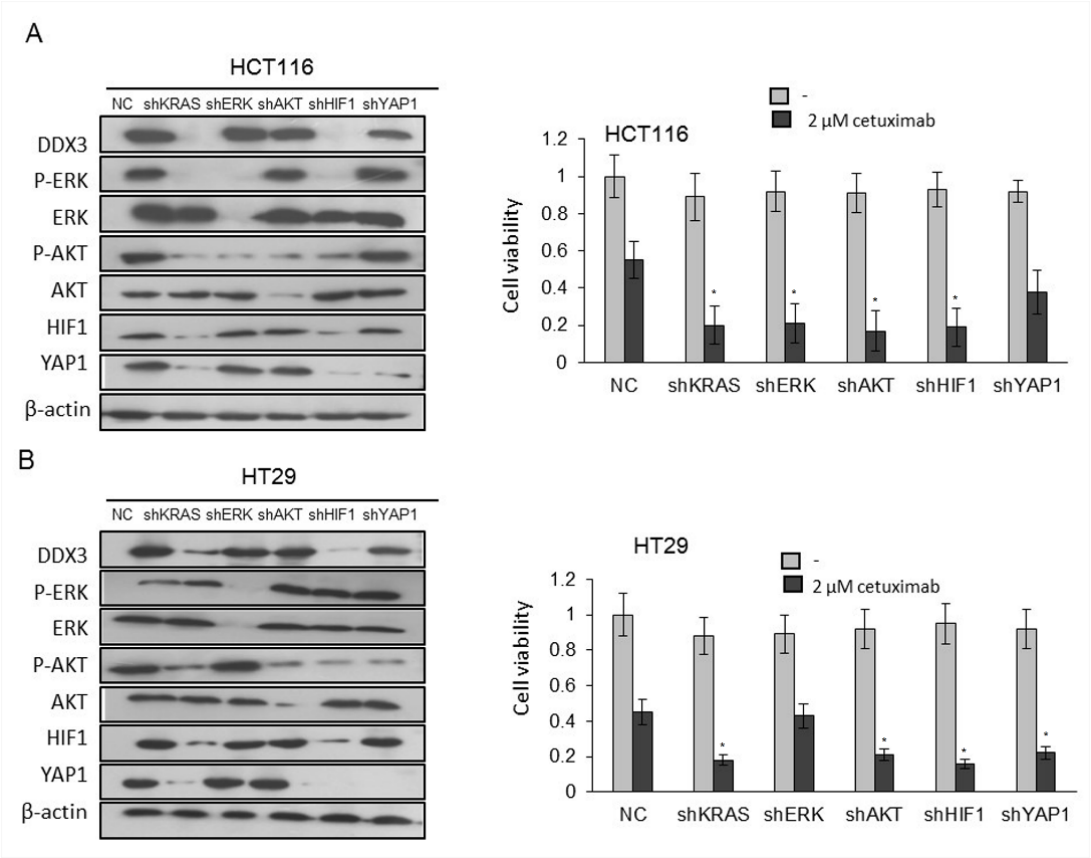

Figure S6. ERK and YAP1 signaling may be responsible for CTX resistance in *KRAS*-mutated and *KRAS*-WT cells, respectively. (A, B) HCT116 and HT29 cells were respectively transfected with KRAS shRNA, ERK shRNA, AKT shRNA shHIF1A, and shYAP1 for 24 h. After transfections, these cells were treated with or without 2 μM CTX for 72 h. The protein expressions as indicated and the cell viability were evaluated by western blotting and MTT assay, respectively. P value was calculated by the Student's *t*-test. The significant differences in experimental groups were compared to VC or NC (*P < 0.05).

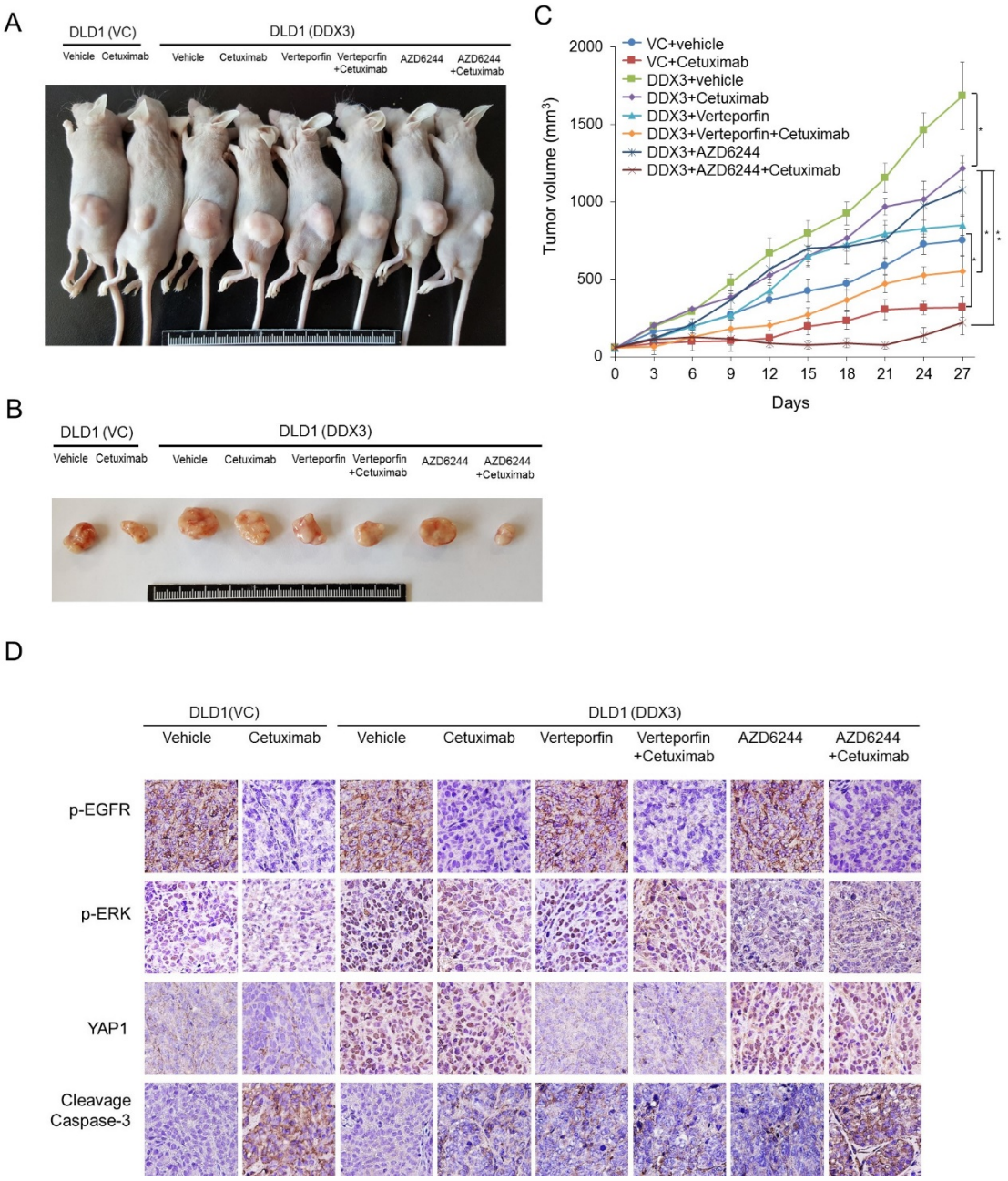

Figure S7. The combination of a MEK/ERK inhibitor (AZD6244) with CTX almost completely suppresses the tumor burden induced by tail vein injection of a stable DDX3-overexpressing DLD1 clone in nude mice. (A and B) The DLD1 xenografts were treated with vehicle or cetuximab (CTX, 10 mg/kg). The DDX3-overexpressing DLD1 xenografts were treated with vehicle, CTX (10 mg/kg),

verteporfin (10 mg/kg), AZD6244 (10 mg/kg), or the combinations as indicated. The representative tumor burdens in the eight groups are illustrated. (C) The tumor volumes in the 8 groups of nude mice were measured at 3-day intervals from Day 3 to Day 27. Mean ± SD values (mm^3) were calculated from the tumor volumes of five nude mice in each group. (D) A representative immunostaining results of p-EGFR, p-ERK, YAP1, and cleavage caspase-3 in tumors of each group of nude mice. . P value was calculated by the Student's t-test. The significance was signed with "*" ($P < 0.05$). N.s., non-significance.

Table S1. The correlation between chemotherapeutic response with DDX3, KRAS, YAP1, and SIX2 expressions in chemotherapeutic group of patients with colorectal cancer.

		Tumor response		
	No	Unfavorable	Favorable	P
All study population				
DDX3				
Low	51	12(24)	39(77)	<0.001
High	30	19(63)	11(37)	
KRAS				
Low	40	6(15)	34(85)	<0.001
High	41	25(61)	16(39)	
YAP1				
Low	27	5(19)	22(81)	<0.001
High	26	19(73)	7(27)	
SIX2				
Low	47	12(26)	35(74)	0.006
High	34	19(56)	15(44)	
***KRAS*-WT**				
DDX3				
Low	30	7(23)	23(77)	0.034
High	13	8(62)	5(38)	
KRAS				
Low	19	2(11)	17(89)	0.003
High	24	13(54)	11(46)	
YAP1				
Low	15	1(7)	14(93)	<0.001
High	15	12(80)	3(20)	
SIX2				
Low	26	5(19)	21(81)	0.008
High	17	10(59)	7(41)	
***KRAS* mutation**				
DDX3				
Low	21	5(24)	16(76)	0.011
High	17	11(65)	6(35)	
KRAS				
Low	21	4(19)	17(81)	0.001
High	17	12(71)	5(29)	
YAP1				
Low	12	4(33)	8(67)	0.146
High	11	7(64)	4(36)	
SIX2				
Low	21	7(33)	14(67)	0.224
High	17	9(53)	8(47)	

The responses were categorized as follows: Complete Response (CR): a complete

disappearance of all the tumors; Partial Response (PR): a decrease in size or number of the tumor lesions by 50% or more; Progressive Disease (PD): at least 25% increase in size or number of the tumor lesions; and Stable Disease (SD): neither sufficient shrinkage to qualify for partial response nor sufficient increase to qualify for progressive disease. Therefore, a favorable response (CR and PR) is a decrease in tumor size of least 50% or more.

Table S2. The correlation between chemotherapeutic response with YAP1/SIX2 expressions in chemotherapeutic group of patients with colorectal cancer.

		Tumor response		
	No	Unfavorable	Favorable	P
All study population				
YAP1/SIX2				
Others	35	9(26)	26(74)	<0.001
High/high	18	15(83)	3(17)	
***KRAS*-WT**				
YAP1/SIX2				
Others	21	4(19)	17(81)	<0.001
High/high	9	9(100)	0(0)	
***KRAS* mutation**				
YAP1/SIX2				
Others	14	5(36)	9(64)	0.214
High/high	9	6(67)	3(33)	

The responses were categorized as follows: Complete Response (CR): a complete disappearance of all the tumors; Partial Response (PR): a decrease in size or number of the tumor lesions by 50% or more; Progressive Disease (PD): at least 25% increase in size or number of the tumor lesions; and Stable Disease (SD): neither sufficient shrinkage to qualify for partial response nor sufficient increase to qualify for progressive disease. Therefore, a favorable response (CR and PR) is a decrease in tumor size of least 50% or more.