Supplemental material

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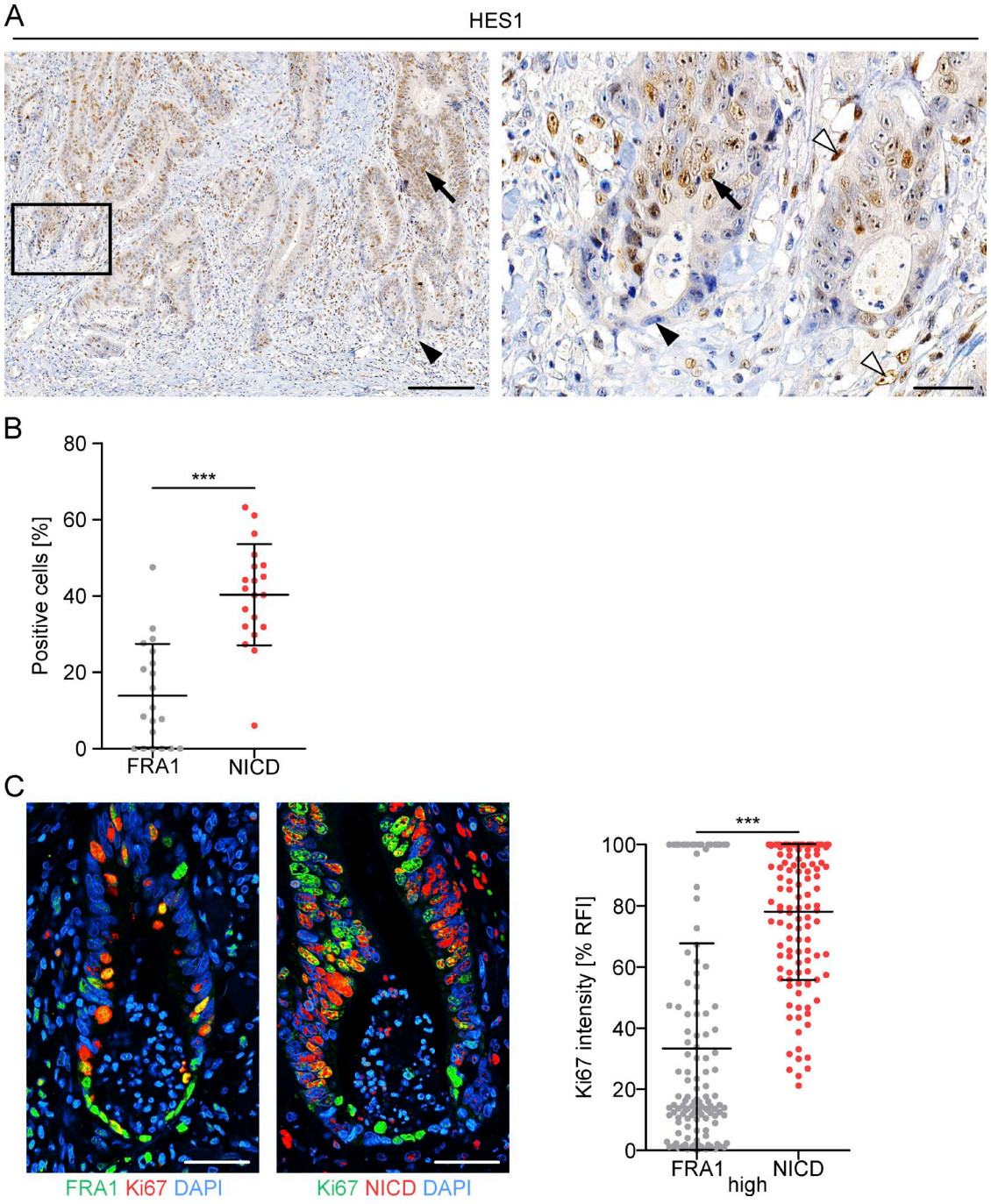

Figure S1. **Distribution of HES1 expression and characteristics of FRA1- and NICD-positive tumor cells in colorectal cancer. (A)** Representative immunostaining for HES1 in primary colon cancer tissue. Right panel shows higher magnification of area boxed in the left panel. Arrowheads indicate tumor cells at the leading tumor edge, and arrows indicate tumor cells toward the tumor center; open arrowheads indicate endothelial cells. **(B)** Quantification of FRA1- and NICD-positive tumor cells in $n = 20$ different primary colon cancers. **(C)** Double immune fluorescence (left panels) and quantification of co-immune fluorescence signals (right panel) for Ki67 and FRA1 or NICD. Relative fluorescence intensities (% RFI) for Ki67 in individual tumor cells with high (upper quartile) FRA1 and NICD staining intensity are shown. Data are derived from $n \geq 500$ tumor cells in $n = 10$ different colorectal cancer cases. Error bars indicate mean ± SD. ***, $P < 0.001$ by *t* test. Bars: 200 µm (A, left); 20 µm (A, right); 50 µm (C).

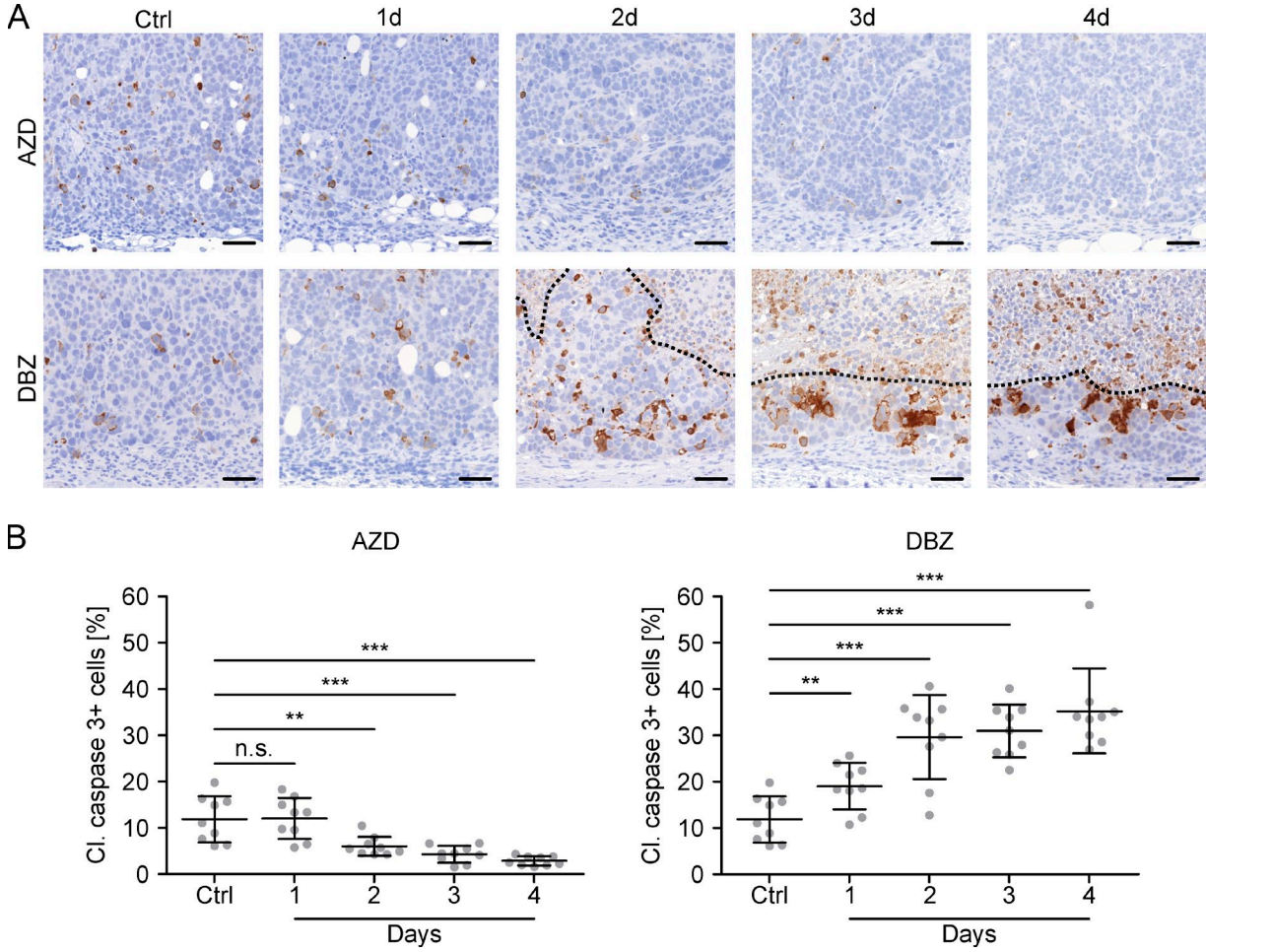

Figure S2. **Effects of short-term AZD and DBZ treatment on apoptosis in colon cancer xenografts. (A and B)** Representative immunostainings (A) and quantification (B) of cleaved (Cl.) caspase-3 in SW480 xenografts. Vehicle-treated tumors (Ctrl) and AZD- or DBZ-treated tumors at indicated time points were analyzed. Areas above dotted lines are tumor necrosis. Bars, 50 µm. Error bars are mean ± SD. **, P < 0.01; ***, P < 0.001 by *t* test; n.s., not significant. $n \geq 3$ independent biological replicates.

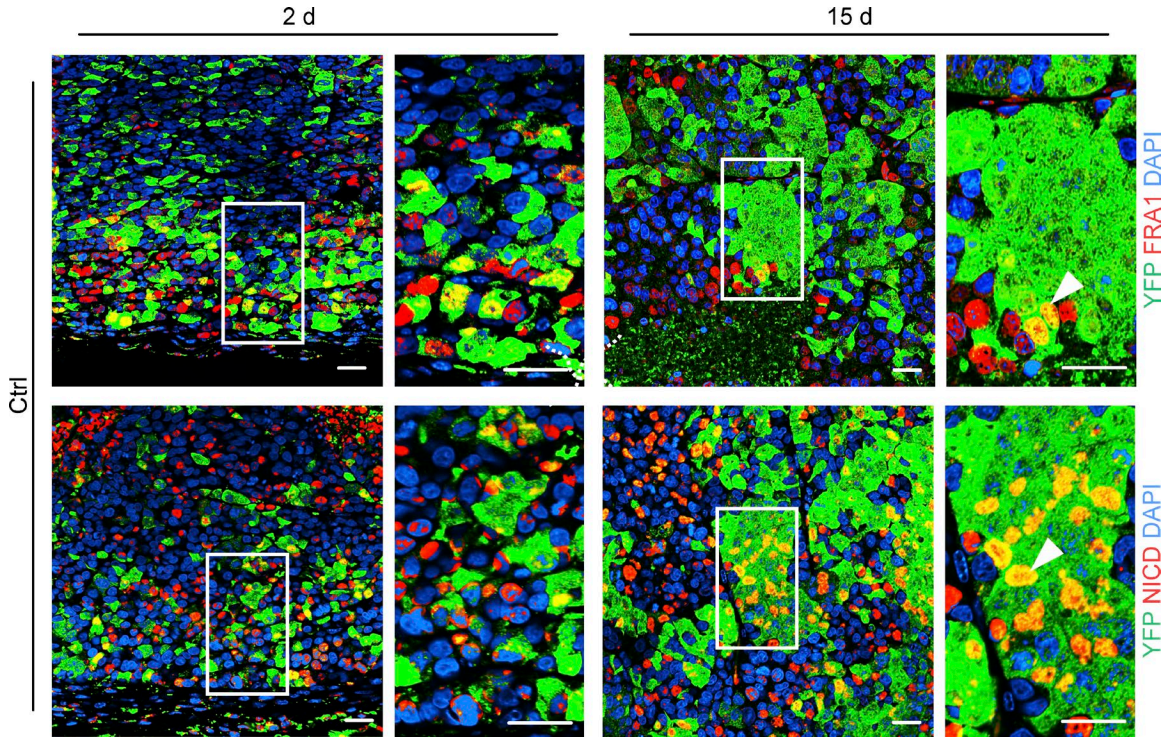

Figure S3. **Lineage tracing of tumor cells in control colon cancer xenografts.** Representative double immune fluorescence images for YFP, FRA1, and NICD at 2 and 15 d after recombination in vehicle (Ctrl)-treated SW480 xenografts, as indicated. Narrow panels are higher magnifications of areas boxed in squared panels. Arrowheads point to FRA1- and NICD-positive tumor cells within single YFP-positive clones at 15 d after recombination. Bars, 25 µm.

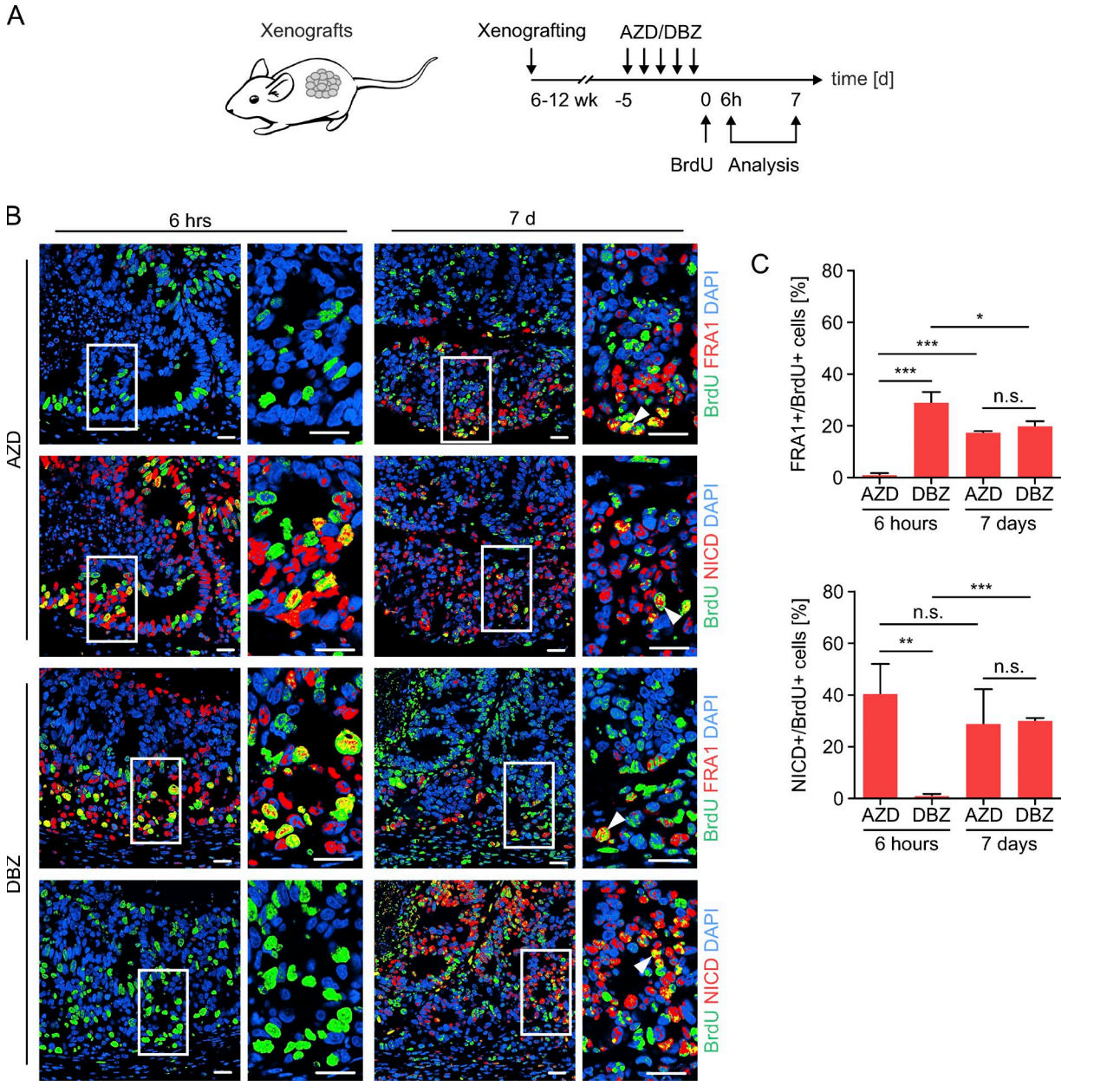

Figure S4. **BrdU tracing of colon cancer cells after MAPK and NOTCH inhibition. (A)** Schema and experimental schedule for BrdU pulse labeling and chasing in patient-derived PDX1 colon cancer xenografts that were treated with AZD or DBZ. **(B)** Double immune fluorescence for BrdU, FRA1, and NICD at indicated time points after BrdU pulse labeling. Arrowheads in right panels point to BrdU staining in reappeared FRA1- and NICD-positive tumor cells at 7 d. Narrow panels are higher magnifications of areas boxed in squared panels. Representative data from more than three biological replicates are shown. Bars, 25 µm. **(C)** Quantification of FRA1−/BrdU− and NICD−/BrdU−double positive tumor cells in AZD- and DBZ-treated SW480 xenografts at indicated time points after BrdU pulse labeling. Data are mean and error bars indicate SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by *t* test; n.s., nonsignificant. $n \geq 3$ independent biological replicates.

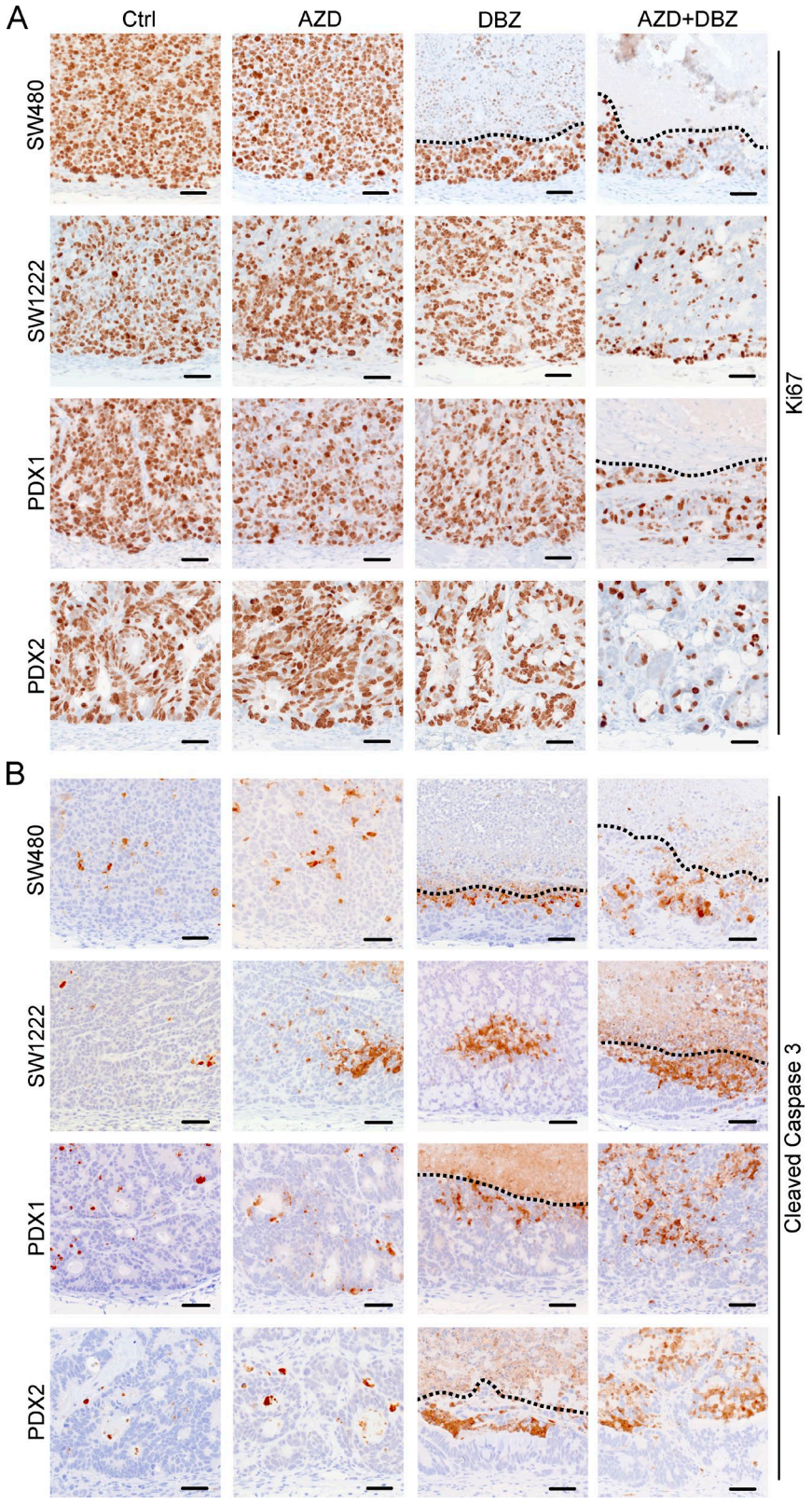

Figure S5. **Impact of long term MAPK and NOTCH repression on proliferation and apoptosis in colon cancer xenografts. (A and B)** Representative immunostainings of Ki67 (A) and cleaved caspase-3 (B) in SW480-, SW1222-, and patient derived–PDX1 and PDX2 colon cancer xenografts after treatment with vehicle (Ctrl), AZD, and/or DBZ. Bars, 50 µm.

Table S1. **Clinical data of FRA1 and NICD expression in UICC stage II colorectal cancer.**

Characteristics	Total	FRA1			NICD			FRA1/NICD combination		
		Negative	Positive	P	Low	High	P	Negative/low	Others	P
All patients	225	53 (23.6)	172 (76.4)		51 (22.7)	174 (77.3)		20 (8.9)	205 (91.1)	
Age (y, median 69)										
≤68	112	29 (25.9)	83 (74.1)	0.411	24 (21.4)	88 (78.6)	0.659	10 (8.9)	102 (91.1)	0.983
≥69	113	24 (21.2)	89 (78.8)		27 (23.9)	86 (76.1)		10 (8.8)	103 (91.2)	
Gender										
Male	121	33 (27.3)	88 (72.7)	0.156	27 (22.3)	94 (77.7)	0.892	11 (9.1)	110 (90.9)	0.909
Female	104	20 (19.2)	84 (80.8)		24 (23.1)	80 (76.9)		9 (8.7)	95 (91.3)	
T-stage (UICC)										
T3	186	41 (22.0)	145 (78.0)	0.243	43 (23.1)	143 (76.9)	0.724	16 (8.6)	170 (91.4)	0.741
T4	39	12 (30.8)	27 (69.2)		8 (20.5)	31 (79.5)		4 (10.3)	35 (89.7)	
Tumor grade										
Low grade	201	42 (20.9)	159 (79.1)	0.007	40 (19.9)	161 (80.1)	0.004	13 (6.5)	188 (93.5)	<0.001
High grade	24	11 (45.8)	13 (54.2)		11 (45.8)	13 (54.2)		7 (29.2)	17 (70.8)	
KRAS Exon 2										
Wild type	139	38 (27.3)	101 (72.7)	0.089	34 (24.5)	105 (75.5)	0.414	14 (10.1)	125 (89.9)	0.428
Mutated	86	15 (17.4)	71 (82.6)		17 (19.8)	69 (80.2)		6 (7.0)	80 (93.0)	

Row percent values are given in parentheses. P-values indicate χ^2 test results.

Table S2. **Multivariate analysis of disease free survival in UICC stage II colorectal cancer**

Variables	Disease free survival		
	HR	(95% confidence interval)	P
Age (≥ vs. < median)	1.0	(0.64-1.66)	0.902
Gender (F vs. M)	1.0	(0.63-1.64)	0.938
T-stage	2.9	(1.73-4.82)	<0.001
Tumor grade	0.8	(0.32-2.03)	0.642
KRAS Exon 2 (mutated vs. wild type)	1.3	(0.78-2.04)	0.348
FRA1/NICD combination (others vs. negative/low)	9.9	(1.35-72.74)	0.024

Table S3. **Clinical data of FRA1 and NICD expression in a case-control collection of colon cancers with and without distant metastasis**

Characteristics	**Total**	**FRA1**			**NICD**			**FRA1/NICD combination**		
		Negative	Positive	P	Low	High	P	Negative/low	Others	P
All patients	92	22 (23.9)	70 (76.1)		13 (14.1)	79 (85.9)		5 (5.4)	87 (94.6)	
Age (y, median 68)										
≤68	48	12 (25.0)	36 (75.0)	0.799	7 (14.6)	41 (85.4)	0.896	4 (8.3)	44 (91.7)	0.200
≥69	44	10 (22.7)	34 (77.3)		6 (13.6)	38 (86.4)		1 (2.3)	43 (97.7)	
Gender										
Male	45	11 (24.4)	34 (75.6)	0.907	6 (13.3)	39 (86.7)	0.830	2 (4.4)	43 (95.6)	0.682
Female	47	11 (23.4)	36 (76.6)		7 (14.9)	40 (85.1)		3 (6.4)	44 (93.6)	
T-stage (UICC)										
T2	8	1 (12.5)	7 (87.5)	0.369	1 (12.5)	7 (87.5)	0.640	1 (12.5)	7 (87.5)	0.437
T3	69	19 (27.5)	50 (72.5)		11 (15.9)	58 (84.1)		4 (5.8)	65 (94.2)	
T4	15	2 (13.3)	13 (86.7)		1 (6.7)	14 (93.3)		0 (0.0)	15 (100.0)	
Nodal status										
N0	39	14 (35.9)	25 (64.1)	0.021	8 (20.5)	31 (79.5)	0.132	4 (10.3)	35 (89.7)	0.080
N+	53	8 (15.1)	45 (84.9)		5 (9.4)	48 (90.6)		1 (1.9)	52 (98.1)	
Metastasis (Liver)										
M0	46	17 (37.0)	29 (63.0)	0.003	10 (21.7)	36 (78.3)	0.036	5 (10.9)	41 (89.1)	0.021
M1	46	5 (10.9)	41 (89.1)		3 (6.5)	43 (93.5)		0 (0.0)	46 (100.0)	
Tumor grade										
Low grade	30	6 (20.0)	24 (80.0)	0.540	2 (6.7)	28 (93.3)	0.153	1 (3.3)	29 (96.7)	0.536
High grade	62	16 (25.8)	46 (74.2)		11 (17.7)	51 (82.3)		4 (6.5)	58 (93.5)	

Row percent values are given in parentheses. P-values indicate χ^2 test results.

Table S4. **Primary antibodies used for immunoblotting (WB), immunohistochemistry (IHC), and immunofluorescence (IF)**

Antibody	Species	Manufacturer	WB	IF	IHC
BrdU (IIB5)	Mouse	Santa Cruz		1:100	
β-catenin (610154)	Mouse	BD Biosciences		1:200	
Cleaved Caspase-3 (Asp175)	Rabbit	Cell Signaling			1:100
Cleaved Notch1 (Val1744)	Rabbit	Cell Signaling		1:100	1:100
E-cadherin (sc-8426)	Mouse	Santa Cruz		1:50	1:200
E-cadherin (24E10)	Rabbit	Cell Signaling	1:1,000		
FRA1 (sc-28310)	Mouse	Santa Cruz		1:50	1:50
GFP (4B10)	Mouse	Cell Signaling		1:100	
GFP (2555S)	Rabbit	Cell Signaling		1:100	
HES1 (D6P2U)	Rabbit	Cell Signaling	1:1,000		1:50
Ki67 (M7240)	Mouse	Dako			1:150
Ki67 (D2H10)	Rabbit	Cell Signaling		1:100	
Laminin-5-γ2 (clone D4B5)	Mouse	Merck Millipore		1:200	
Phospho p44/42 MAPK (Thr202/Tyr204)	Rabbit	Cell Signaling	1:1,000		
Tubulin (DM1A)	Mouse	Sigma-Aldrich	1:50,000		
Vimentin (M0725)	Mouse	Dako			1:150

Catalogue numbers and/or clones are given in parentheses.