Ubiquitination of DDX21 by HERC2 induces a dormancy-like phenotype via the NUCKS1-p21/p27 axis to promote radioresistance in colorectal cancer cells

Supplementary figures and tables

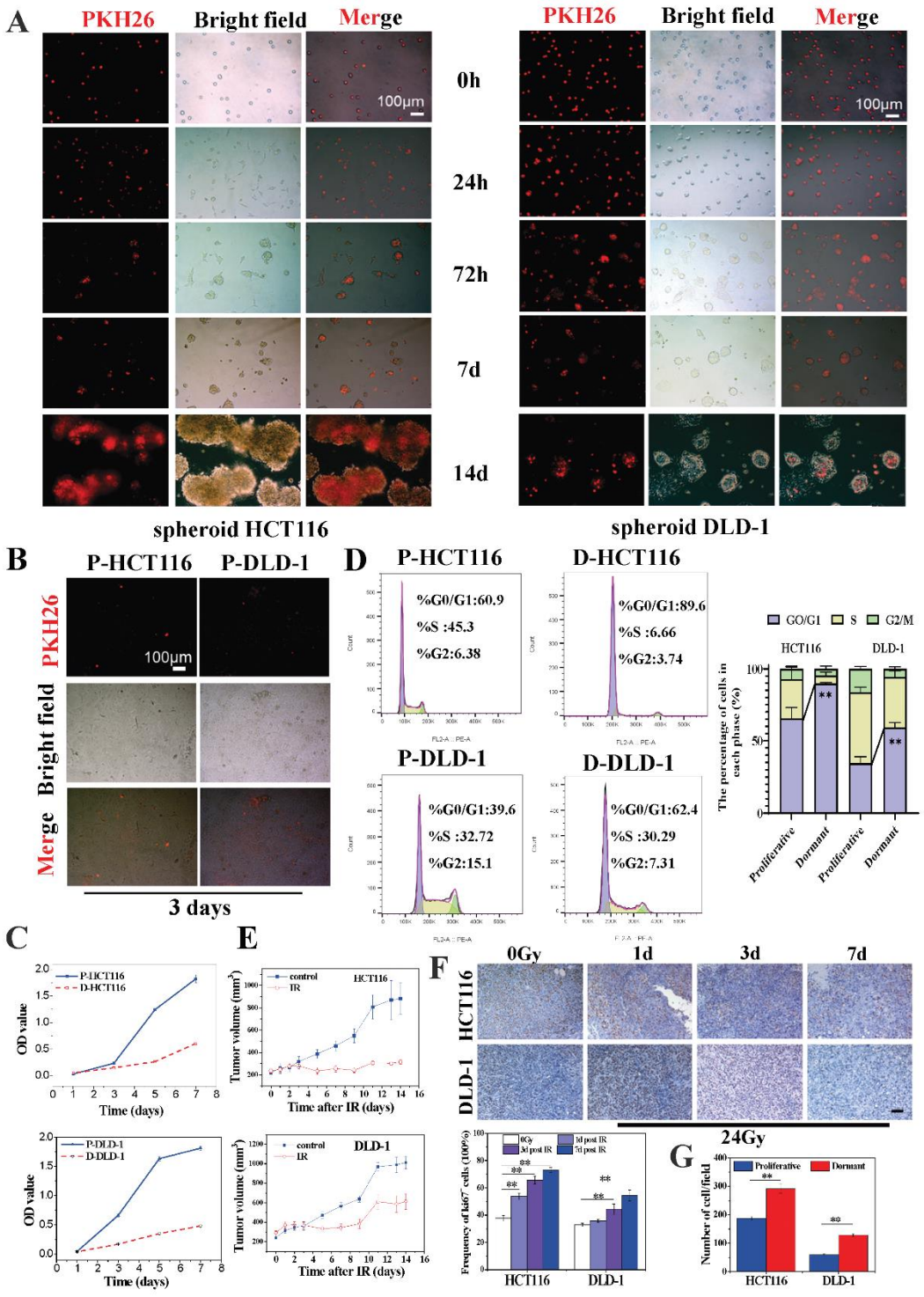


Figure S1. Label-retaining cells within colon cancer spheroids exhibit a dormancy-like state. (A) Representative images of PKH26-stained HCT116 and DLD-1 spheroids over a 14-day period. **(B)** Representative images of PKH26-stained HCT116 and DLD-1 cells cultured in standard medium at day 3. **(C)** The growth curves of proliferative and dormancy-like CRC cells. **(D)** Cell cycle analysis of proliferative and dormancy-like CRC cells. *P* values indicate the statistical significance relative to the percentages of G0/G1 phase. **(E)** The growth curves of xenograft tumor of HCT116 (above) and DLD-1 (below) ((n=6 or 8/group). **(F)** IHC quantification of Ki67-negative (dormancy-like) CRC cells in tumor tissues at days 1, day3, and day7 post-irradiation. Scale bar, 100 μm. **(G)** Statistical column plots of the migration assay of the dormancy-like HCT116 and DLD-1 cells compared with their proliferative counterparts. D/P indicates the cells at dormancy-like or proliferative state**.** The data are presented as means ± SD, ** $P<0.01$.

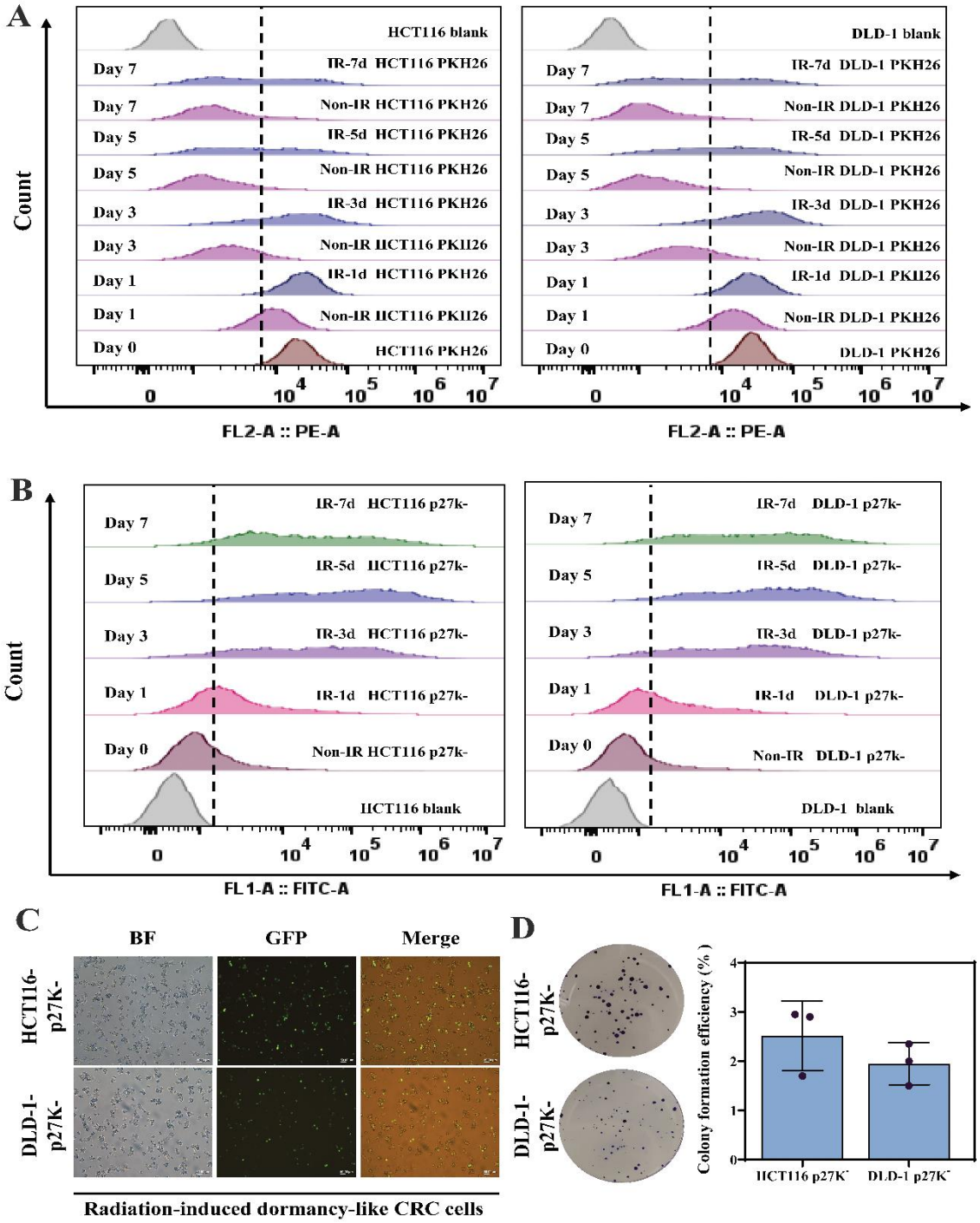


Figure S2. Radiation induces CRC cells into a dormancy-like state. (A) PKH26 dye retention in HCT116 and DLD-1 cells on days 0, 1, 3, 5, and 7 post-irradiation following membrane labeling with PKH26. **(B)** EGFP fluorescence intensity in HCT116 and DLD-1 cells stably expressing the p27K⁻ reporter on days 0, 1, 3, 5, and 7 post-irradiation. **(C)** Fluorescence images of sorted radiation-induced dormancy-like populations from HCT116 and DLD-1 cells on day 5 post-irradiation, captured 24 h after sorting. Scale bar, 50 μm. **(D)** Representative images (left) and quantification (right) of colony formation by sorted radiation-induced dormancy-like CRC cells. Cells were seeded at 1000 cells per well and cultured for 10 days. Colonies were fixed, stained with crystal violet, and counted. Colony formation efficiency was calculated as (number of colonies / number of seeded cells) × 100%. The data are presented as means ± SD.

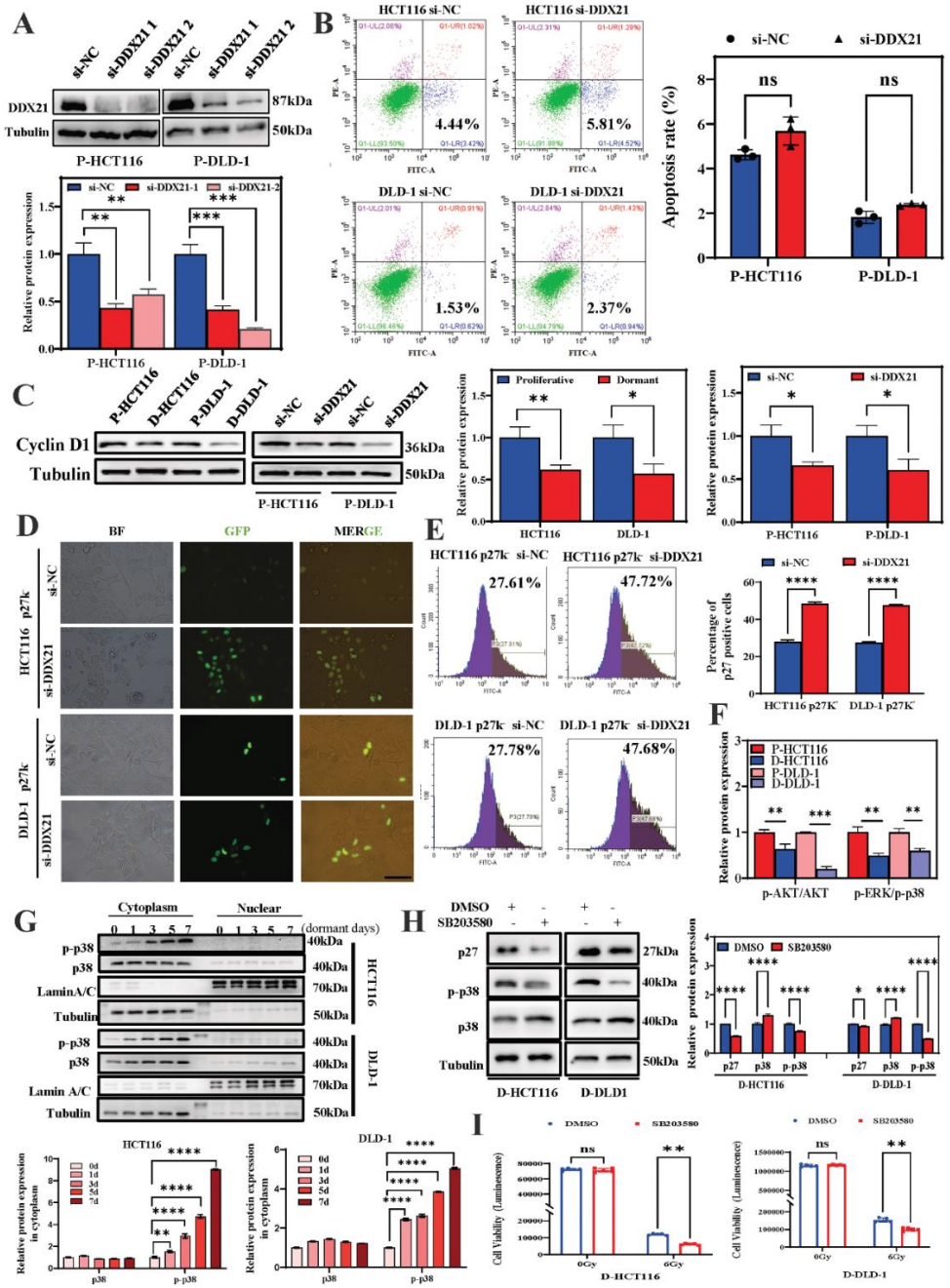


Figure S3. Down-regulation of DDX21 induces CRC cells into dormancy-like state. (A) Western blot assay showing the interference efficiency of siRNA-mediated DDX21 knockdown in the proliferative HCT116 and DLD-1 cells. **(B)** Apoptosis rates of proliferative HCT116 and DLD-1 cells after DDX21 knockdown. **(C)** Western blot assay of Cyclin D1 in proliferative and dormancy-like CRC cells with or without si-DDX21 transfection. **(D)** Representative images of EGFP-p27K$^-$ fluorescence (a dormancy marker) in proliferative CRC cells after DDX21 knockdown. Scale bar, 100 μm. **(E)** Flow cytometry analysis (left) and quantification (right) of EGFP-p27K$^-$ positive dormancy-like cells following DDX21 knockdown in CRC cells. **(F)** Western blot assay of p-AKT, AKT, p-ERK, ERK, p-p38, and p38 proteins in the proliferative versus dormancy-like CRC cells. The p-ERK/p-p38 ratio was calculated by first normalizing p-ERK to total ERK and p-p38 to total p38, then dividing the normalized p-ERK value by the normalized p-p38 value. Quantitative results are presented as bar graphs. **(G)** Western blot assay of p38 and p-p38 in the cytoplasm and nuclear lysates of CRC cells at different time points during dormancy-like induction process. **(H)** Western blot assay of p27, p38, and p-p38 in dormancy-like CRC cells treated with or without 10 μM SB203580. **(I)** Cell viability of dormancy-like CRC cells pretreated with or without 10 μM SB203580 for 2 hours followed by 6 Gy irradiation. D/P indicates the cells at dormancy-like or proliferative state. The data are presented as means ± SD, *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

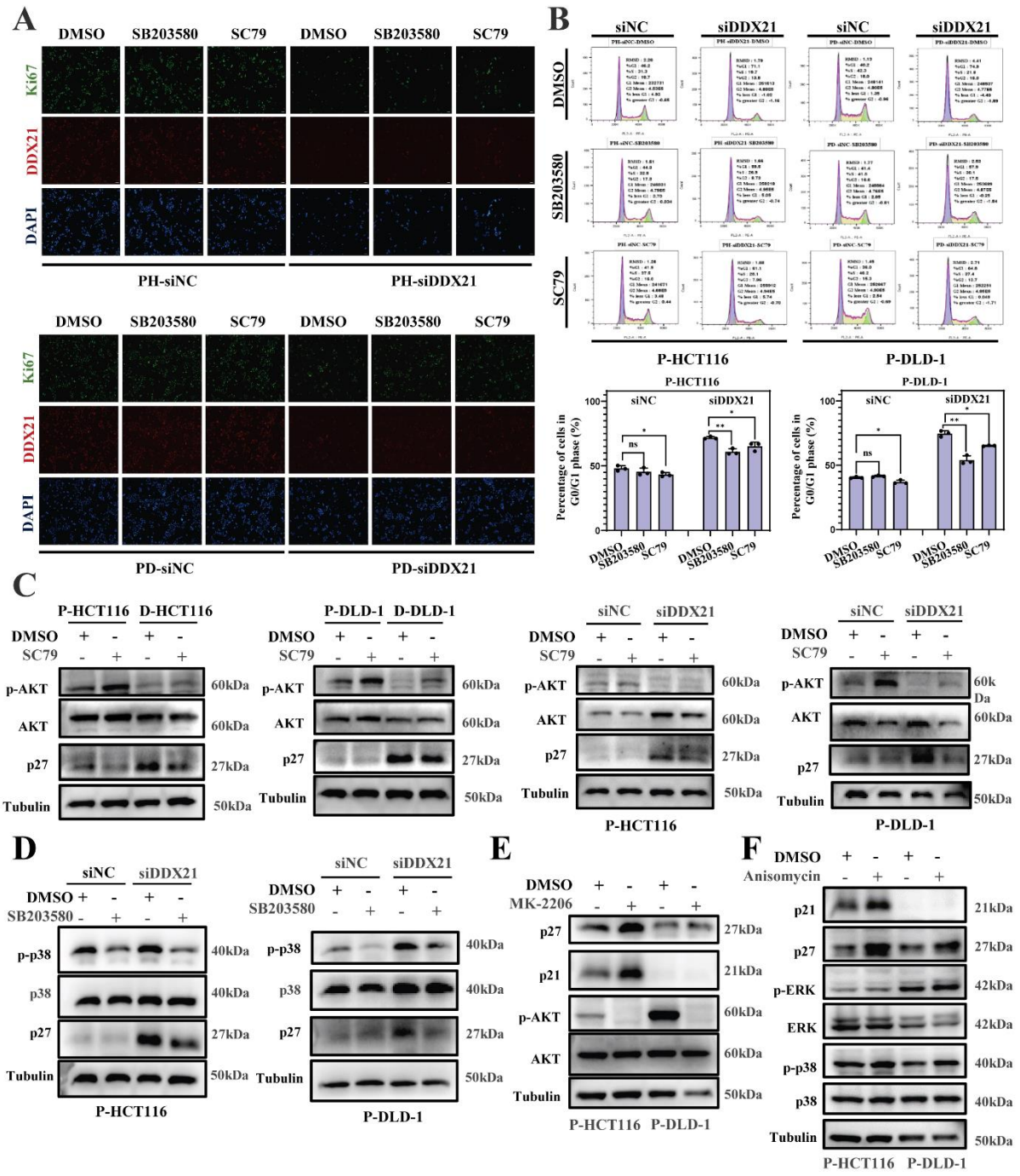


Figure S4. DDX21 knockdown induces a dormancy-like state in CRC cells via the p38MAPK/AKT signaling pathway. (A) Representative immunofluorescence images of Ki67 (green), DDX21 (red), and DAPI (blue) in proliferative CRC cells following DDX21 knockdown, with or without treatment with the p-p38 inhibitor SB203580 or the p-AKT activator SC79. Cells treated with 0.1% DMSO served as a control. Scale bar, 50 μm. **(B)** Cell cycle distribution of proliferative CRC cells under the same conditions as in (A). *P* values indicate statistical significance compared to the G0/G1 phase percentage of the control group. **(C)** Western blot assay of p-AKT, total AKT, and p27 in proliferative, dormancy-like, and DDX21-KD CRC cells, with or without SC79 treatment. **(D)** Western blot assay of p-p38, total p38, and p27 in DDX21-KD CRC cells, with or without SB203580 treatment. **(E)** Western blot assay of p-AKT, total AKT, p21, and p27 in proliferative CRC cells treated with the p-AKT inhibitor MK-2206. **(F)** Western blot assay of p-p38, total p38, p-ERK, total ERK, p21, and p27 in proliferative CRC cells with the p-p38 activator Anisomycin. D/P indicates the cells at dormancy-like or proliferative state. The data are presented as means ± SD, **P* < 0.05, ***P* < 0.01, *** *P* < 0.001.

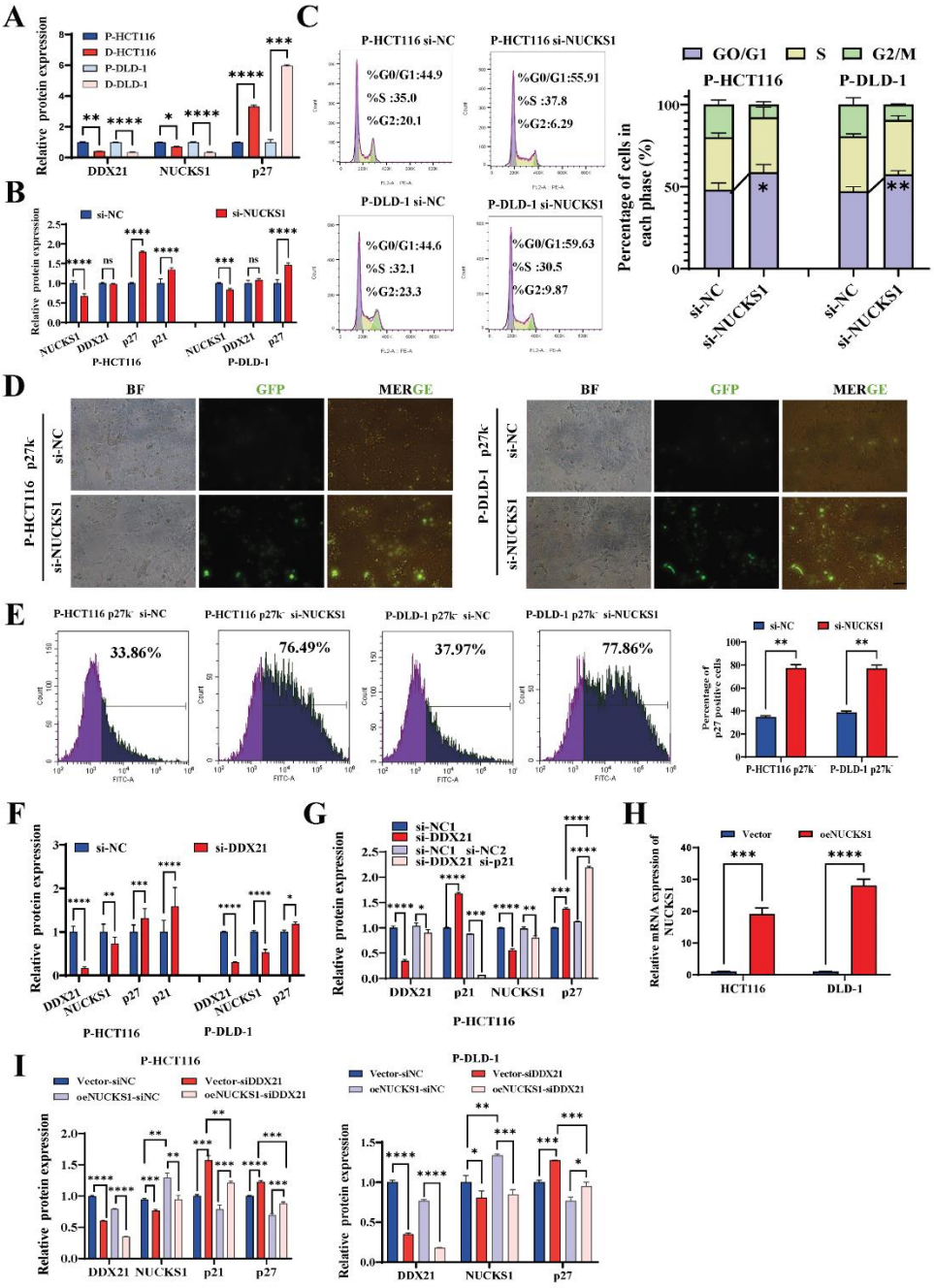
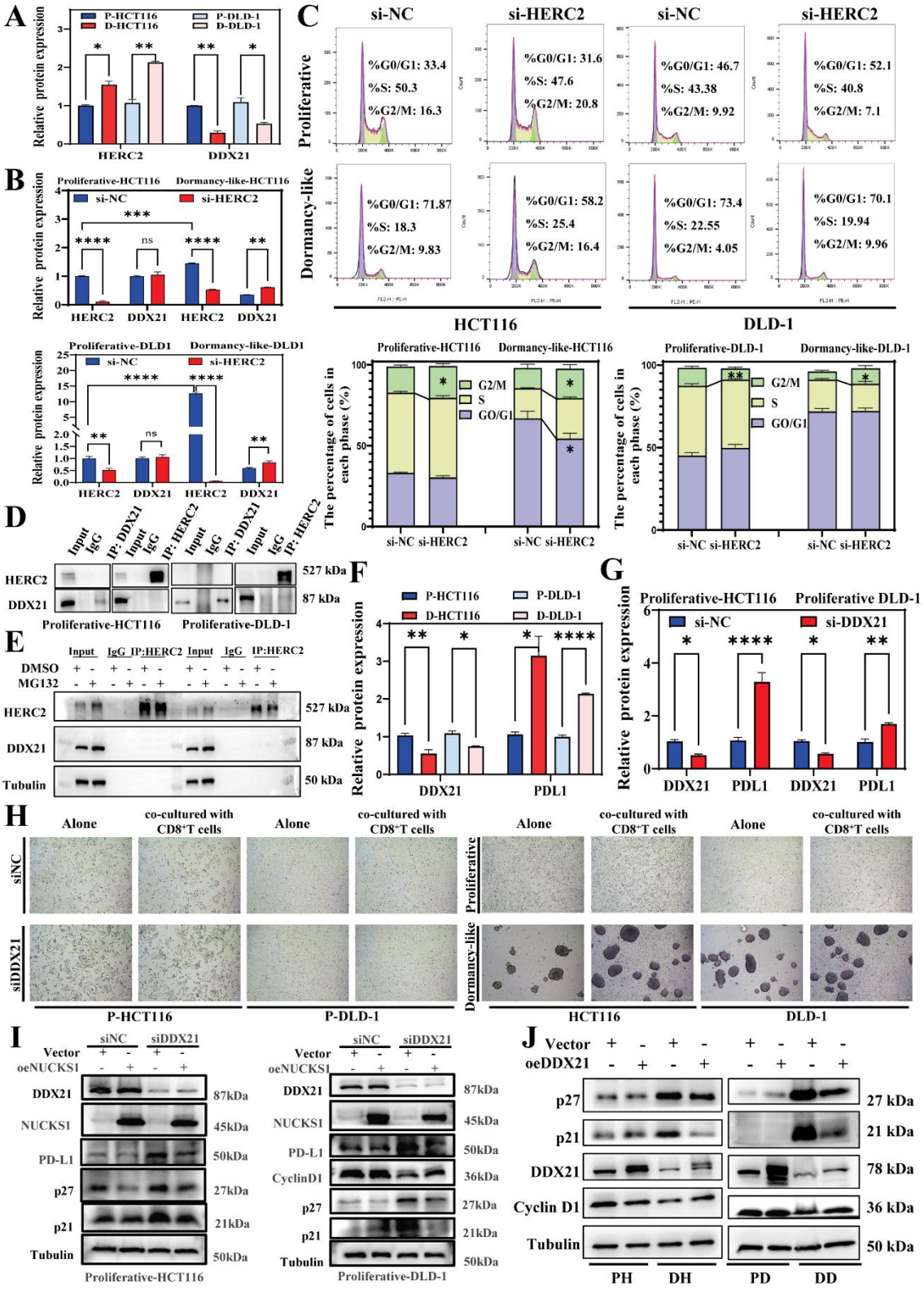


Figure S5. Low expression of DDX21 induces CRC cells into dormancy through DDX21-NUCKS1-p27/p21 axis. (A) Relative protein expression of DDX21, NUCKS1 and p27 in proliferative and dormancy-like CRC cells. **(B)** Relative protein expression of DDX21, NUCKS1, p21, and p27 in proliferative CRC cells following NUCKS1-KD. **(C)** Cell cycle analysis of the proliferative CRC cells with NUCKS1 siRNA transfection. *P* values indicate the statistical significance relative to the percentages of G0/G1 phase. **(D)** Representative images of EGFP-p27K$^-$ fluorescence in proliferative CRC cells after NUCKS1 knockdown. Scale bar, 50 μm. **(E)** Flow cytometry analysis (left) and quantification (right) of EGFP-p27K$^-$positive dormancy-like cells following NUCKS1 knockdown in CRC cells. **(F)** Relative protein expression of DDX21, NUCKS1, p27, and p21 in proliferative CRC cells following DDX21 knockdown. **(G)** Relative protein expression of DDX21, NUCKS1, p21, and p27 in proliferative HCT116 cells after combined treatment of p21 knockdown and DDX21 knockdown. **(H)** Relative mRNA expression of *NUCKS1* in proliferative HCT116 and DLD-1 cells transfected with NUCKS1 overexpression plasmid or empty vector. Data normalized to ACTB and presented as fold change relative to control (Vector). **(I)** Relative protein expression of DDX21, NUCKS1, p21, and p27 in proliferative CRC cells following DDX21-KD with or without transfected with NUCKS1 overexpression plasmid or empty vector. D/P indicates the cells at dormancy-like or proliferative state. The data are presented as means ± SD, **P* < 0.05, ***P* < 0.01, *** *P* < 0.001, **** *P* < 0.0001.


A
P-HCT116
D-HCT116
P-DLD-1
D-DLD-1
Relative protein expression
HERC2
DDX21
B
Proliferative-HCT116
Dormancy-like-HCT116
si-NC
si-HERC2
Relative protein expression
HERC2
DDX21
HERC2
DDX21
Proliferative-DLD1
Dormancy-like-DLD1
si-NC
si-HERC2
Relative protein expression
HERC2
DDX21
HERC2
DDX21
C
si-NC
si-HERC2
si-NC
si-HERC2
Proliferative
%G0/G1: 33.4
%S: 50.3
%G2/M: 16.3
%G0/G1: 31.6
%S: 47.6
%G2/M: 20.8
%G0/G1: 46.7
%S: 43.38
%G2/M: 9.92
%G0/G1: 52.1
%S: 40.8
%G2/M: 7.1
Dormancy-like
%G0/G1: 71.87
%S: 18.3
%G2/M: 9.83
%G0/G1: 58.2
%S: 25.4
%G2/M: 16.4
%G0/G1: 73.4
%S: 22.55
%G2/M: 4.05
%G0/G1: 70.1
%S: 19.94
%G2/M: 9.96
HCT116
DLD-1
Proliferative-HCT116
Dormancy-like-HCT116
The percentage of cells in each phase (%)
G2/M
S
GO/G1
si-NC si-HERC2
si-NC si-HERC2
Proliferative-DLD-1
Dormancy-like-DLD-1
The percentage of cells in each phase (%)
G2/M
S
GO/G1
si-NC si-HERC2
si-NC si-HERC2
D
Input
IgG
IP: DDX21
Input
IgG
IP: HERC2
Input
IgG
IP: DDX21
Input
IgG
IP: HERC2
HERC2
DDX21
527 kDa
87 kDa
Proliferative-HCT116
Proliferative-DLD-1
E
Input
IgG
IP:HERC2
Input
IgG
IP:HERC2
DMSO
MG132
HERC2
DDX21
Tubulin
527 kDa
87 kDa
50 kDa
F
P-HCT116
D-HCT116
P-DLD-1
D-DLD-1
Relative protein expression
DDX21
PDL1
G
Proliferative-HCT116
Proliferative DLD-1
si-NC
si-DDX21
Relative protein expression
DDX21
PDL1
DDX21
PDL1
H
Alone
co-cultured with CD8+T cells
Alone
co-cultured with CD8+T cells
Alone
co-cultured with CD8+T cells
Alone
co-cultured with CD8+T cells
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Tubulin
87kDa
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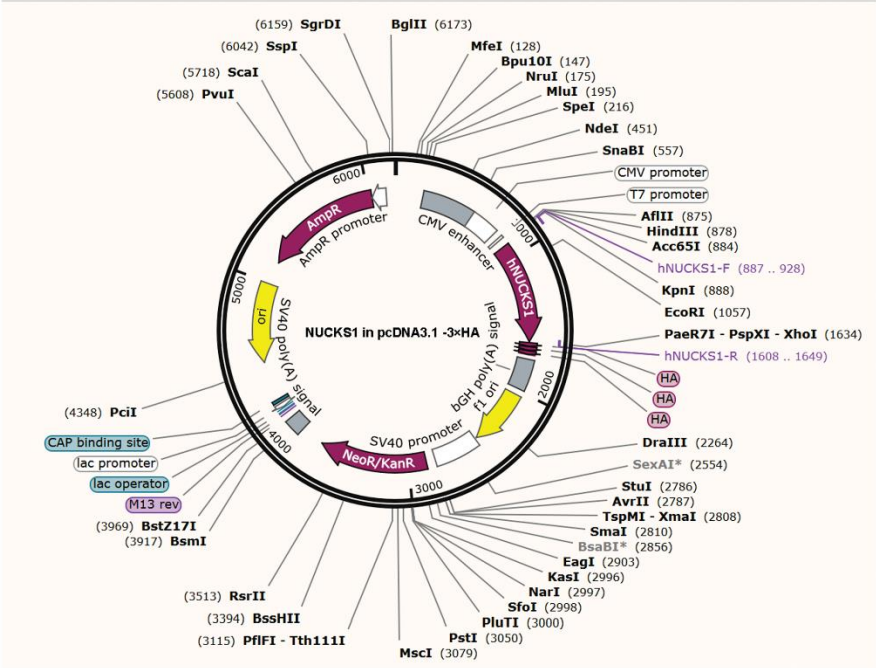
p21
DDX21
Cyclin D1
Tubulin
27 kDa
21 kDa
78 kDa
36 kDa
50 kDa
PH
DH
PD
DD

Figure S6. HERC2-mediated degradation of DDX21 promotes immune evasion and radio-resistance in dormancy-like CRC cells. (A) Relative protein expression of HERC2 and DDX21 in the proliferative and dormancy-like CRC cells. (B) Relative protein expression of HERC2 and DDX21 in the proliferative and dormancy-like CRC cells following HERC2-KD. (C) Cell cycle analysis of proliferative and dormancy-like CRC cells following HERC2-KD. (D) Co-immunoprecipitation and Western blot assay of HERC2 and DDX21 proteins in the whole cell lysates of proliferative CRC cells. (E) Co-immunoprecipitation and Western blot assay of HERC2 and DDX21 proteins in the whole cell lysates of proliferative CRC cells with the treatment of MG132. (F) Relative protein expressions of DDX21 and PD-L1 in the proliferative and dormancy-like CRC cells. (G) Relative protein expressions of DDX21 and PD-L1 in the proliferative CRC cells following DDX21-KD. (H) Representative images of proliferative CRC cells with or without DDX21 knockdown (left), and of proliferative versus dormancy-like CRC cells (right), after 48 h of co-culture with activated $CD8^+$ T cells. Scale bar, 50 μm. (I) Western blot assay of DDX21, NUCKS1, PD-L1 and dormant-related proteins in CRC proliferative cells transfected with NUCKS1 overexpression plasmid or empty vector with or without the knockdown of DDX21. (J) Western blot assay of DDX21, Cyclin D1, p21, and p27 proteins in the proliferative and dormancy-like CRC cells transfected with DDX21 overexpression plasmid or empty vector. D/P indicates the cells at dormancy-like or proliferative state. The data are presented as means ± SD, $*P < 0.05$, $**P < 0.01$, $**** P < 0.0001$.

A



B

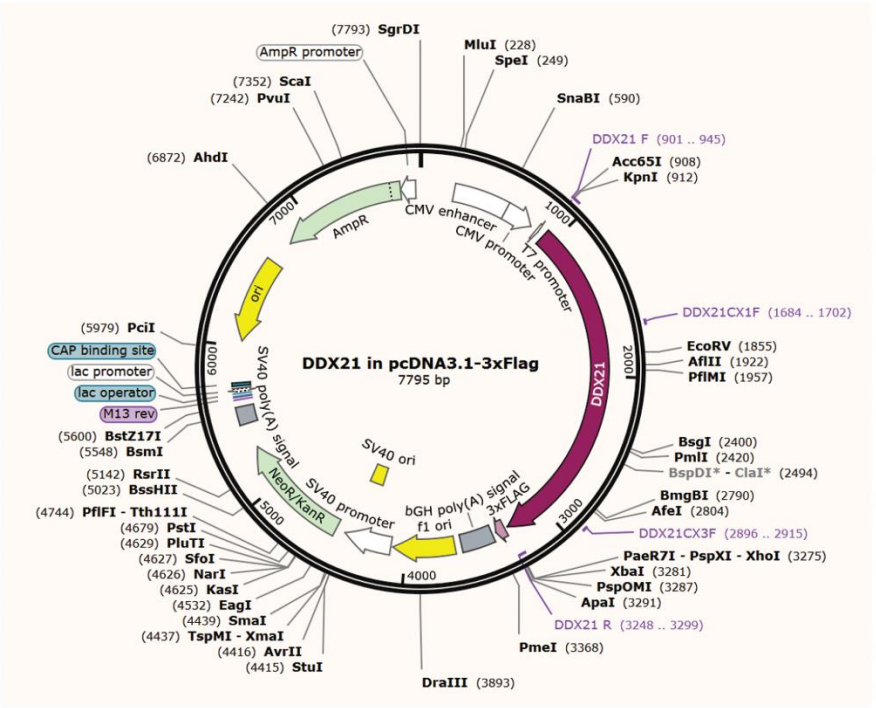


Figure S7. Plasmid maps of key constructs. (A) NUCKS1 overexpression vector. Full-length human NUCKS1 cDNA was PCR-amplified and cloned into pcDNA3.1(+) (Invitrogen). All constructs were validated by restriction digestion and sequencing. **(B)** DDX21 overexpression vector. Full-length human DDX21 cDNA was PCR-amplified and cloned into pcDNA3.1(+) (Invitrogen). All constructs were validated by restriction digestion and sequencing.

Table S1. Clinical information of 10 different tumor regression grades (TRG) colorectal cancer patients for immunofluorescence and immunohistochemistry assay.

No.	Pathology number	Sex	Age	T stage	Lymph node metastasis	Distant metastasis	Neoplasm stage	Tumor regression grades (TRG)
1	814556	male	61	3	2	0	III	0
2	792269	male	65	3	2	x	III	3
3	828805	male	64	3	0	0	II	0
4	791353	male	55	3	x	0	II	1
5	827175	male	56	4	2	0	III	2
6	802659	male	55	2	1	0	III	3
7	606146	female	51	0	0	0	0	0
8	556330	male	66	3	+	0	III	1
9	537367	female	45	3	2	x	III	2
10	614705C	male	55	3	+	x	III	3

Table S2. Antibodies, recombinant protein, and drugs information.

Name	Supplier	Cat.no	Application
DDX21	Santa Cruz Biotechnology	sc-376953	1:1000
phosphorylated AKT	Cell Signaling Technology	4060T	1:1000
Total AKT	ProteinTech	10176-2-AP	1:1000
phosphorylated p38	Cell Signaling Technology	4511T	1:1000
Total p38	Cell Signaling Technology	8690T	1:1000
Phospho-ERK1-T202/Y204 + ERK2-T185/Y187 Rabbit mAb	Abclonal	AP0974	1:1000
ERK1/2 Rabbit mAb	Abclonal	A4782	1:1000
Ubi	Cell Signaling Technology	3936T	1:1000
NUCKS1	ProteinTech	12023-2-AP	1:1000
Cyclin D1	Abclonal	A19038	1:1000
CDKN1B/Kip1 p27	Santa Cruz Biotechnology	sc-1641	1:1000
CDKN1A/p21CIP1	Abclonal	A27846	1:1000
PDL1	Abclonal	A19135	1:1000
Phosphorylated ATM	Cell Signaling Technology	D25E5	1:1000
ATM	Cell Signaling Technology	2873S	1:1000
RAD51	Abclonal	A26856	1:1000
p-Histone H2AX	Santa Cruz Biotechnology	sc-517348	1:1000
caspase-3	Santa Cruz Biotechnology	sc-56053	1:1000
Cleaved-caspase3	Cell Signaling Technology	9661	1:1000
Bcl-2	Santa Cruz Biotechnology	sc-7382	1:1000
Bax	ProteinTech	50599-2-Ig	1:1000
Herc2	Santa Cruz Biotechnology	sc-515891	1:1000
Tubulin	Beyotime	AT819-1	1:1000
Lamin A/C	Abclonal	A19524	1:50000
HA-Tag(26D11) mAb	Abmart	M20003S	1:5000
anti DDDDK-Tag pAb	ABclonal	AE004	1:2000
VeriBlot for IP Detection Reagent (HRP)	Abcam	ab131366	1:3000
HRP-labeled Goat Anti-Mouse IgG(H+L)	Beyotime	A0216	1:3000
HRP-labeled Goat Anti-Rabbit IgG(H+L)	Beyotime	A0208	1:3000
anti-CD3	BioLegend	300438	5 μg/mL
anti-CD28	BioLegend	302934	5 μg/mL
recombinant human IL-2	ABclonal	RP01039	100 U/mL
MG132	MedChemExpress	HY-13259	10 μM
Cyclohexamide	MedChemExpress	HY-12320	200 μg/ml
SC79	TargetMol	T2274	10 μM
Anisomycin	TargetMol	T6758	10 nM
MK-2206	TargetMol	T1952	10 μM
WAY-600	TargetMol	T6730	3 μM
SB202190	MedChemExpress	HY-10295	10 μM
SB203580	MedChemExpress	HY-10256	10 μM

Table S3. The primer sequences of qRT-PCR.

Gene	Primer sequences (5’ to 3’)
DDX21	F: AATGTTGCTGCACGTGGGTTAG R: CCCGGATCGATGAATGTAGGAC
ACTB	F: AGGATTCCTATGTGGGCGAC R: GATAGCACAGCCTGGATAGCAA
CDKN1A（p21)	F: GCGACTGTGATGCGCTAATG R: GTGGTAGAAATCTGTCATGCTGG
NUCKS1	F: GGCCTGTCAGAAATAGGAAGGT R: TTTAGCTTCTCGGGGAGATGAT
HERC2	F: GCGCTGTCTTTTGCCTTTG R: AGGAACCTGGTCGCTCTCTC

Table S4. Small interfering RNA sequences.

Gene	Primer sequences (5’ to 3’)
DDX21#1	GCGGAGTTTCAGTAAAGCATT
DDX21#2	CGCTCCTTGATCAACTCAAAT
NUCKS1	GTTGTTGATTACTCACAGTTT
CDKN1A（p21)	CGCTCTACATCTTCTGCCTTA
HERC2	CCUGGCAUAAUGGAGUCAUUU

Table S5. Differential expression genes between si-NC and si-DDX21 in CRC cells.

Cell type	Gene Name	si-DDX21	si-NC	log2FoldChange	Fold Change	*P* value	Regulated type
P-HCT116	DDX21	769	8020	-3.567402433504	0.084353841	5.53E-54	down
	NUCKS1	2622	10841	-2.2327848	0.212747665	3.19E-25	down
	HMGB1	4789	14392	-1.772526574	0.292695694	5.14E-17	down
	KRT7	54	247	-2.375697944	0.192683115	3.54E-16	down
	RDX	1916	5564	-1.72303921	0.302909932	5.53E-16	down
	PIP4K2A	1279	3233	-1.52284359	0.347999324	9.33E-13	down
	TOP2A	3347	8247	-1.486048156	0.356989079	1.62E-12	down
	MYCBP	192	418	-1.306885455	0.404192525	6.01E-08	down
	TMOD2	233	512	-1.320404317	0.400422704	2.30E-08	down
	SULT2B1	4	32	-3.143157205	0.113191913	8.97E-07	down
	HIST1H2BK	57	142	-1.499766857	0.35361053	7.63E-07	down
	C2orf76	55	118	-1.284312496	0.41056641	3.94E-05	down
	TPM4	9954	1789	2.290963771	4.893829268	3.06E-26	up
	ADAM15	3282	996	1.535162441	2.898210616	7.61E-13	up
	SCG2	373	99	1.7274015	3.311308669	3.69E-11	up
	TSC22D1	7052	2319	1.41939992	2.674742335	1.61E-11	up
P-DLD-1	DDX21	1712	12696	-3.139648916	0.113467504	5.42E-45	down
	NUCKS1	3693	13032	-2.068283864	0.238442967	3.59E-22	down
	TOP2A	4128	13377	-1.945335515	0.259654383	5.86E-20	down
	RDX	1282	4307	-1.997303463	0.250467711	1.83E-20	down
	SULT2B1	112	322	-1.771456824	0.292912807	7.15E-12	down
	HMGB1	9299	19820	-1.340927643	0.394766742	1.27E-10	down
	HIST1H2BK	184	427	-1.462983012	0.362742323	1.47E-09	down
	MYCBP	317	699	-1.389558531	0.38168158	1.26E-09	down
	TMOD2	37	110	-1.817250641	0.283761223	5.42E-08	down
	C2orf76	35	97	-1.715873327	0.304418232	5.03E-07	down
	PIP4K2A	668	1152	-1.035201488	0.487947729	2.11E-06	down
	KRT7	7	32	-2.419203579	0.186959336	4.17E-05	down
	SCG2	12	30	-1.560311297	0.33907791	0.004744033	down
	ADAM15	4217	1740	1.027950537	2.039125453	1.00E-06	up
	TSC22D1	3088	1282	1.019080458	2.02662682	1.44E-06	up
	TPM4	10641	2573	1.798933817	3.479629781	2.42E-17	up