Supplementary information

Supplementary Table ST1: Key resources.

Supplementary Table ST2: Clinicopathological characteristics of CRC patients included in the study.

Supplementary Figure S1: $1,25(OH)_2D_3$ opposes Wnt-induced β-catenin nuclear accumulation and signalling in carcinoma cells.

(S1A) Confocal immunostaining imaging of the increase in nuclear SIRT1 content in response to $1,25(OH)_2D_3$ (100 nM, 24 h) in HCT 116 cells. Images corresponding to single nuclear staining using DAPI and to merged DAPI + anti-SIRT1 staining are shown. Scale bars: 25 μm.

(S1B) Western blot analysis of SIRT expression in human cell lines from pancreatic cancer (Panc-1, PL-45) and melanoma (A-375, 501mel) treated with $1,25(OH)_2D_3$ (100 nM) for 24 h. TBP and Lamin B1 were used as loading controls.

(S1C) $1,25(OH)_2D_3$ reduces the level of nuclear β-catenin lysine acetylation. Nuclear extracts (NE) from HCT 116 or HT-29 CRC cells were immunoprecipitated (IP) using anti-β-catenin antibodies and analysed by western blotting using anti-acetyl-lysine antibody. TBP in the flow through (FT) was used as control.

(S1D) Representative western blots and statistical analysis of $1,25(OH)_2D_3$ effects on the nuclear β-catenin content in HCT 116 and HT-29 cells. TBP was used as loading control. The full distribution of the data from at least 3 independent experiments is shown. Mean ± SEM of replicates is displayed to illustrate the representability of the western blot shown.

(S1E) Left, confocal immunostaining imaging of nuclear β-catenin in HCT 116 cells treated with Wnt3A in the presence or absence of $1,25(OH)_2D_3$ for 24 h. Scale bars: 25 μm. Right, quantification of fluorescence intensity in 3 independent experiments (2-3 fields/each) using ImageJ software. Mean ± SD is displayed to show variability.

(S1F) RT-qPCR analysis of *MYC* RNA and *CCND1* RNA levels in HCT 116 cells treated Wnt3A (100 ng/ml) in the presence or absence of $1,25(OH)_2D_3$ (100 nM) for 24 h. Values were normalized to those of 18S rRNA and are presented as Mean ±SD.

(S1G) Western blot analysis of the levels of SIRT7 and HDAC-1 in nuclear extracts (NE) of HCT 116 cells treated with $1,25(OH)_2D_3$ (100 nM) or vehicle for 24 h.

(S1H) Effect of pre- and post-treatment with $1,25(OH)_2D_3$ on the high nuclear content of β-catenin protein induced by LiCl-mediated Wnt signalling. Western blot of β-catenin protein in nuclear extracts (NE) of HCT 116 cells. Lamin A/C was used as loading control. Left: cells were treated or not with $1,25(OH)_2D_3$ (100 nM) for 24 h and then treated with LiCl (40 mM) for additional 24 h. Right, cells were first treated with LiCl and 24 h later with $1,25(OH)_2D_3$ or vehicle under the same conditions. Mean ± SEM of triplicates is displayed.

Statistical analysis of at least 3 independent experiments by One-Way ANOVA (panel S1D) or Student t-test (panels S1E, S1F and S1H); $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Supplementary Figure S2: Acetylation of SIRT1 substrates in human colon carcinomas and established cell lines.

(S2A) - (S2C) Western blot analyses of the activation by $1,25(OH)_2D_3$ of SIRT1 deacetylase activity on several substrates. HCT 116 CRC cells were treated with $1,25(OH)_2D_3$ (100 nM) or vehicle in the presence or absence of the EX527 SIRT1 inhibitor (10 μM) or the SRT1720 SIRT1 activator (10 μM) for 24 h. Levels of AceH3K9 and AceH4 (K5, 8, 12, 16) (S2A) and of p53 in nuclear extracts (NE) (S2B) and of Acep53K382 in whole cell extracts (WCE) (S2C) are shown.

(S2D) Western blot analyses of the level of AceH4 (K5, 8, 12, 16) and of Acep53K382 in nuclear extracts (NE) of HCT 116 cells that were transfected with expression vectors for Myc-tagged WT SIRT1 or the constitutively active K610R SIRT1 mutant or the catalytically dead H363Y SIRT1 mutant, or with an empty vector (-). Expression of the exogenous SIRT1 proteins was analysed using anti-Myc antibody. TBP and Tubulin were used as loading controls.

(S2E) Representative immunostaining micrographs (40X) of consecutive tumour sections from CRC patients using antibodies against SIRT1 (brown) and its substrates β-catenin (brown) and AceH3K9, AceH4 (K5,8,12,16) and Acep53 (K382) (magenta). Two

examples of patients with high expression and two with low expression of SIRT1 are shown. Scale bars, 20 μm.

(S2F) Representative micrographs (10X) to illustrate example cut-offs from immunostaining of CRC patient samples for both nuclear and cytoplasmic β-catenin (brown).

Supplementary Figure S3: Effect of $1,25(OH)_2D_3$ on the expression of β-catenin and Wnt target genes in the presence of SIRT1 modulators

(S3A-B) Effect of the general sirtuin inhibitor nicotinamide (NAA) on the effect of $1,25(OH)_2D_3$ on β-catenin protein expression in CRC cells. (S3A) Confocal immunostaining imaging of β-catenin (green) in HCT 116 cells treated with $1,25(OH)_2D_3$ (100 nM) or vehicle in the presence or absence of NAA (300 μM) for 24 h. Lamin B antibody (red) marked the nuclear envelope. (S3B) Western blot analysis of β-catenin protein content in nuclear extracts (NE) of HCT 116 or HT-29 cells treated with $1,25(OH)_2D_3$ or vehicle in the presence or absence of NAA (300 μM) for 24 h. TBP was used as loading control (S3C) Comparison of the efficiency to deplete SIRT1 protein expression of siRNAs from Santa Cruz and Dharmacon. Representative western blot analyses of nuclear extracts (NE) of HCT 116 and HT-29 cells. TBP was used as loading control.

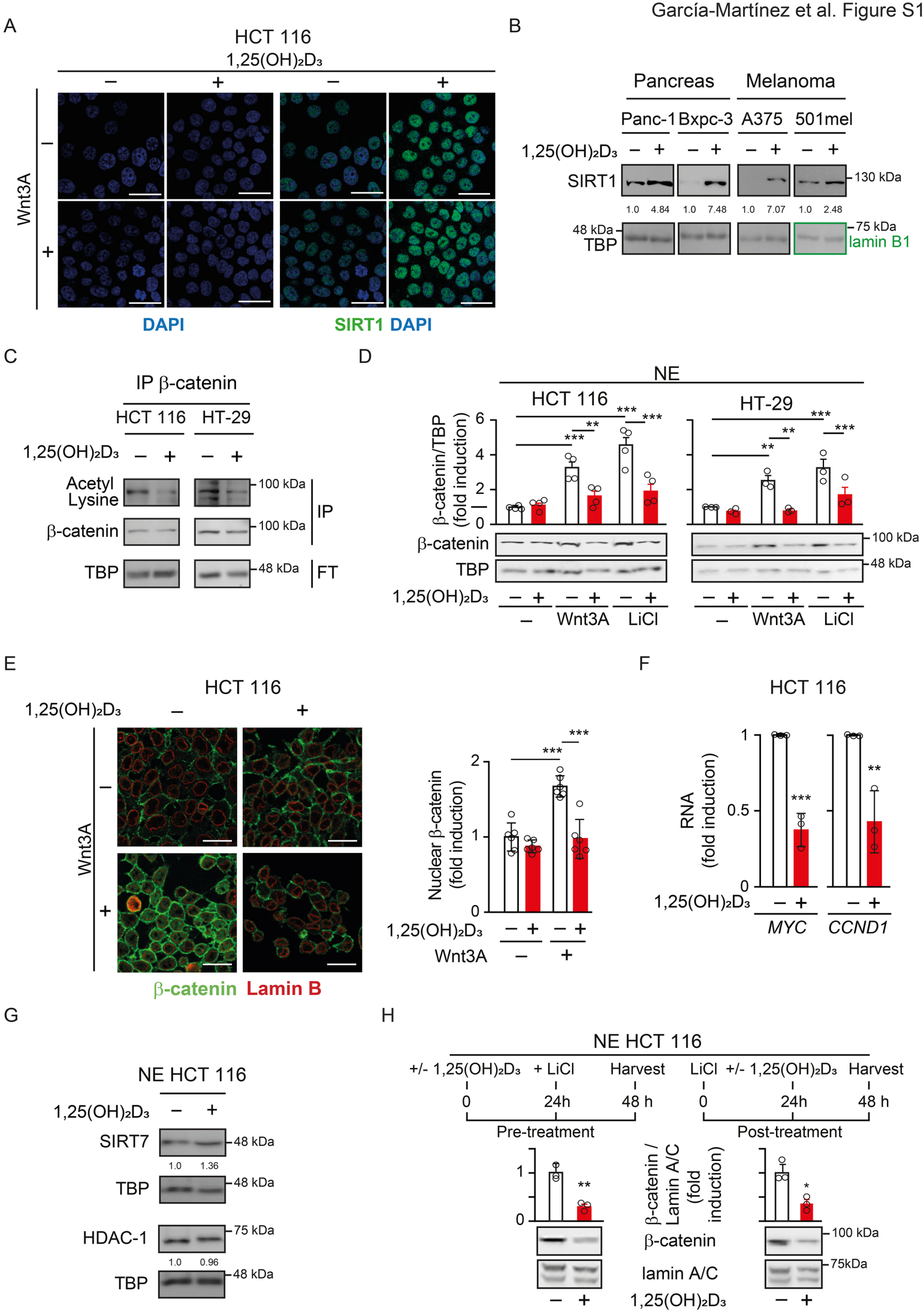
(S3D) Western blot analysis of the effect of SIRT1 depletion (siRNA from Dharmacon) on the nuclear levels of β-catenin in HT-29 CRC cells. TBP served as loading control.

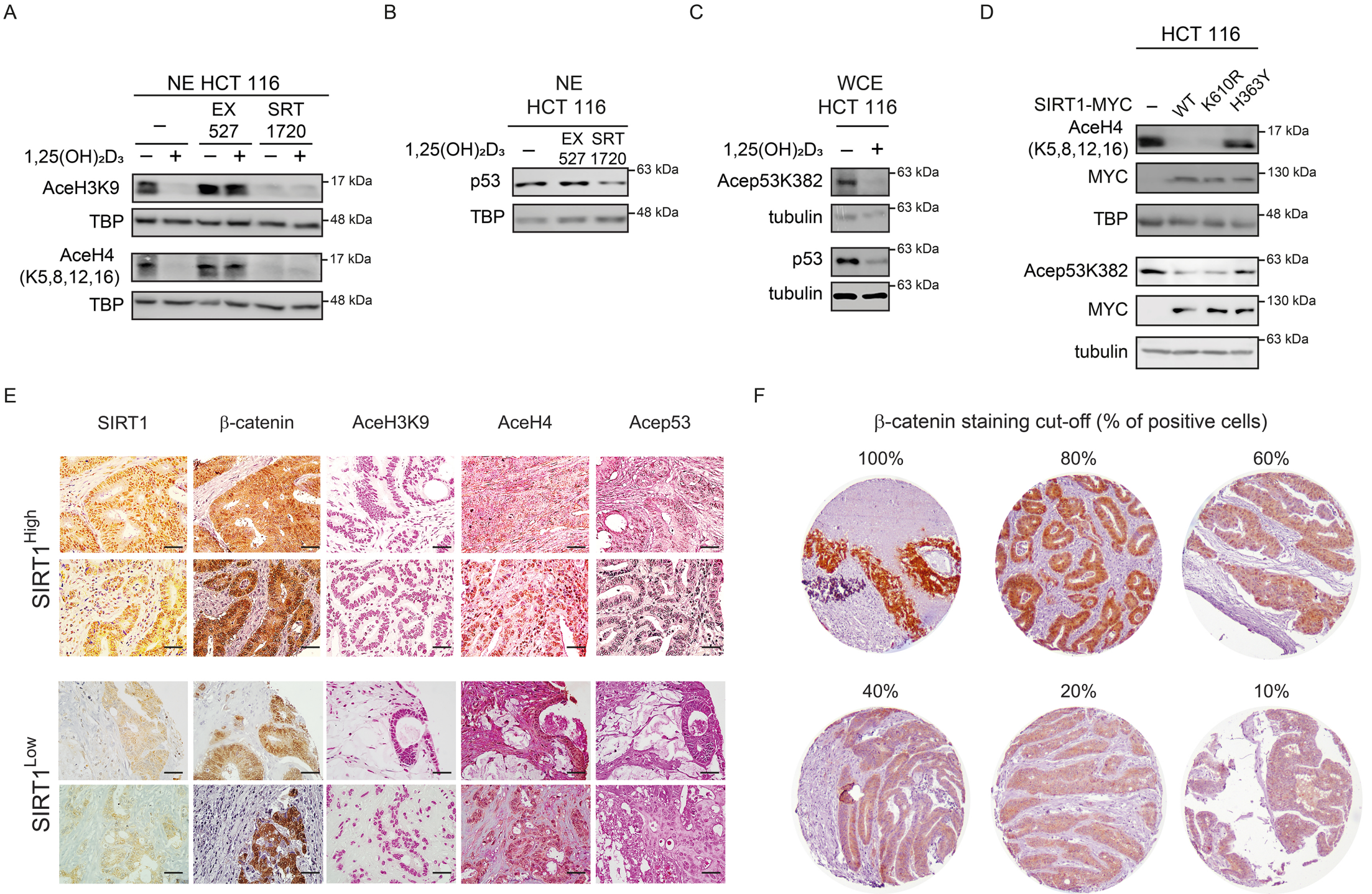
(S3E-F) SIRT1 mediates $1,25(OH)_2D_3$ action on Wnt signalling. RT-qPCR analysis of the regulation of *AXIN 2* (S3E) and *DKK1* (S3F) RNA levels in HCT 116 cells by treatment with $1,25(OH)_2D_3$ (100 nM) in the presence or absence of the EX527 SIRT1 inhibitor (10 μM) or the SRT1720 SIRT1 activator (10 μM) for 24 h.

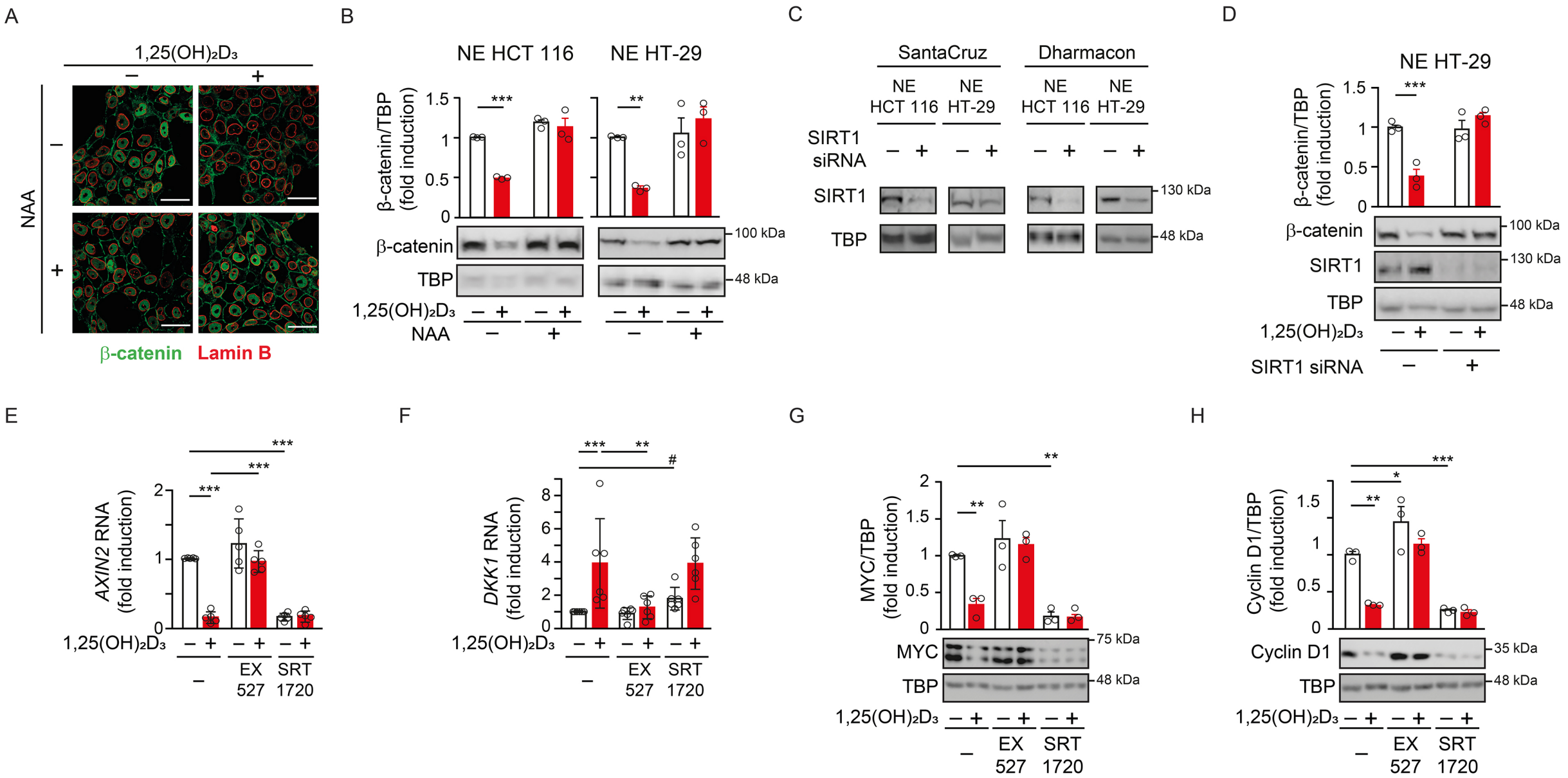
(S3G-H) SIRT1 mediates $1,25(OH)_2D_3$ action on Wnt target proteins. Western blot analyses of the regulation of Myc and Cyclin D1 proteins in HCT 116 cells by treatment with $1,25(OH)_2D_3$ (100 nM) in the presence or absence of the EX527 SIRT1 inhibitor (10 μM) or the SRT1720 SIRT1 activator (10 μM) for 24 h. TBP was used as loading control.

Mean ± SEM of triplicates is displayed for western blots in panels B, D, G and H. Median ± SD is shown for RT-qPCR in panels E and F. Statistical analysis by One-Way ANOVA

of at least 3 independent experiments is performed; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Specific pairs of variables were additionally compared using Student t-test (#): #$P < 0.05$.


A
HCT 116
1,25(OH)₂D₃
Wnt3A
DAPI
SIRT1 DAPI
B
Pancreas
Melanoma
Panc-1
Bxpc-3
A375
501mel
1,25(OH)₂D₃
SIRT1
130 kDa
1.0 4.84 1.0 7.48 1.0 7.07 1.0 2.48
48 kDa
TBP
75 kDa
lamin B1
C
IP β-catenin
HCT 116
HT-29
1,25(OH)₂D₃
Acetyl Lysine
β-catenin
TBP
100 kDa
48 kDa
IP
FT
D
NE
HCT 116
HT-29
β-catenin/TBP (fold induction)
β-catenin
TBP
100 kDa
48 kDa
1,25(OH)₂D₃
Wnt3A
LiCl
E
HCT 116
1,25(OH)₂D₃
Wnt3A
β-catenin Lamin B
Nuclear β-catenin (fold induction)
F
HCT 116
RNA (fold induction)
MYC
CCND1
G
NE HCT 116
1,25(OH)₂D₃
SIRT7
48 kDa
1.0 1.36
TBP
HDAC-1
75 kDa
1.0 0.96
H
NE HCT 116
+/- 1,25(OH)₂D₃
+ LiCl
Harvest
LiCl
0
24h
48 h
Pre-treatment
Post-treatment
β-catenin / Lamin A/C (fold induction)
β-catenin
lamin A/C
100 kDa
75kDa


A
NE HCT 116
–
EX 527
SRT 1720
1,25(OH)2D3 – + – + – +
AceH3K9
TBP
AceH4 (K5,8,12,16)
TBP
17 kDa
48 kDa
17 kDa
48 kDa
B
NE HCT 116
1,25(OH)2D3 – EX 527 SRT 1720
p53
TBP
63 kDa
48 kDa
C
WCE HCT 116
1,25(OH)2D3 – +
Acep53K382
tubulin
p53
tubulin
63 kDa
63 kDa
63 kDa
63 kDa
D
HCT 116
SIRT1-MYC – WT K610R H363Y
AceH4 (K5,8,12,16)
MYC
TBP
Acep53K382
MYC
tubulin
17 kDa
130 kDa
48 kDa
63 kDa
130 kDa
63 kDa
E
SIRT1
β-catenin
AceH3K9
AceH4
Acep53
SIRT1High
SIRT1Low
F
β-catenin staining cut-off (% of positive cells)
100%
80%
60%
40%
20%
10%


A
1,25(OH)₂D₃
–
+
NAA
–
+
β-catenin
Lamin B
B
NE HCT 116
NE HT-29
β-catenin/TBP (fold induction)
1.5
1
0.5
0
***
**
β-catenin
TBP
100 kDa
48 kDa
1,25(OH)₂D₃ – + – + – + – +
NAA – + – +
C
SantaCruz
Dharmacon
NE HCT 116
NE HT-29
NE HCT 116
NE HT-29
SIRT1 siRNA – + – + – + – +
SIRT1
TBP
130 kDa
48 kDa
D
NE HT-29
β-catenin/TBP (fold induction)
1.5
1
0.5
0
***
β-catenin
SIRT1
TBP
100 kDa
130 kDa
48 kDa
1,25(OH)₂D₃ – + – +
SIRT1 siRNA – +
E
AXIN2 RNA (fold induction)
2
1
0
***
***
***
1,25(OH)₂D₃ – + – + – +
–
EX 527
SRT 1720
F
DKK1 RNA (fold induction)
8
6
4
2
0
***
**
#
1,25(OH)₂D₃ – + – + – +
–
EX 527
SRT 1720
G
MYC/TBP (fold induction)
2
1.5
1
0.5
0
**
**
MYC
TBP
75 kDa
48 kDa
1,25(OH)₂D₃ – + – + – +
–
EX 527
SRT 1720
H
Cyclin D1/TBP (fold induction)
1.5
1
0.5
0
***
*
**
Cyclin D1
TBP
35 kDa
48 kDa
1,25(OH)₂D₃ – + – + – +
–
EX 527
SRT 1720

Supplementary Table ST1. Key resources

Reagent or Resource	Source	Identifier
Antibodies		
Rabbit Polyclonal anti-Acetylated-Lysine	Cell Signaling	Cat# 9441
Rabbit Polyclonal anti-β-Catenin (C18)	Santa Cruz Biotechnology	Cat# SC-1496-R
Mouse monoclonal anti-β-Catenin	BD	Cat# 610154
Rabbit Polyclonal anti-SIRT1	Santa Cruz Biotechnology	Cat# SC-15404
Rabbit Polyclonal anti-SIRT1 (D1D7)	Cell Signaling	Cat# 9475
Rabbit monoclonal anti-Histone H3K9-Ace (C5B11)	Cell Signaling	Cat # 9649
Rabbit Polyclonal anti-Histone H3	Cell Signaling	Cat # 9715
Rabbit Polyclonal anti-Vitamin D_3 receptor (D2K6W)	Cell Signaling	Cat #12550
Rabbit Polyclonal anti-MYC (N-262)	Santa Cruz Biotechnology	Cat# SC-764
Mouse monoclonal anti-Cyclin D1 (R-124):	Santa Cruz Biotechnology	Cat# SC-6281
Rabbit monoclonal anti-TBP (D5C9H)	Cell Signaling	Cat#44059
Rabbit Polyclonal anti-Histone H4K5,8,12,16-Ace	Millipore	Cat# 06-598
Rabbit monoclonal anti-K382p53 (10 H13L14)	Thermo Scientific	Cat# 701270
Mouse monoclonal anti-p53 (DO-1)	Santa Cruz Biotechnology	Cat# sc-126
Goat Polyclonal anti-Lamin B (C20)	Santa Cruz Biotechnology	Cat# sc-6216
Mouse monoclonal anti-Lamin A/C (4C11)	Cell Signaling	Cat# 4777
Mouse Monoclonal anti-MYC (9E10)	Santa Cruz Biotechnology	Cat# SC-40
Mouse Monoclonal anti-Tubulin B-5-1-2	Sigma-Aldrich	Cat# T5168
Mouse Monoclonal anti-SIRT7 (C-3)	Santa Cruz Biotechnology	Cat# sc- 365344
Rabbit Polyclonal anti-HDAC-1	Upstate	Cat# 06-720
Goat Anti-Rabbit IgG (H+L) HRPO	BIO-RAD	Cat #170-6515

Goat Anti-Mouse IgG (H+L) HRPO	BIO-RAD	Cat #170-6516
Rabbit-Anti Goat IgG (H+L) HRPO	BIO-RAD	Cat #172-1034
Donkey Anti-Rabbit AlexaFluor488	Invitrogen	Cat# A21206
Donkey Anti-Mouse AlexaFluor488	Invitrogen	Cat# A21202
Donkey Anti-Goat AlexaFluor647	Invitrogen	Cat# A21447
Bacterial and Virus Strains		
E. coli-5α	New England Biolabs	NEB5α
Chemicals, Peptides, and Recombinant Proteins		
1α,25-Dihydroxyvitamin D_3	Sigma-Aldrich	17936
Lithium Chloride	Sigma-Aldrich	Cat# L9650
Recombinant Murine Wnt3A	Peprotech	Cat# 315-20
Glucose	Sigma-Aldrich	G8769
BSA	Sigma-Aldrich	A7906
Nicotinamide (NAA)	Sigma-Aldrich	N3376
SRT1720	Selleckchem	S1129
TRIzol reagent	Invitrogen	Cat#15596026
COMPLETE Protease inhibitor Cocktail	Roche	Cat#04693132001
7-AAD	Santa Cruz Biotechnology	SC-221210
DAPI (4',6-diamidino-2-fenilindol, dilactato)	Invitrogen	10184322
3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazol (MTT)	Sigma-Aldrich	Cat# M5655
DMEM medium	Lonza	Cat# 12-604F
Bovine Fetal Serum	Sigma	Cat# F7524
JetPEI PolyPlus reagent	Genycell Biotech	Cat# 101-10N
JetPRIME PolyPlus reagent	Genycell Biotech	Cat # 114-01

DYNAbeads Protein A	Invitrogen	Ref 10002D
DYNAbeads Protein G	Invitrogen	Ref 10004D
Experimental Models: Cell Lines		
HCT 116 (Male) colorectal carcinoma	ATCC	Cat# CCL-247, RRID:CVCL_0291
HT-29 (Female) rectosigmoid adenocarcinoma	ATCC	Cat# HTB−38, RRID:CVCL_0320
HCT 116 ShControl	In house	PMID: 21858154
HCT 116 ShVDR	In house	PMID: 21858154
PANC-1 Pancreas Epithelioid Carcinoma.	ATCC	Cat# CRL-1469
PL-45. Pancreatic Ductal Adenocarcinoma	ATCC	Cat# CRL-2558
A375M (Male). Human melanoma cell line	ATCC	Cat# CVCL_2765
501mel (Female). Human melanoma cell line	Obtained from Ruth Halaban, Yale	(Zakut et al., 1993)
Oligonucleotides		
Human *SIRT1* K610R mutagenesis primers F 5′-3′: GGTTCTAGTACTGGGGAGAGGAATG AAAGAACTT CAGTGG R 5′-3′: CCAGCCACTGAAGTTCTTTCATTCCTC TCCCCAGT ACTAG	Sigma	This study
Human CYCLIN D1 (*CCND1*) qPCR primers F 5′-3′: AAGATCGTCGCCACCTGG R 5′-3′: GGAAGACCTCCTCCTCGCAC	Sigma	This study
Human *MYC* qPCR primers F 5′-3′: CTTCTCTCCGTCCTCGGATTCT R 5′-3′: GAAGGTGATCCAGACTCTGACCTT	Sigma	This study
Human 18s *rRNA* qPCR primers F 5′-3′: AGTCCCTGCCCTTTGTACACA R 5′-3′: GCCTCACTAAACCATCCAATCG	Sigma	This study
Human *DKK1* TaqMan® probe	Applied Biosystems	Hs00167999_m1
Human *AXIN2* TaqMan® probe	Applied Biosystems	Hs00610344_m1

Sirt1 siRNA	Santa Cruz Biotechnology	Cat# sc-45313
ON-TARGETplus Human SIRT1 (23411) siRNA	Dharmacon	Cat# SO-2917221G
siRNA Control	Qiagen	Cat# 1027281
Recombinant DNA		
pcDNA3.1-SIRT1-Myc-His	Gift from Prof. Colin R Goding	N/A
pcDNA3.1-SIRT1H363Y- Myc-His	Gift from Prof. Colin R Goding	N/A
pcDNA3.1-SIRT1 K601R- Myc-His	This study	N/A
Software and Algorithms		
LAS AF software	Leica	SP5
3730xl Analyzer	Applied Biosystem	ABI 3730XL
GraphPad Prism software	GraphPad Software	https://www.graphpad.com
Typhoon scanner control 3.0	Applied Biosystem	Typhoon 9210
ImageLab	Bio-Rad	ChemiDoc XRS+ System
CXP software	Becton-Dickinson	FACSCalibur
ImageJ software	https://imagej.nih.gov/ij/download.html	N/A
SPSS Statistics version 26	IBM	N/A
The Human Protein Atlas	https://www.proteinatlas.org/ENSG00000096717-SIRT1/pathology/colorectal+cancer	N/A

Supplementary Table ST2. Clinicopathological characteristics of CRC patients included in the study.

Patient characteristics	Number (%)	
	Stage II	Stage IV
Gender		
Male	57 (60%)	27 (48%)
Female	38 (40%)	23 (41%)
N/A	0	6 (11%)
Tumor Location		
Cecum	13 (14%)	3 (5%)
Right	25 (26%)	10 (18%)
Transverse	7 (8%)	3 (5%)
Left	5 (5%)	2 (4%)
Sigma	24 (25%)	21 (38%)
Rectum	21 (21%)	12 (21%)
N/A	0	5 (9%)
Grade Primary Tumor		
Well differentiated	18 (19%)	15 (27%)
Moderately differentiated	69 (73%)	27 (48%)
Poorly differentiated	8 (8%)	5 (9%)
N/A	0	9 (16%)
Paired Liver Metastasis		54 (96%)
N/A		2 (4%)
Grade Metastatic Tumor		
Well differentiated		12 (21%)
Moderately differentiated		29 (52%)
Poorly differentiated		4 (7%)
N/A		11 (20%)
Metastasis at diagnosis		
Synchronous		35 (62%)
Metachronous		21 (38%)
Pt		
T1	3 (3%)	2 (4%)
T2	30 (32%)	5 (9%)
T3	61 (64%)	42 (75%)
T4	1 (1%)	4 (7%)
N/A		3 (5%)
pN		
N0	95 (100%)	27 (48%)
N1		16 (29%)
N2		9 (16%)
N3		1 (2%)
N/A		3 (5%)