Supplemental Figure Legends

Figure S1

Western blot analysis of SIRT6 expression in (A and B) colon cancer tissue and adjacent noncancerous tissue from patients, as well as (C) the colon cancer cell lines or normal cancer cell line. (A) Representative Western Blot results of SIRT6 protein in normal colon specimens from health person or in CRC tissues and in matched adjacent non-cancerous tissues from the same patients respectively. (B) Quantitation analysis of SIRT6 protein bands density from western in CA and NCA (**P<0.01, paired t test). (C) The expression of SIRT6 in FHC normal colon epithelial cell line and the indicated colon cancer cell lines.

Figure S2

Akt but not p53 is involved in SIRT6 induction. (A and C) The immunoblot bands of p-Akt and SIRT6 in (A) Figure 2C and in (C) Figure 2E were quantified by densitometry analysis and the ratio to Akt and β-actin were calculated, respectively. (B and D) Real-time PCR analysis of SIRT6 mRNA induction (B) during time course of BKM120 treatment in DLDs cells or in (D) LoVo and DLD1 cells without Akt expression by shRNA knockdown. *P<0.05, **P<0.01 vs. 0h in A, *P<0.05, **P<0.01 vs. shVector in B. (E and F) Western blot analysis of p53, SIRT6 and cleaved-caspase3 in (E) DLD1 cells after different time of BKM120 stimulation or in (F) WT or $p53^{-/-}$ HCT-116 cells following BKM120 treatment. Data represent the mean ± SEM of four independent experiments.

Figure S3

The effect of Akt inhibition on FoxO3a activation and SIRT6 induction in colon cancer cells. (A) The immunoblot bands of p-FoxO3a and SIRT6 in Figure 3F were quantified by densitometry

analysis and the ratio to FoxO3a and β-actin were calculated, respectively. (B) Real-time PCR analysis of FoxO3a expression in colon tissue from normal person and in tumor tissue or adjacent tissue from colorectal cancer (CRC) patients after BKM120 treatment. (C) The effects of Akt activity on SIRT6 induction in DLD1 cells transfected with Myr-Akt or WT-Akt constructs after 24 hours BKM120 treatment. (D) DLD1 cells transfected with Myr-Akt (a constitutively-active Akt expression construct) were treated in the presence/absence of BKM120. Akt inhibitor VIII was added to mimic the effect of BKM120. Akt inhibitor VIII was added to mimic the effect of BKM120. The levels of p-Akt (S473), p-FoxO3a (S253) and p53, SIRT6 were analyzed by Western blotting. (E) Western blot analysis the levels of p-Akt (Ser473), p-FoxO3a (S253) and SIRT6 in DLD1 cells treated with Akt inhibitor VIII (5 μM) or MK-2206 (5μM) for 24 hours.

Figure S4

Regulation SIRT6 expression by the binding of FoxO3a but not SIRT1 to the SIRT6 promoter (related to Fig. 4) (A and B) Knockdown of FoxO3a or SIRT1 by shRNA blocked (A) SIRT6 promoter activity in LoVo cells revealed by luc reporter assay or (B) endogenous SIRT6 mRNA expression in DLD1 cells as revealed by RT-PCR. (C) The absence of SIRT1 did not change SIRT6 mRNA level induced by BKM120. Semi-Quantitative PCR analysis showing SIRT6 mRNA expression in immortalized WT and $SIRT1^{-/-}$ cells treated with/without BKM120 for 24h. (D and E) Effect of (D) RNAi-mediated knockdown of NRF1 or (E) NRF1 ectopic expression on SIRT6 promoter reporter. (F) Left, schematic representation of fragments (Frag) A to F of the SIRT6 promoter used in the luciferase experiment. right, LoVo cells were transient transfected with different luciferase reporters, followed by BKM120 treatment for 24h. (G) The amount of

FoxO3a and SIRT1 bound to SIRT6 promoter in the presence or absence of BKM120 stimulation. (H and I) The effect of siRNA-mediated knockdown of either (H) FoxO3a or (I) SIRT1 on recruitment of them to the SIRT6 promoter. (J) SIRT1 did not bind to FoxO3a in response to BKM120 treament. Co-immunoprecipitation with an anti-SIRT1 antibody was used to pull down SIRT1, western blotting for FoxO3a shows the amount of FoxO3a binding to SIRT1.

Figure S5

The effects of combination treatment of LoVo cells. (A) The combination treatment of 5-Fu and BKM120 induced much more apoptosis upon Akt inhibition. The expression of p53, P-Akt, Akt and cleaved-caspase3 were detected by western blotting after the treatment of 20 mg/mL 5-FU and/or 2.5μM BKM120 for 24 hours in LoVo cells. (B and C) Inhibition of ERK by UO126 activated FoxO3a. (B) FoxO3a nuclear translocation was determined by western blot. LaminA/C and GAPDH were used as the nucleus and cytoplasm marker for loading, respectively. (C) p-FoxO3a (S253) and SIRT6 expression after UO126 or BKM120 treatment were detected by Western blot analysis. (D) Effect of FoxO3a on SIRT6 induction in combination treatment. SIRT6 promoter activity in shVector or shFoxO3a LoVo cells with different drug treatment revealed by luc reporter assay. (E and F) Effect of SIRT6 on cell viability in combination treatment. Cells viability was analyzed using Cell Counting Kit-8 at 24 hours after 5μM BKM120 treatment combined with (E) cisplatin or with (F) regorafenib in LoVo cells. Error bars are SEM from four independent experiments; P* < 0.05, P**<0.01.

Figure S6

Loss of SIRT6 blocked Cyto c release and mitochondrial pathway apoptosis. Quantitative

analysis of (A) cleaved-Caspase-3 or (B) cleaved-caspase-9 expression corresponding to the images in Fig6F. Data represent the mean ± S.D. of four independent experiments. (C) WT and Bax-KO HCT-116 cells were treated with BKM-120 for 48 hours. Apoptosis was determined by nuclear staining with Hoechst 33342. Results were expressed as means±SD of three independent experiments. * $P < 0.05$. (D and E) Cells viability was analyzed using Cell Counting Kit-8 at 24 hours after 5μM BKM120 treatment in shVector or shSIRT6 (D) LoVo or (E) DLD1 cells. Error bars are SEM from four independent experiments; P* < 0.05, P**<0.01. (F) The effect of over-expression SIRT6 on the level of H3K9 acetylation. Western blot analysis the levels of Ac-H3K9 and histone H3 level in the nucleus fraction of cells transfected with flag-SIRT6. (G) Western blot analysis of survivin, SIRT6 and c-caspase3 expression in DLD1 cells after BKM120 treatment. (H and I) Level of Survivin mRNA and protein expression in LoVo cells (H) transfected with SIRT6 following BKM120 treatment or (I) during time course of BKM120 treatment by real-time PCR.

Figure S7

Quantitative analysis of (A) ki67 and (B) active Caspase-3 staining by IHC or SIRT6 mRNA expression by real-time PCR (C) in shVector and shSIRT6 tumors with/without BKM120 from xenograft mouse. Data represent the mean ± S.D. of three independent experiments. Six samples from three tumors in each group were used.

Table S1. The information of antibodies.

Figure S1

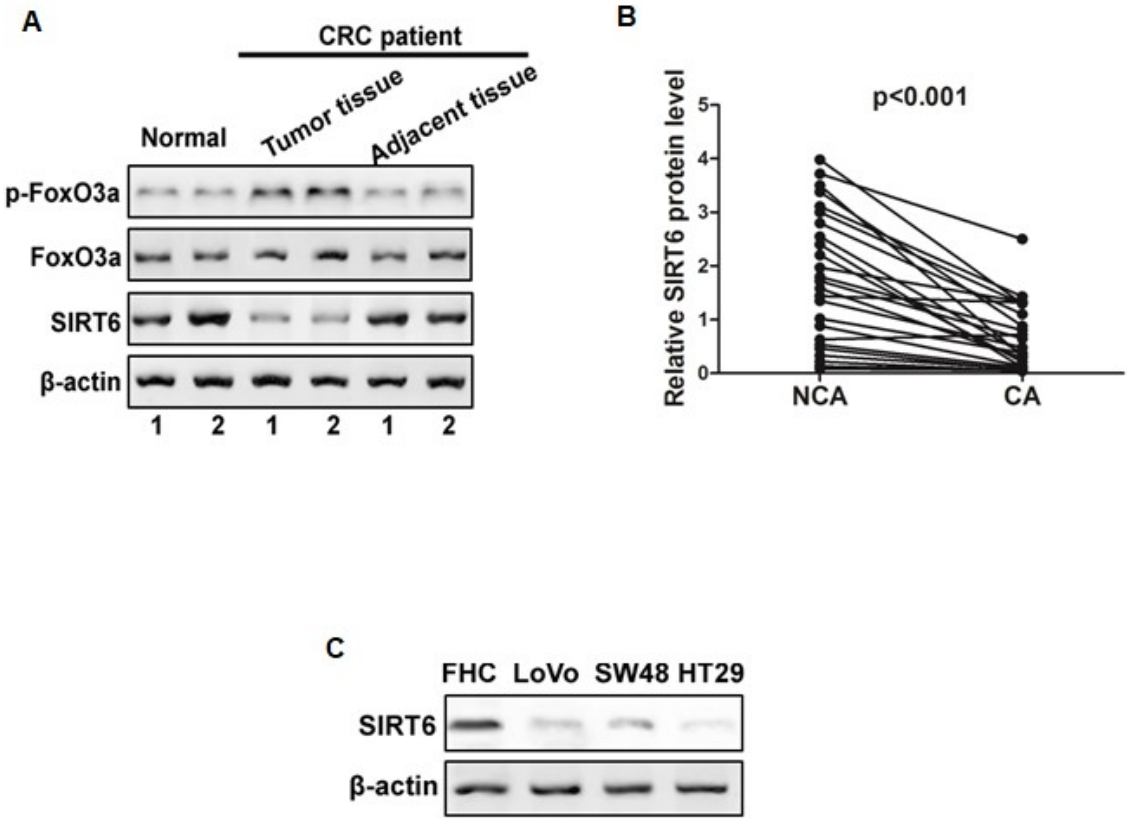

A
CRC patient
Normal
Tumor tissue
Adjacent tissue
p-FoxO3a
FoxO3a
SIRT6
β-actin
1 2 1 2 1 2
B
p<0.001
Relative SIRT6 protein level
0
1
2
3
4
5
NCA
CA
C
FHC LoVo SW48 HT29
SIRT6
β-actin

Figure S2

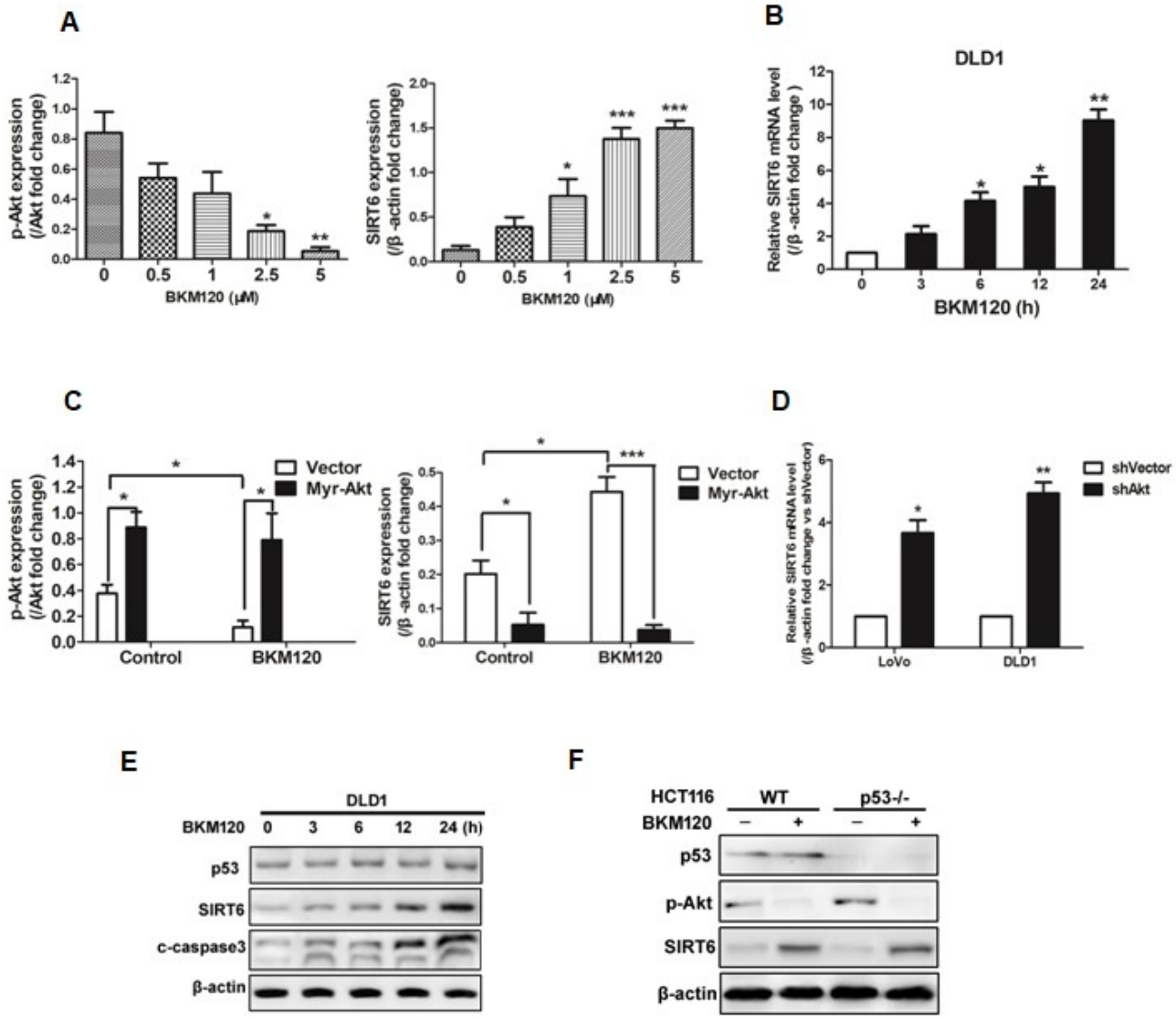

A
p-Akt expression (/Akt fold change)
BKM120 (μM)
SIRT6 expression (/β -actin fold change)
BKM120 (μM)
B
DLD1
Relative SIRT6 mRNA level (/β -actin fold change)
BKM120 (h)
C
Vector
Myr-Akt
p-Akt expression (/Akt fold change)
Control
BKM120
SIRT6 expression (/β -actin fold change)
D
shVector
shAkt
Relative SIRT6 mRNA level (/β -actin fold change vs shVector)
LoVo
DLD1
E
DLD1
BKM120 0 3 6 12 24 (h)
p53
SIRT6
c-caspase3
β-actin
F
HCT116 WT p53-/-
BKM120 – + – +
p53
p-Akt
SIRT6
β-actin

Figure S3

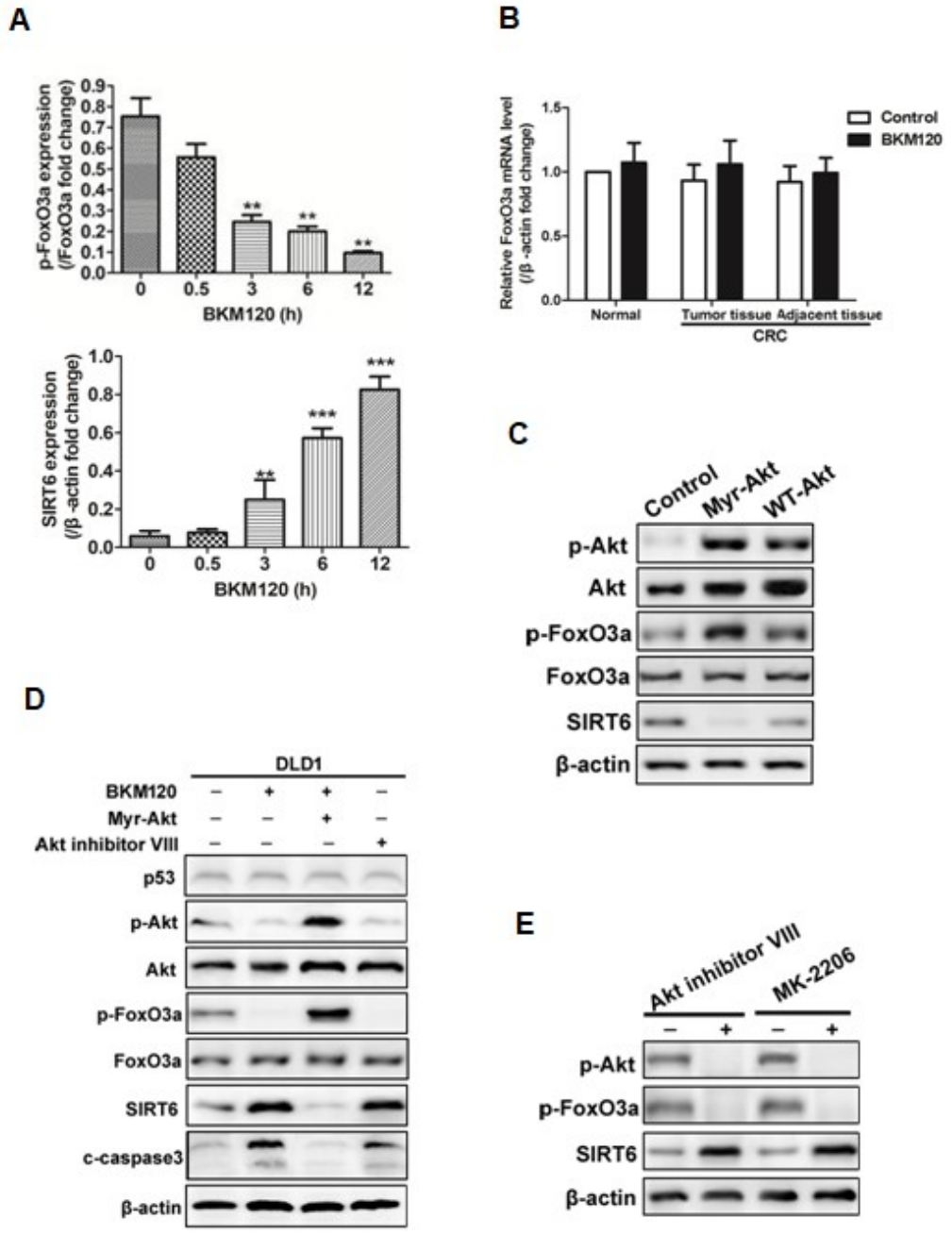

A
p-FoxO3a expression (/FoxO3a fold change)
BKM120 (h)
SIRT6 expression (/β -actin fold change)
BKM120 (h)
B
Relative FoxO3a mRNA level (/β -actin fold change)
Control
BKM120
Normal
Tumor tissue
Adjacent tissue
CRC
C
Control
Myr-Akt
WT-Akt
p-Akt
Akt
p-FoxO3a
FoxO3a
SIRT6
β-actin
D
DLD1
BKM120
Myr-Akt
Akt inhibitor VIII
p53
p-Akt
Akt
p-FoxO3a
FoxO3a
SIRT6
c-caspase3
β-actin
E
Akt inhibitor VIII
MK-2206
p-Akt
p-FoxO3a
SIRT6
β-actin

Figure S4

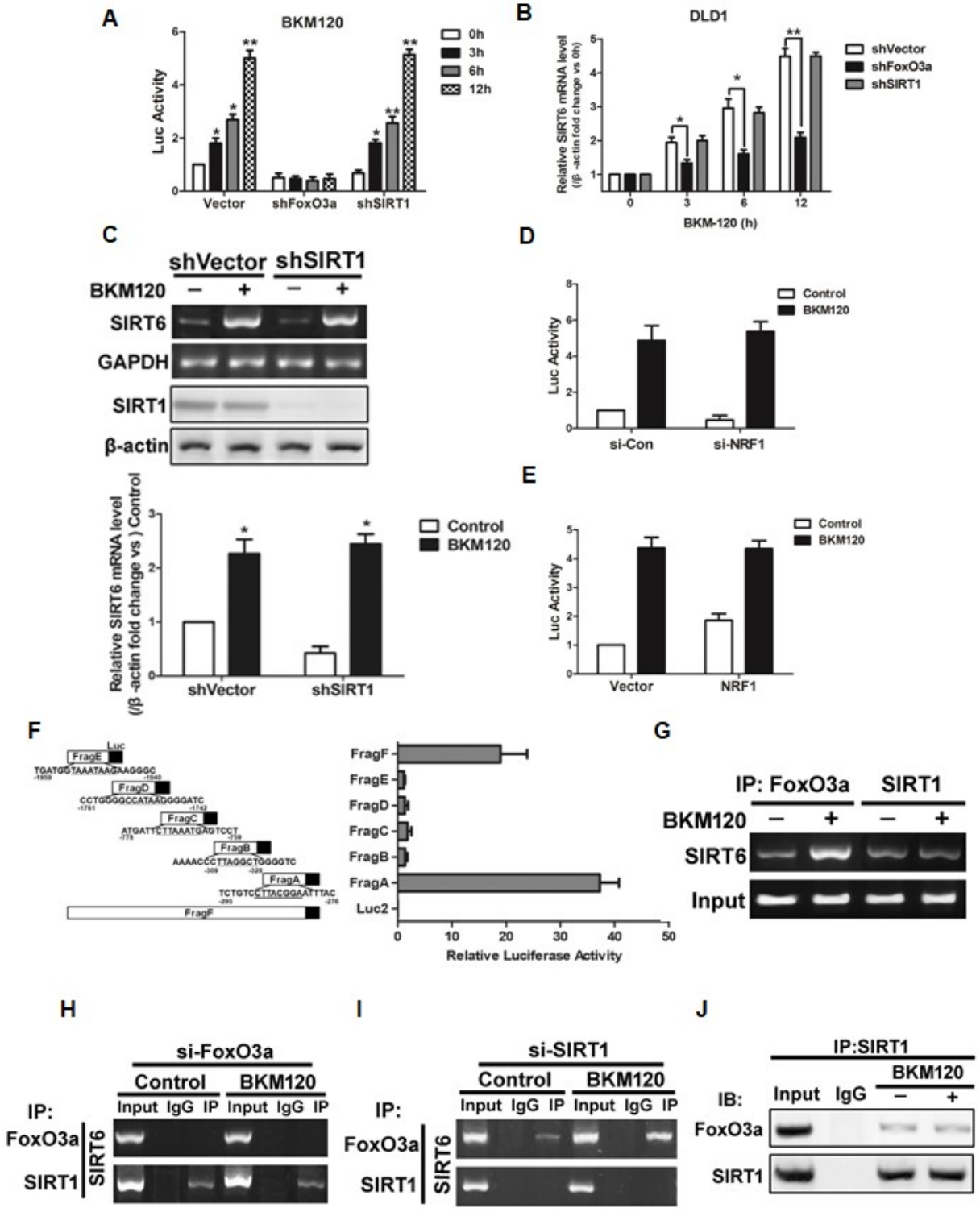

A
BKM120
Luc Activity
0h
3h
6h
12h
Vector
shFoxO3a
shSIRT1
B
DLD1
Relative SIRT6 mRNA level (/β -actin fold change vs 0h)
shVector
shFoxO3a
shSIRT1
BKM-120 (h)
C
shVector
shSIRT1
BKM120
SIRT6
GAPDH
SIRT1
β-actin
Relative SIRT6 mRNA level (/β -actin fold change vs) Control
Control
BKM120
D
Luc Activity
si-Con
si-NRF1
E
Luc Activity
Vector
NRF1
F
Luc
FragE
FragD
FragC
FragB
FragA
FragF
Luc2
Relative Luciferase Activity
G
IP: FoxO3a
SIRT1
BKM120
SIRT6
Input
H
si-FoxO3a
Control
BKM120
IP:
Input IgG IP Input IgG IP
FoxO3a
SIRT1
SIRT6
I
si-SIRT1
Control
BKM120
IP:
Input IgG IP Input IgG IP
FoxO3a
SIRT1
SIRT6
J
IP:SIRT1
BKM120
IB:
Input IgG
FoxO3a
SIRT1

Figure S5

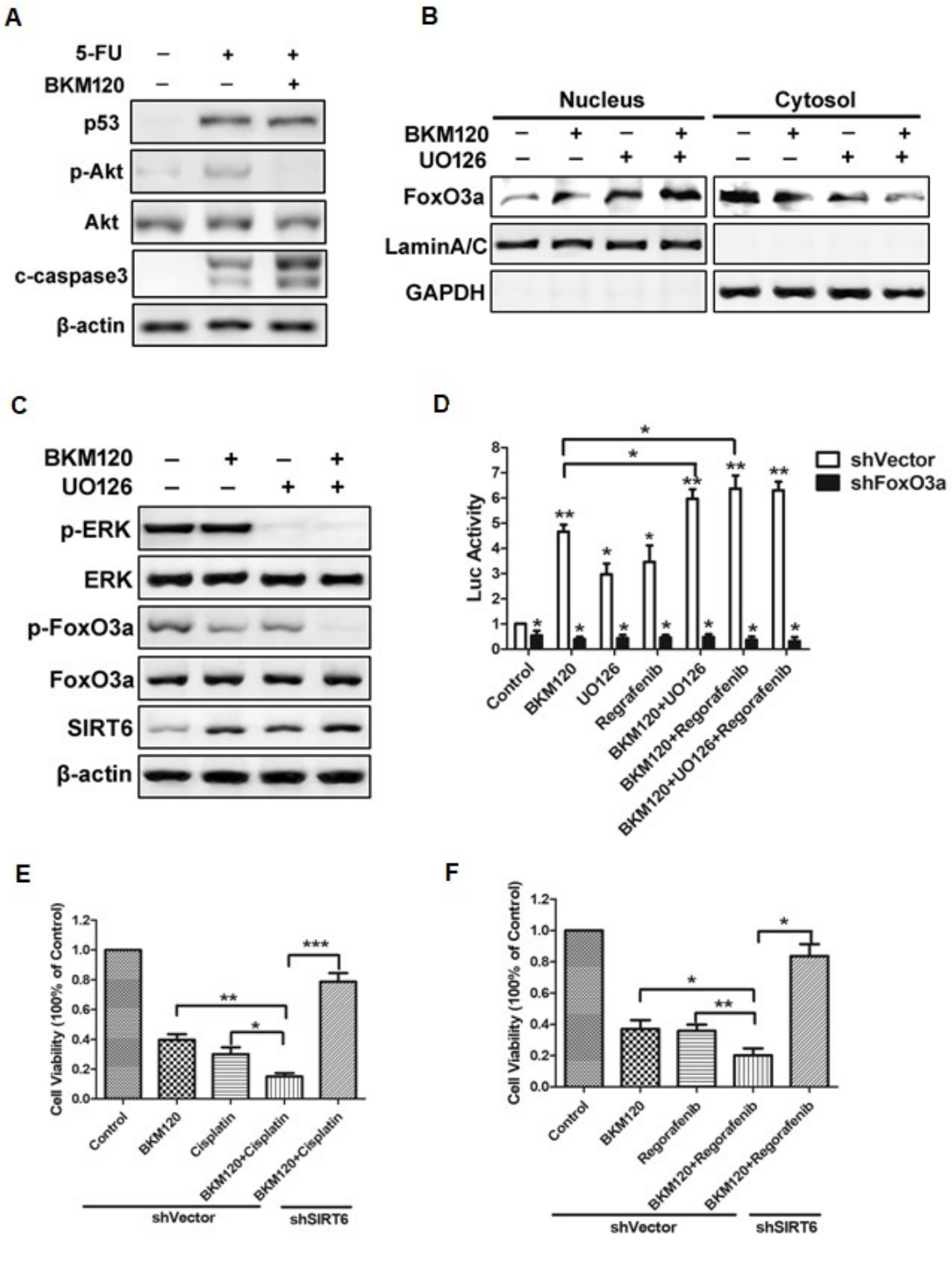

A
5-FU
BKM120
p53
p-Akt
Akt
c-caspase3
β-actin
B
Nucleus
Cytosol
BKM120
UO126
FoxO3a
LaminA/C
GAPDH
C
BKM120
UO126
p-ERK
ERK
p-FoxO3a
FoxO3a
SIRT6
β-actin
D
shVector
shFoxO3a
Luc Activity
Control
BKM120
UO126
Regrafenib
BKM120+UO126
BKM120+Regorafenib
BKM120+UO126+Regorafenib
E
Cell Viability (100% of Control)
Control
BKM120
Cisplatin
BKM120+Cisplatin
BKM120+Cisplatin
shVector
shSIRT6
F
Cell Viability (100% of Control)
Control
BKM120
Regorafenib
BKM120+Regorafenib
BKM120+Regorafenib
shVector
shSIRT6

Figure S6

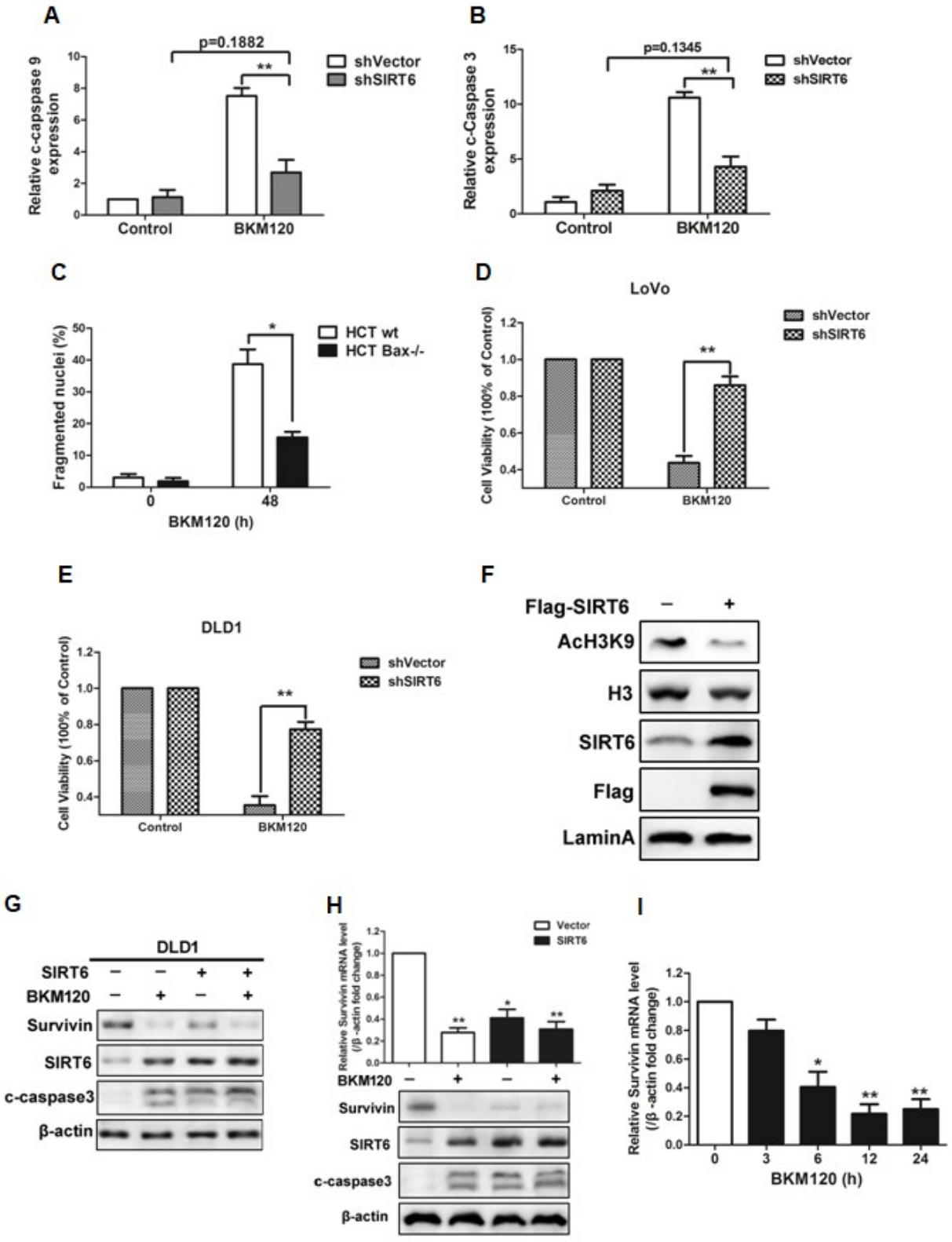
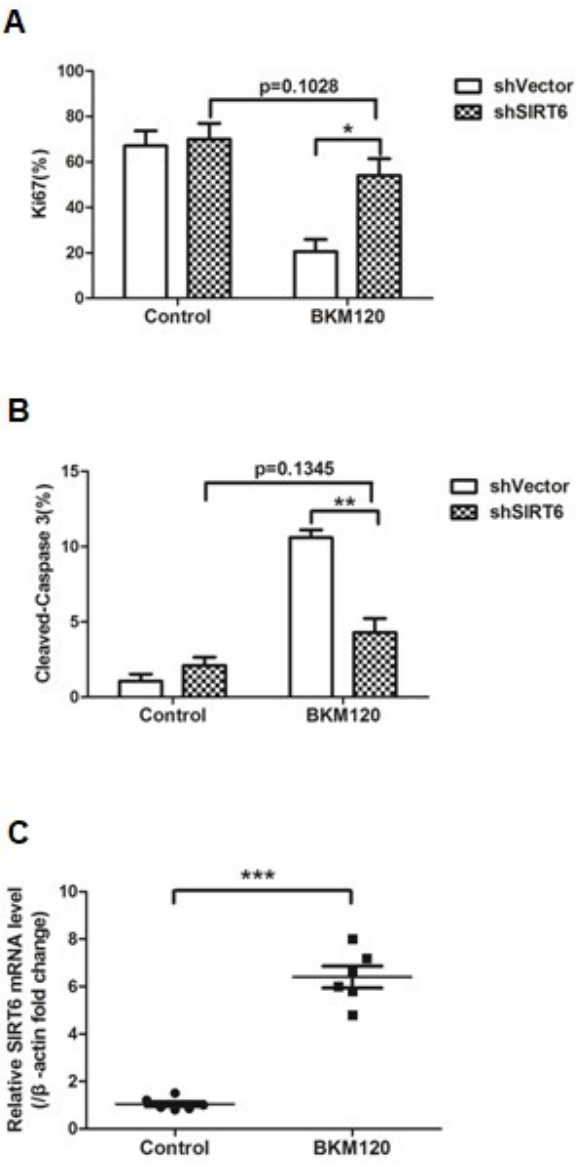

A
p=0.1882
**
shVector
shSIRT6
Relative c-capspase 9 expression
Control
BKM120
B
p=0.1345
**
shVector
shSIRT6
Relative c-Caspase 3 expression
Control
BKM120
C
*
HCT wt
HCT Bax-/-
Fragmented nuclei (%)
0
48
BKM120 (h)
D
LoVo
shVector
shSIRT6
**
Cell Viability (100% of Control)
Control
BKM120
E
DLD1
shVector
shSIRT6
**
Cell Viability (100% of Control)
Control
BKM120
F
Flag-SIRT6 – +
AcH3K9
H3
SIRT6
Flag
LaminA
G
DLD1
SIRT6 – – + +
BKM120 – + – +
Survivin
SIRT6
c-caspase3
β-actin
H
Vector
SIRT6
Relative Survivin mRNA level (/β -actin fold change)
**
*
**
BKM120 – + – +
Survivin
SIRT6
c-caspase3
β-actin
I
Relative Survivin mRNA level (/β -actin fold change)
*
**
**
0
3
6
12
24
BKM120 (h)

Figure S7


A
Ki67(%)
p=0.1028
*
shVector
shSIRT6
Control
BKM120
B
Cleaved-Caspase 3(%)
p=0.1345
**
shVector
shSIRT6
Control
BKM120
C
Relative SIRT6 mRNA level
(/β -actin fold change)
***
Control
BKM120

Supplemental Table

Antibodies	Company
p-Akt(S473)	Cell signaling, Abcam
Akt	Cell signaling
SIRT6	Cell signaling
P53	Cell signaling, Santa Cruz
FoxO3a	Cell signaling
p-FoxO3a(S315)	Cell signaling
p-FoxO3a(T32)	Cell signaling
p-FoxO3a(S253)	Cell signaling
SIRT1	Cell Signaling, Millipore
caspase3	Cell signaling
c-caspase3	Cell signaling
c-caspase9	Abcam
β-actin	Cell signaling
LaminA/C	Sigma
GAPDH	Santa Cruz
Ac-K	Abcam
p-ERK(Thr202/Tyr204)	Cell signaling
ERK	Cell signaling
Ki67	Millipore