Supplementary Methods

Clonogenic cell survival assay

Cells were seeded at 1,000 cells/well in 6-well plates. Next day, the cells were treated with alisertib (range: 0.01 – 0.25 µmol/L) in 2% FBS-DMEM medium. After 24 hrs, the medium was replaced by drug-free 10% FBS-DMEM medium and cells were allowed to grow for 10 days. Cell colonies were fixed with 2% paraformaldehyde solution for 10 min and stained overnight with crystal violet (0.05% crystal violet in 50% methanol). Cells were gently washed with 1X PBS and images were taken for quantification. Cell survival was determined by quantifying the dye signal in each well utilizing the ImageJ software (NIH).

Cell cycle distribution

Cells were treated for 24, 48, and 72 hrs, then were fixed in 75% ethanol, treated with RNase A, and were resuspended in 1 ml Propidium Iodide (PI) solution (PI 50µg/ml and RNase 1 µg/ml in 1x Phosphate Buffered Saline) and incubated at room temperature in the dark for 15-30 min. Subsequently, cell cycle distribution was measured and analyzed with BD LSR III Flow Cytometer (BD Biosciences, San Jose, CA) and the data were processed with BD FACS Diva software.

In vitro kinase assay

The in vitro kinase assay was performed using active human recombinant AURKA and RPS6KB1 proteins (Cell Sciences). Briefly, reaction was carried out in 20 µL assay buffer (50 mmol/L HEPES [pH7.4], 3 mmol/L $MgCl_2$, 3 mmol/L $MnCl_2$, 1 mmol/L dithiothreitol, 3 µmol/L Na-orthovanadate, 0.5 mmol/L adenosine triphosphate) containing a constant amount of RPS6KB1 (0.2 µg) and increasing amounts of recombinant AURKA (from 0.001 µg to 0.02 µg). The glutathione peroxidase 7 protein (Enzo Life Sciences, Inc., Farmingdale, NY) was used as a negative control for kinase activity. Reaction mixtures were incubated at 30℃ for 30 min. In parallel, a range of concentrations of alisertib (500 nmol/L, 100 nmol/L, 10 nmol/L, 5 nmol/L) were added to a mixture of recombinant AURKA (0.1 µg)

and RPS6KB1 (0.2 µg) and proteins were subjected to in vitro kinase assay followed by Western blot analysis.

Establishment of Tet-One™ inducible AURKA stable expression cell lines

Flag-tagged coding sequence of AURKA was subcloned into EcoR I and BamH I sites of pTetOne Vector (Clontech). The target cell lines (AGS, SW480, HCT116) were seeded in a single well of a 6-well plate at a density sufficient to reach near confluence at 48 hr after transfection. Cells were co-transfected with 2 µg pTetOne-AURKA and 100 ng hygromycin Selection Marker. Transfection of cells was achieved by using PolyJet transfection reagent (SignaGen Laboratories) according to the manufacturer's instructions. For selection of positive cells, hygromycin was added at the concentration 50-200 ng, optimized for each cell line for two weeks. Cells were maintained using 10% doxycycline free FBS (Invitrogen). For induction of AURKA, doxycycline was added for 72h (100 ng/ml).

Lentiviral infection

KRAS-G12D Lentiviral Plasmid (Human) (CMV) was purchased from Applied Biological Materials, KRAS knockdown constructs (pLV[shRNA]-Puro-U6>hKRAS) (shRNA-KRAS #5: VB160511-1123dwt and shRNA-KRAS #2: VB160511-1124fzb) were purchased from VectorBuilder (Santa Clara, CA). Lentivirus particles were produced by co-transfection of the lentiviral vectors with the 2nd Generation Packaging System Mix vector (Applied Biological Materials) into 293FT cells. Cells were selected with 1 µg/ml puromycin at least 48 hrs prior to experiments. Supernatants containing viral particles were collected after transfection for 2 days.

AURKA and RPS6KB1 silencing by small interfering RNA (siRNA)

Cells were seeded at 60% confluency in 10% FBS DMEM for 24 hrs in p60 plates. AURKA or RPS6KB1 were transiently silenced by using validated siAURKA (Invitrogen), and siRPS6KB1 (Santa Cruz) for 48 or 72 hrs. A negative siRNA control (Ambion, Austin, TX) was used in each experiment. Transfection of cells was achieved by using LipoJet reagent (SignaGen) according to the

manufacturer's instructions. Following 24 hrs transfection, medium was replaced with DMEM or RPMI 1640 media, supplemented with 10% FBS and antibiotics. Validation of AURKA and RPS6KB1 knockdown was assessed by Western blot analyses.

Immunofluorescence

Double immunofluorescence was used to co-localize AURKA and RPS6KB1. Cells were washed with PBS and fixed with a fresh 4% paraformaldehyde solution for 20 min in a humidified chamber; and then blocked in PBS with 0.5% Triton-X for 15 min and followed by incubation in 10% normal goat serum blocking solution (Thermo Fisher Scientific, Waltham, MA) for 20 min at room temperature. Cells were then incubated in the specific primary antibody against-Rabbit AURKA (Cell Signaling) diluted in blocking solution (1:100) and against-Mouse RPS6KB1 (Cell Signaling) in blocking solution (1:100) overnight at 4℃. Cells were washed 3 times in PBS and incubated in Alexa Fluor 568-conjugated goat anti-mouse (1:500) (Life Technologies) and Alexa Fluor 488-conjugated goat anti-Rabbit (1:500) (Life technologies) secondary antibody diluted in PBS for 45 min at room temperature. The cells were then washed in PBS, mounted with Vectashield/40'6-diamidino- 2-phenylindole (Vector Laboratories, Burlingame, CA), and visualized with a Zeiss LSM880 with AiryScan Confocal microscope (Carl Zeiss Microscopy, Thornwood, NY).

Statistical analysis

Data are presented as means +/- standard error of mean (SEM). Statistical significance of difference between control groups and treatment groups was determined using One-way ANOVA Test. Statistical analyses were carried out using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA), nonlinear regression. The correlation between two parameters was determined by two-tailed Student's test. The differences were considered statistically significant when the $P \leq 0.05$.

Supplementary Table S1. Histopathology, AURKA expression, and molecular correlatives of colon cancer tissue microarrays

ID	AURKA Cytolsoic CES Score	AURKA Nuclear CES Score	Clinical Stage	MSI Status	KRAS	BRAF	PIK3CA
CRC1	8	12	2A	Unknown	NA	NA	NA
CRC2	8	12	2A	MSS	wt	wt	wt
CRC3	7	12	3B	Unknown	NA	NA	NA
CRC4	8	11	3B	MSS	wt	wt	wt
CRC5	12	6	2A	MSS	NA	NA	NA
CRC6	12	6	2A	MSS	NA	NA	NA
CRC7	7	11	3C	MSS	p.Q61H; c.183A>	wt	wt
CRC8	7	11	3C	Unknown	NA	NA	NA
CRC9	7	11	2B	Unknown	wt	wt	wt
CRC10	10	8	2A	MSI-H	NA	NA	NA
CRC11	8	10	2A	Unknown	NA	NA	NA
CRC12	8	10	3C	MSS	p.G12A;c.35G>C	not tested	not tested
CRC13	8	10	3B	MSS	p.G12A; c.35G>C	wt	wt
CRC14	12	5	2A	MSS	NA	NA	NA
CRC15	6	11	3B	MSS	wt	wt	wt
CRC16	6	11	2B	MSS	p.G13D; c.38G>A	wt	wt
CRC17	7	10	3B	MSS	wt	p.G596R; c.1786G>C	wt
CRC18	7	10	2A	MSI-H	wt	p.V600E; c.1799T>A	wt
CRC19	7	7	3B	MSS	wt	wt	wt
CRC20	8	6	2B	MSI-H	NA	NA	NA
CRC21	10	6	2A	Unknown	p.G12D; c.35G>A	wt	wt
CRC22	6	10	2B	MSS	p.Q61L; c.182A>T	wt	wt
CRC23	8	5	2A	Unknown	NA	NA	NA
CRC24	7	6	2A	Unknown	NA	NA	NA
CRC25	7	6	2B	Unknown	NA	NA	NA
CRC26	12	0	NA	NA	NA	NA	NA
CRC27	12	0	2A	Unknown	NA	NA	NA
CRC28	12	0	2A	Unknown	wt	wt	wt

CRC29	12	0	3B	MSS	wt	wt	wt
CRC30	0	12	2A	MSI-H	p.G13D; c.38G>A	wt	wt
CRC31	5	10	2A	Unknown	NA	NA	NA
CRC32	12	0	3B	MSS	p.G12V; c.35G>T	wt	wt
CRC33	12	0	2A	MSI-H	wt	wt	wt
CRC34	12	0	2A	MSI-H	wt	p.V600E; c.1799T>A	wt
CRC35	12	0	2B	MSI-H	wt	wt	wt
CRC36	0	12	2B	MSS	wt	wt	wt
CRC37	0	12	2A	Unknown	NA	NA	NA
CRC38	12	0	2A	MSI-H	p.G13D; c.38G>A	wt	p.E542K; c.1624G>A
CRC39	12	0	2B	Unknown	wt	p.V600E; c.1799T>A	wt
CRC40	0	12	3B	MSI-H	p.G12A; c.35G>C	p.D594G; c.1781A>G	wt
CRC41	12	0	3B	MSS	wt	wt	wt
CRC42	12	0	3C	Unknown	NA	NA	NA
CRC43	12	0	2A	Unknown	p.G13D; c.38G>A	wt	wt
CRC44	6	6	2B	MSS	NA	NA	NA
CRC45	6	6	2A	MSS	p.G13D; c.38G>A	wt	wt
CRC46	6	6	2A	Unknown	p.G12D; c.35G>A	wt	p. H1047R; c.3140A>C
CRC47	6	6	3B	Unknown	NA	NA	NA
CRC48	6	6	3A	Unknown	wt	wt	wt
CRC49	11	0	2B	Unknown	wt	wt	wt
CRC50	11	0	2B	Unknown	wt	wt	wt
CRC51	11	0	2A	Unknown	wt	wt	wt
CRC52	0	11	3B	MSS	NA	NA	NA
CRC53	0	11	2A	MSS	p.G12D; c.35G>A	wt	wt
CRC54	0	11	3B	MSS	p.G12V; c.35G>T	wt	p.H1047R; c.3140A>G
CRC55	0	11	2A	MSS	p.G13D; c.38G>A	wt	wt
CRC56	11	0	3C	Unknown	NA	NA	NA
CRC57	0	11	2A	Unknown	NA	NA	NA
CRC58	11	0	3B	MSS	wt	wt	wt
CRC59	0	11	3B	MSS	wt	wt	wt
CRC60	0	11	3B	Unknown	wt	wt	wt
CRC61	11	0	3C	MSS	NA	NA	NA
CRC62	3	6	2A	MSS	p.G12V; c.35G>T	wt	p.E545K; c.1633G>A

CRC63	6	5	2B	MSS	p.G12C; c.34G>T	wt	wt
CRC64	6	5	3B	MSI-H	wt	p.V600E; c.1799G>A	not tested
CRC65	8	0	2A	MSS	p.G12V; c.35G>T	wt	wt
CRC66	0	8	2B	MSI-H	NA	NA	NA
CRC67	8	0	3B	Unknown	NA	NA	NA
CRC68	8	0	3C	MSS	wt	wt	wt
CRC69	8	0	2A	MSS	wt	wt	wt
CRC70	8	0	3B	MSS	NA	NA	NA
CRC71	8	0	2A	MSS	wt	wt	wt
CRC72	8	0	3B	MSI-H	NA	NA	NA
CRC73	8	0	3B	MSI-low	NA	NA	NA
CRC74	8	0	2B	Unknown	NA	NA	NA
CRC75	8	0	2B	MSI-H	p. G12V; c.35G>T	wt	p.E545K; c.1633G>A
CRC76	8	0	3B	MSS	p.Q61L; c.182A>T	wt	wt
CRC77	8	0	2A	Unknown	NA	NA	NA
CRC78	10	0	3B	MSI-H	wt	p.V600E; c.1799T>A	wt
CRC79	10	0	3B	MSS	wt	wt	wt
CRC80	0	10	2C	MSS	NA	NA	NA
CRC81	0	10	2A	Unknown	NA	NA	NA
CRC82	0	10	2A	Unknown	p.G13D; c.38G>A	wt	wt
CRC83	0	10	2B	Unknown	NA	NA	NA
CRC84	0	10	2A	Unknown	NA	NA	NA
CRC85	0	10	2A	MSS	p.G13D; c.38G>A	wt	wt
CRC86	10	0	3C	unknown	wt	wt	wt
CRC87	10	0	2A	MSS	p.G12D;c.35G>A	wt	wt
CRC88	2	6	2A	Unknown	NA	NA	NA
CRC89	0	10	2B	Unknown	NA	NA	NA
CRC90	0	7	2A	Unknown	NA	NA	NA
CRC91	7	0	3B	MSS	wt	wt	wt
CRC92	7	0	3A	MSS	wt	wt	wt
CRC93	0	7	3C	Unknown	NA	NA	NA
CRC94	0	7	3B	MSS	NA	NA	NA
CRC95	7	0	2A	MSS	NA	NA	NA
CRC96	7	0	2A	MSI-H	wt	p.V600E; c.1799T>A	wt

CRC97	0	7	2A	Unknown	NA	NA	NA
CRC98	7	0	3B	MSS	p.G12V;c.35G>T	wt	wt
CRC99	0	7	2A	MSS	NA	NA	NA
CRC100	7	0	2A	Unknown	NA	NA	NA
CRC101	0	7	3C	MSS	NA	NA	NA
CRC102	7	0	2A	MSS	wt	wt	wt
CRC103	7	0	3A	MSS	p.Q61R; c.182A>	wt	wt
CRC104	10	0	2A	MSS	p.G13D; c.38G>A	wt	wt
CRC105	0	6	3C	Unknown	NA	NA	NA
CRC106	6	0	2A	Unknown	p.G12V; c.35G>T	wt	wt
CRC107	0	6	3B	Unknown	NA	NA	NA
CRC108	0	6	3B	Unknown	wt	wt	wt
CRC109	6	0	2B	MSS	p.G12S; c.34G>A	wt	wt
CRC110	6	0	3B	Unknown	NA	NA	NA
CRC111	6	0	2A	Unknown	NA	NA	NA
CRC112	0	6	3B	MSS	p.G12V; c.35G>T	wt	wt
CRC113	6	0	3B	MSS	wt	wt	p.E542K; c.1624G>A
CRC114	0	6	3B	MSI-H	wt	wt	wt
CRC115	6	0	2A	Unknown	NA	NA	NA
CRC116	6	0	2A	Unknown	NA	NA	NA
CRC117	6	0	2A	MSS	wt	wt	wt
CRC118	6	0	2A	MSS	NA	NA	NA
CRC119	0	6	2A	MSI-H	wt	wt	wt
CRC120	6	0	2A	MSS	p.G13D; c.38G>A	wt	wt
CRC121	6	0	2A	MSS	wt	wt	wt
CRC122	0	6	2A	MSS	wt	wt	wt
CRC123	6	0	3A	MSS	NA	NA	NA
CRC124	0	6	2B	MSI-H	wt	wt	wt
CRC125	0	6	2A	Unknown	NA	NA	NA
CRC126	0	6	3C	Unknown	NA	NA	NA
CRC127	6	0	3B	Unknown	NA	NA	NA
CRC128	4	0	2A	Unknown	NA	NA	NA
CRC129	0	5	3B	MSS	wt	wt	wt
CRC130	5	0	3C	MSS	p.G12A; c.35G>C	wt	wt

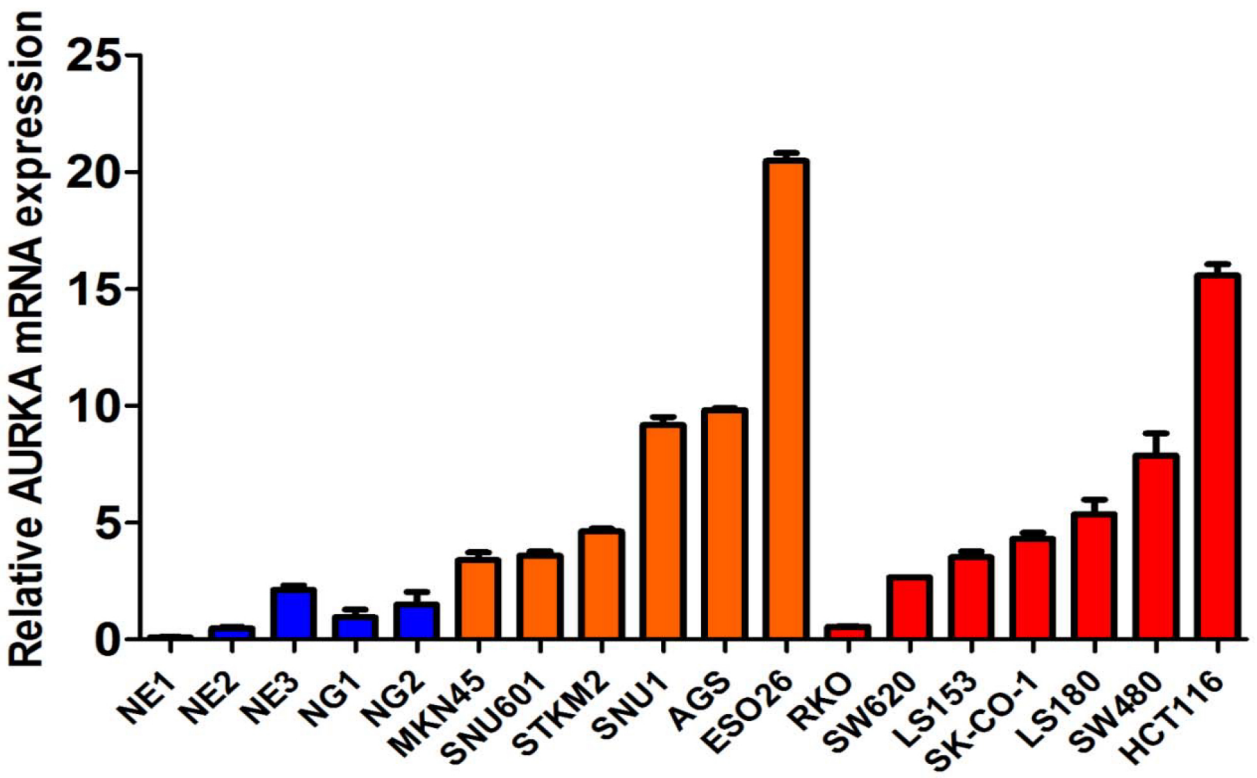
CRC131	5	0	3B	MSS	wt	wt	wt
CRC132	3	0	3B	MSS	NA	NA	NA
CRC133	5	0	2B	MSS	p.G12D; c.35G>A	wt	wt
CRC134	0	5	3B	MSS	NA	NA	NA
CRC135	0	5	2A	Unknown	NA	NA	NA
CRC136	0	2	3B	Unknown	NA	NA	NA
CRC137	0	2	2B	MSI-H	NA	NA	NA
CRC138	1	0	2A	Unknown	wt	wt	wt
CRC139	1	0	3B	Unknown	NA	NA	NA
CRC140	0	1	3B	MSS	p.G12V;c.35G>T	wt	not tested
CRC141	0	0	3C	Unknown	NA	NA	NA
CRC142	0	0	2A	MSS	wt	wt	wt
CRC143	0	0	3C	Unknown	NA	NA	NA
CRC144	0	0	2B	Unknown	wt	p.V600E; c.1799T>A	wt
CRC145	0	0	3B	Unknown	NA	NA	NA
CRC146	0	0	3B	MSS	p.G12V;c.35G>T	wt	wt
CRC147	0	0	3B	MSS	wt	wt	wt
CRC148	0	0	3C	MSS	wt	wt	wt
CRC149	0	0	3B	Unknown	p.G12D; c.35G>A	wt	wt
CRC150	0	0	3B	Unknown	wt	wt	wt
CRC151	0	0	3A	MSS	NA	NA	NA

Supplementary Table S2. Summary of molecular alterations in cell lines.

Cancers	Genes / Cell lines	KRAS	TP53	CTNNB1	APC	TCF7L2	BRAF	PIK3CA	*AURKA fold expression
Upper GI cancer	ESO26	Amplified	MUT	WT	WT	WT	WT	MUT	20.5
	AGS	G12D	WT	MUT	MUT	WT	WT	MUT	9.8
	SNU-601	G12D	MUT	MUT	WT	WT	WT	WT	3.6
	SNU-1	G12D	WT	WT	WT	WT	WT	WT	9.2
	STKM2	WT	WT	WT	WT	WT	WT	WT	4.6
	MKN45	WT	WT	WT	WT	WT	WT	WT	3.4
Colorectal cancer	HCT116	G13D	WT	MUT	WT	WT	WT	MUT	15.6
	SW480	G12V	MUT	WT	MUT	WT	WT	WT	7.9
	SW620	G12V	MUT	WT	MUT	WT	WT	WT	2.7
	LS180	G12D	WT	MUT	MUT	WT	MUT	MUT	5.4
	LS153	G12D	WT	MUT	WT	WT	MUT	WT	3.5
	SK-CO-1	G12V	WT	WT	MUT	WT	WT	WT	4.3
	RKO	WT	MUT	WT	WT	WT	MUT	MUT	0.5

This information was obtained from Cosmic onlline (http://cancer.sanger.ac.uk/cosmic/)

*Fold expression was obtained by using qRT-PCR, normalized to 5 normal gastric & esophageal tissue samples (NG and NE). Data is also summarized in the graph below.



Supplementary Table S3. Histopathology and AURKA expression levels in normal tissues and colon adenomas in tissue microarrays.

ID	Histology	AURKA Cytosolic CES Score	AURKA Nuclear CES Score
N1	NORMAL	0	0
N2	NORMAL	2	0
N3	NORMAL	0	12
N4	NORMAL	0	12
N5	NORMAL	0	12
N6	NORMAL	0	12
N7	NORMAL	0	12
N8	NORMAL	0	12
N9	NORMAL	0	12
N10	NORMAL	0	12
N11	NORMAL	0	2
N12	NORMAL	0	0
A1	ADENOMA	12	0
A2	ADENOMA	8	0
A3	ADENOMA	8	0
A4	ADENOMA/NORMAL	1	0
A5	ADENOMA	6	0
A6	ADENOMA	11	0
A7	ADENOMA/NORMAL	0	0
A8	ADENOMA	0	7
A9	ADENOMA	2	0
A10	ADENOMA	6	0
A11	ADENOMA	12	0
A12	ADENOMA	3	0
A13	ADENOMA	0	1
A14	ADENOMA	0	6
A15	ADENOMA	10	0
A16	ADENOMA	8	10

Supplementary Figure Legends:

Supplementary Figure S1. Quantification data of Western blot data in Figure 2, 3 and 5. A, Quantification data of Western blot data in Figure 2. B, Western blot quantification data of Figure 3A. C, Western blot quantification data of Figure 3B. D, Western blot quantification data of Figure 3C. E, Western blot quantification data of Figure 5A. F, Western blot quantification data of Figure 5B. G, Western blot quantification data of Figure 5C. H, Western blot quantification data of Figure 5D. I, Western blot quantification data of Figure 5E.

Supplementary Figure S2. Alisertib treatment inhibits cell survival and enhances polyploidy.

AGS, SNU-601, and SW480 cells were treated with alisertib (0.2 μM) for 24, 48 and 72 hrs, and cell cycle progression was analyzed with flow cytometry. The data indicated that alisertib treatment enhances polyploidy and alters cell cycle progression.

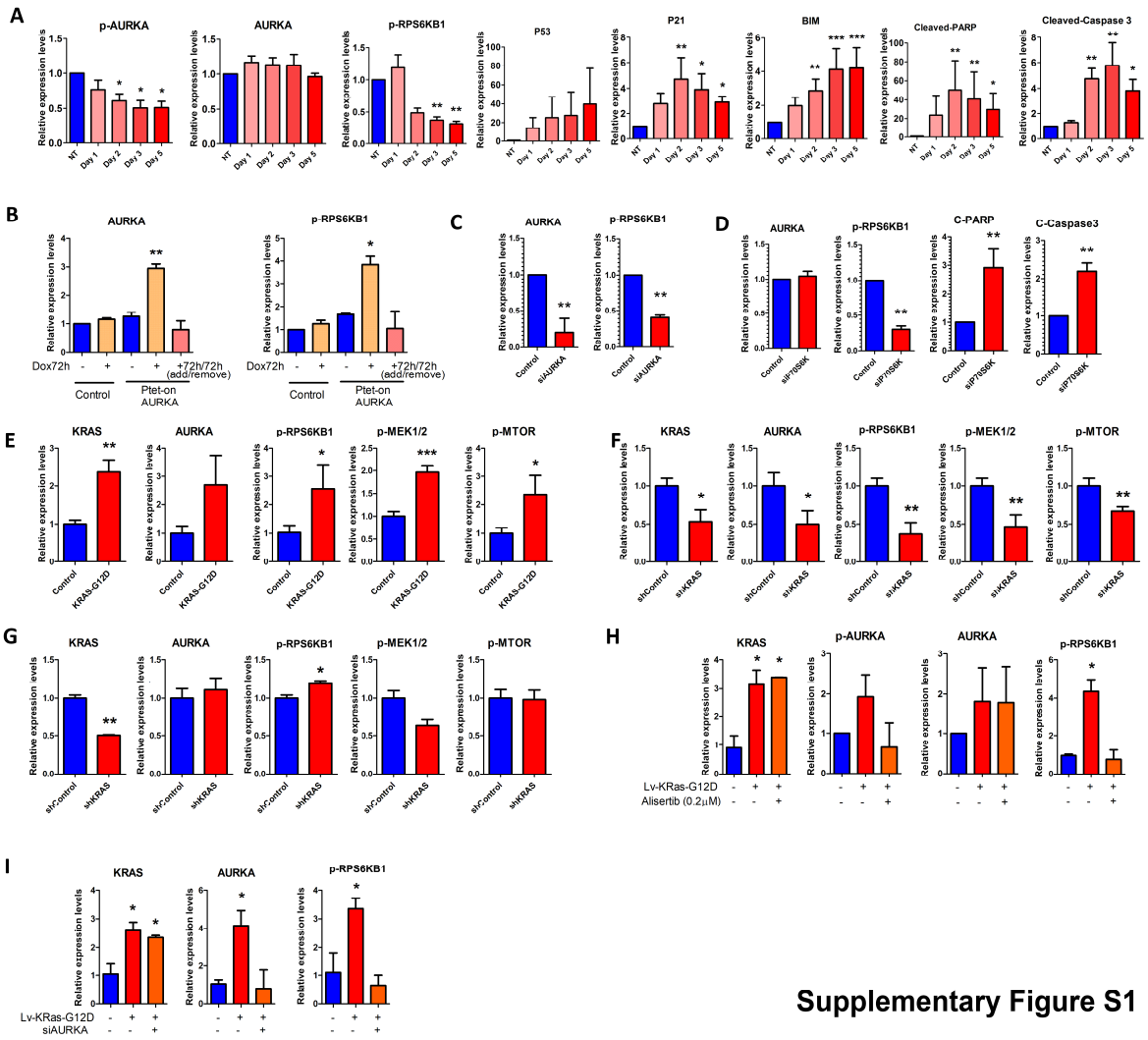
Supplementary Figure S3. The effect of alisertib treatment on KRAS downstream molecules

A, Upper GI cancer (UGC) cells (ESO26, AGS, SNU-1, SNU-601) and colorectal cancer (CRC) cells (HCT116, SW480, SW620) were treated with alisertib (0.2 μM) for 1, 2, 3, and 5 days, and cell lysates were subjected to Western blot analysis of the indicated proteins. The data showed that compared with control, there was no significant change on Phospho-MEK1/2 (Ser217/221) and phosphor-MTOR (Ser2448) with alisertib treatment in tested cell lines; although there was a decreased expression of phosphor-AKT (Ser473) in tested cell lines, the time point of p-AKT downregulation is different and not consistent in different cell lines.

Supplementary Figure S4. AURKA knockdown induced apoptosis and suppressed cell growth in GI cancer cells. UGC (ESO26, AGS, SNU-1, SNU-601) and CRC cells (HCT116, SW480, SW620) were transfected with siControl or siAURKA for 48 or 72 hrs and subjected to Western blot analysis (A)

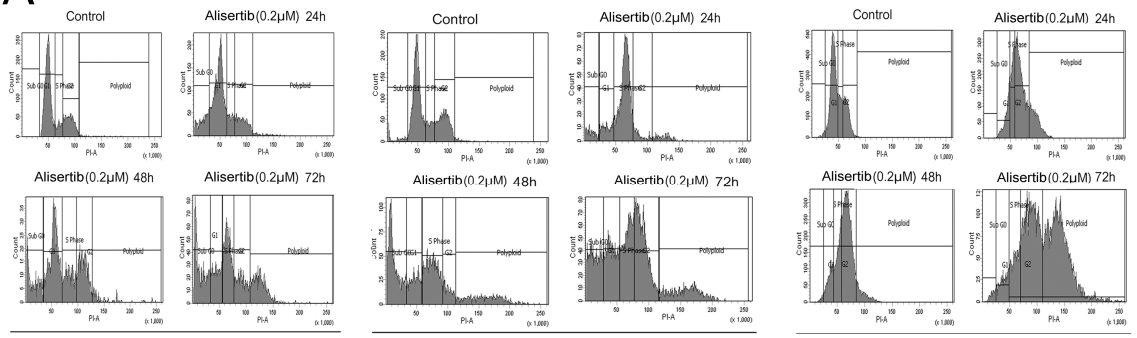
and cell growth CellTiter-Glo analysis (B). The data indicated that knockdown of AURKA upregulated protein expression of p53 (only in p53 WT cell lines), p21, BIM, Cleaved PARP, Cleaved caspase 3, and suppressed cell growth. C, Upper GI cancer cells (AGS, SNU-601, SNU-1) and CRC cells (HCT116, SW480, SW620) were transfected with siControl or siRPS6KB1 for 48 hrs, cells were subjected to cell growth CellTiter-Glo analysis, which were measured every two days and normalized to the corresponding values of day 1. Accordingly, loss of RPS6KB1 suppressed cell growth.

Supplementary Figure S5. AURKA knockdown decreased phosphor-RPS6KB1 expression in mutant KRAS cells. Western blot data analysis of AURKA, phospho-RPS6KB1, RPS6KB1, and Actin expression levels in SNU1 (mutant KRAS, G12D), MKN45 (wild type KRAS), and STKM2 (wild type KRAS) cells.



Supplementary Figure S1

A



AGS

	Control	Alisertib(0.2μM) 24h	48h	72h
Sub-G0	0.1	14.1	16.0	24.7
G1	62.8.	55.0	39.4	15.5
S phashe	8.9	9.3	16.9	25.4
G2-M	24.2	16.3	23.1	14.5
Ploypoid	0.4	3.4	7.8	19.1

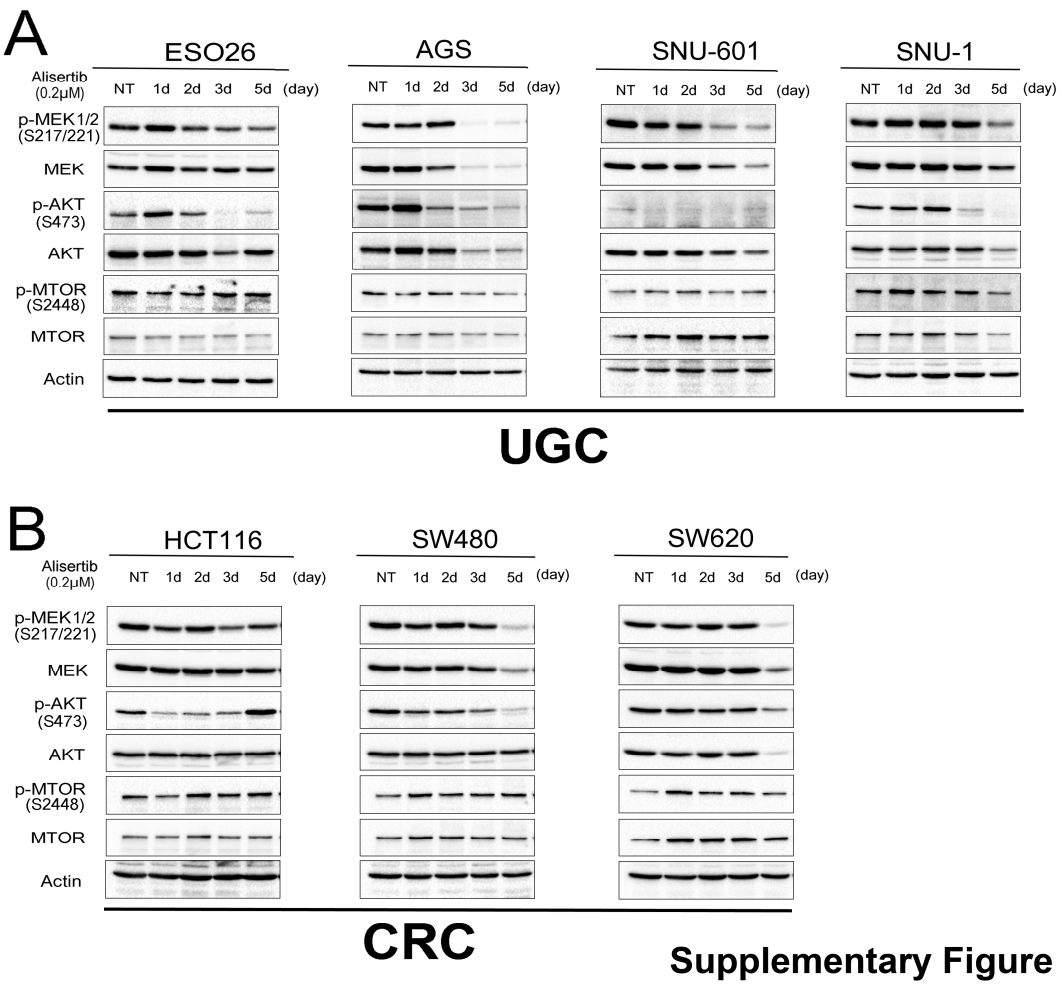
SNU-601

	Control	Alisertib(0.2μM) 24h	48h	72h
Sub-G0	4.2	8.0	26.0	29.0
G1	54.6	18.1	14.8	13.4
S phashe	11.1	9.9	29.4	22.8
G2-M	27.5	47.4	7.3	28.0
Ploypoid	0.6	2.5	6.5	11.7

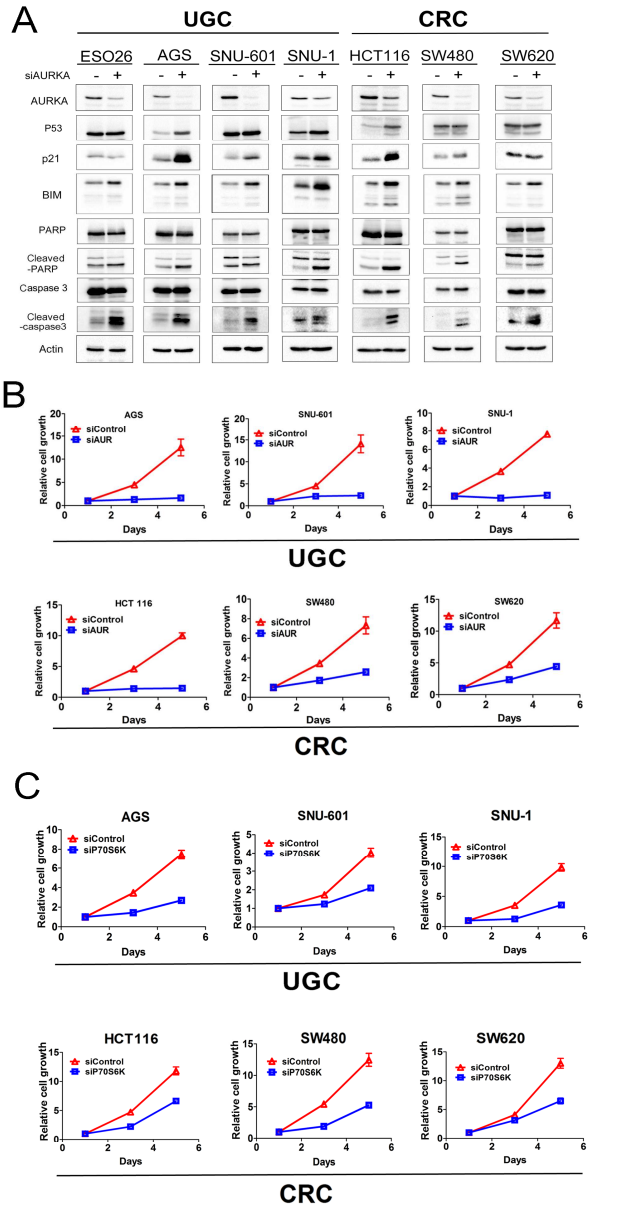
SW480

	Control	Alisertib(0.2μM) 24h	48h	72h
Sub-G0	2.4	0.3	1.5	1.7
G1	58.4	14.8	12.9	5.7
S phashe	16.0	20.6	22.4	10.3
G2-M	23.3	47.1	55.6	35.8
Polyploid	0.6	16.9	11.7	46.4

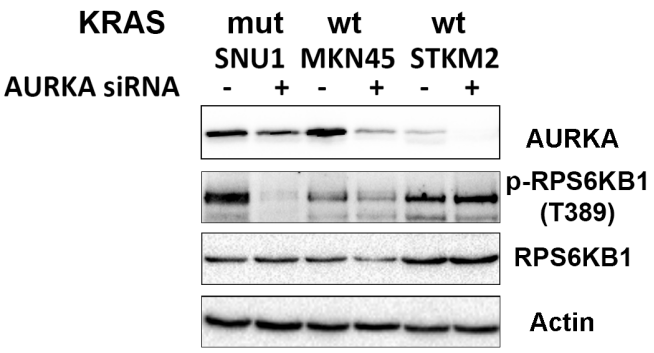
Supplementary Figure S2



Supplementary Figure S3



Supplementary Figure S4



Supplementary Figure S5