

**Supporting information for:**

**Identification of Eph receptor signaling as a regulator of autophagy and a therapeutic target in colorectal carcinoma**

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**Table S1. Primers used for quantitative PCR**

Target	Forward	Reverse
<i>GAPDH</i>	CATgAgAAgTATgACAACAgCCT	AgTCCTTCCACgATACCAAAgT
<i>EFNB1</i>	AAgAACCTggAgCCCgTATC	gCAgATgATgTCCAgCTTgT
<i>EFNB2</i>	TggggTgTTTgATggTTT	CCAgTCCTTgTCCAggTAgAA
<i>EFNB3</i>	CAgCCTggAgCCTgTCTACT	CgATCTgAgggTACAgCACA
<i>EphB1</i>	gCTACCTCCAggATgACACC	gACgATCCCATAgCTCCAAA
<i>EphB2</i>	TgTgTAACAgACggggTTT	CCTCgTAggTgAAAAGgATCg
<i>EphB3</i>	TgCCACTCAAgnCTCTACTgC	CTgCTTgCCTTgTAgCTC
<i>EphB4</i>	CTTTggAAgAgACCCTgCTg	TgCACgTCACACACTTCgTA
<i>EphB6</i>	ggCTgTTAATggggTgTCTg	gACAgCAgAgggCACTTCAT
<i>EphA4</i>	TCATTggAgATggggCTAAC	CCgCTTCTTgTTTggCTTTA

**Table S2. Lentiviral Constructs**

Target	Name	TRC <sup>A</sup> number Cat. no.	Target region <sup>B</sup>	Target sequence
<i>EphB4</i>	#774	TRCN0000001774	CDS	TGATCTGAAGTGGGTGACATT
<i>EphB4</i>	#651	TRCN0000010651	CDS	CTGGAGTTACGGGATTGTGAT
<i>EphB4</i>	#826	TRCN0000314826	CDS	ACAGCATGCCCGTCATGATT
<i>EphB4</i>	#407	TRCN0000195407	CDS	CGTCATGATTCTCACAGAGTT
<i>EphB4</i>	#827	TRCN0000314827	3UTR	CACCACCAAACTCAATCATT
<i>EphB2</i>	#424	TRCN0000006424	CDS	GCTGTGATTCCAGTGTCAAT
<i>EphB2</i>	#819	TRCN0000199819	CDS	GCGTGTCTTCTACCGCAAGTG
<i>EphB2</i>	#426	TRCN0000006426	CDS	CGGGAGTTGCCAAGGAAATT
<i>EphB2</i>	#423	TRCN0000006423	CDS	GCTAGACAAGATGATCCGCAA
<i>EphB2</i>	#573	TRCN0000196573	CDS	GAAGATCTACATCGATCCTT
<i>EphrinB2</i>	#427	TRCN0000058427	CDS	CTGGTACTATAACCCACAGATA
<i>EphrinB2</i>	#423	TRCN0000058423	CDS	CCAGGAATAAAGATCCAACAA
<i>EphrinB2</i>	#426	TRCN0000058426	CDS	CCTTATTGCAGGGATTGCTT
<i>EphrinB2</i>	#596	TRCN0000285596	3UTR	CTTCCCAGAGGACACCTAAT
<i>EphrinB2</i>	#588	TRCN0000276588	CDS	TCTACATCAAATGGGTCTTG
plKO	Control	SHC001		no insert
non- targeting	Non- targeting	SHC002		CAACAAGATGAAGAGCACCAA
pk-hLC3B	pk-LC3	61460 (Addgene)		

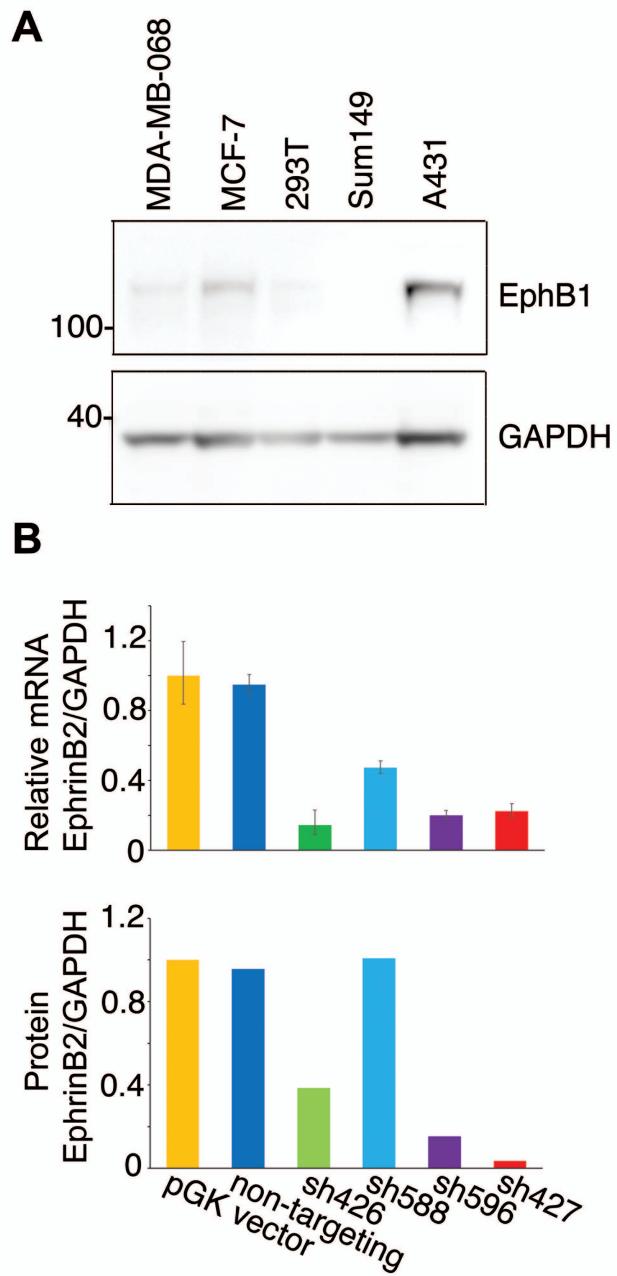
All constructs purchased from Sigma, unless otherwise noted

A, The RNAi Consortium; B, Coding region or 3'UnTranslated Region

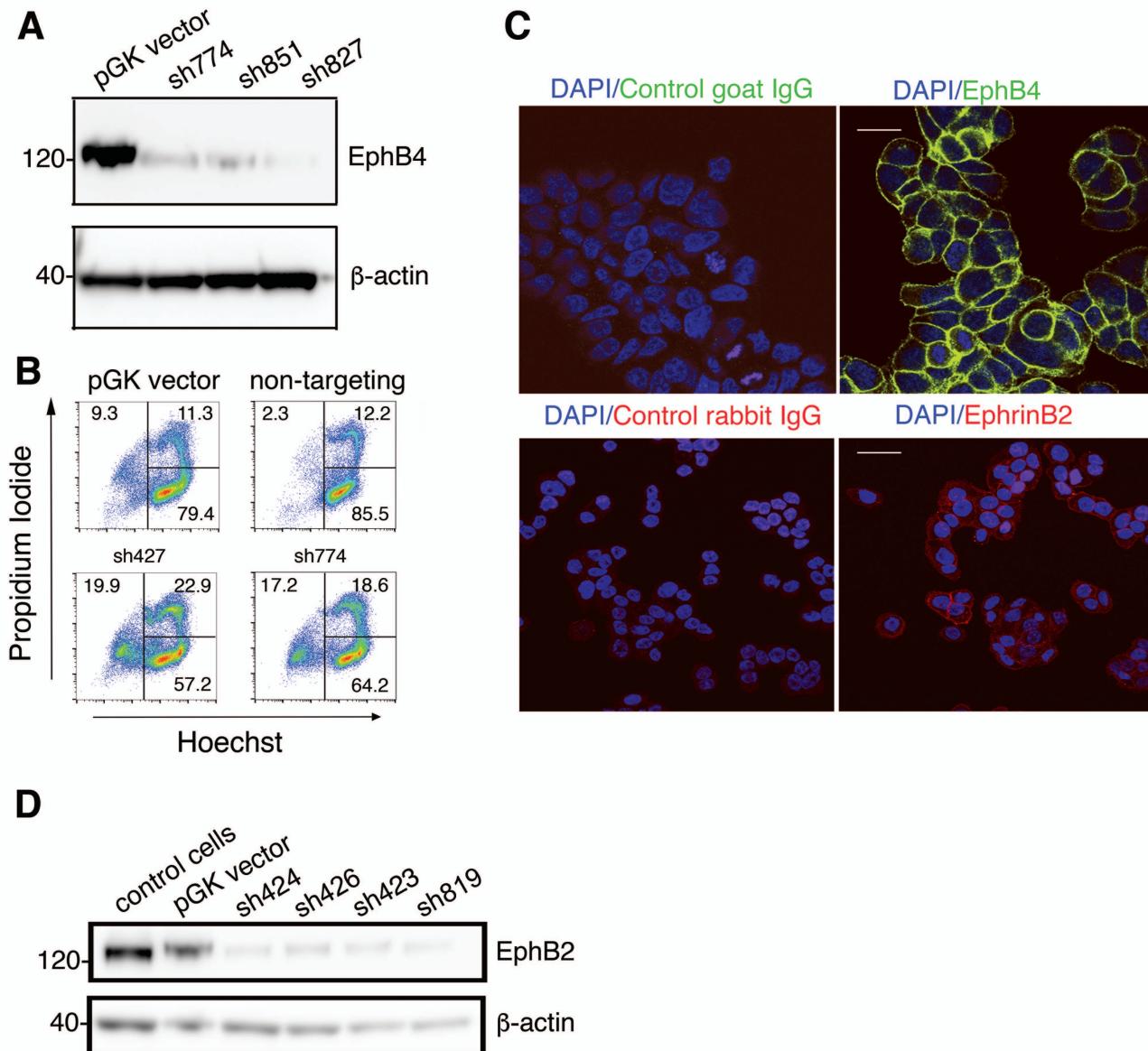
**Table S3. List of antibodies used**

<b>Target</b>	<b>Host</b>	<b>Clonality<sup>A</sup></b>	<b>Source</b>	<b>Catalog number</b>	<b>Western dilution</b>	<b>IF<sup>B</sup> dilution</b>	<b>IP<sup>C</sup> per 250µg lysate</b>
EphrinB2	rabbit	mono	Abcam	ab150411	1:1000	1:100	
EphrinB	rabbit	poly	Santa Cruz	sc-910			1µg
pEphrinB	rabbit	poly	Cell Signaling	3481		1:100	
EphA4	rabbit	poly	Abcam	ab5396	1:1000		
EphB1	rabbit	poly	Stratagene		1:500		
EphB2	goat	poly	R&D Systems	AF467	1µg/mL		
pEphB1+B2 (pY594+pY604)	rabbit	poly	Abcam	ab61791	1:500		
EphB4	goat	poly	R&D Systems	AF3038	1µg/mL	5µg/mL	1µg
GAPDH	mouse	mono	Santa Cruz	sc-47724	1:1000		
B-actin	mouse	mono	Santa Cruz	sc-47778	1:1000		
pTyr (4G10)	mouse	mono	Millipore-Sigma	05-1050	1:1000		
CD31	rat	mono	HistoBiotec	SZ31		1:20	
KI67	rabbit	mono	Cell Signaling	9129		1:400	
cleaved Caspase-3	rabbit	mono	Cell Signaling	9579		1:200	
LC3 A/B	rabbit	mono	Cell Signaling	12741	1:1000		
ATG5	rabbit	mono	Cell Signaling	12994	1:1000		
p-mTor (S2448)	rabbit	poly	Cell Signaling	2971	1:1000		
mTor	mouse	mono	Cell Signaling	4517	1:1000		

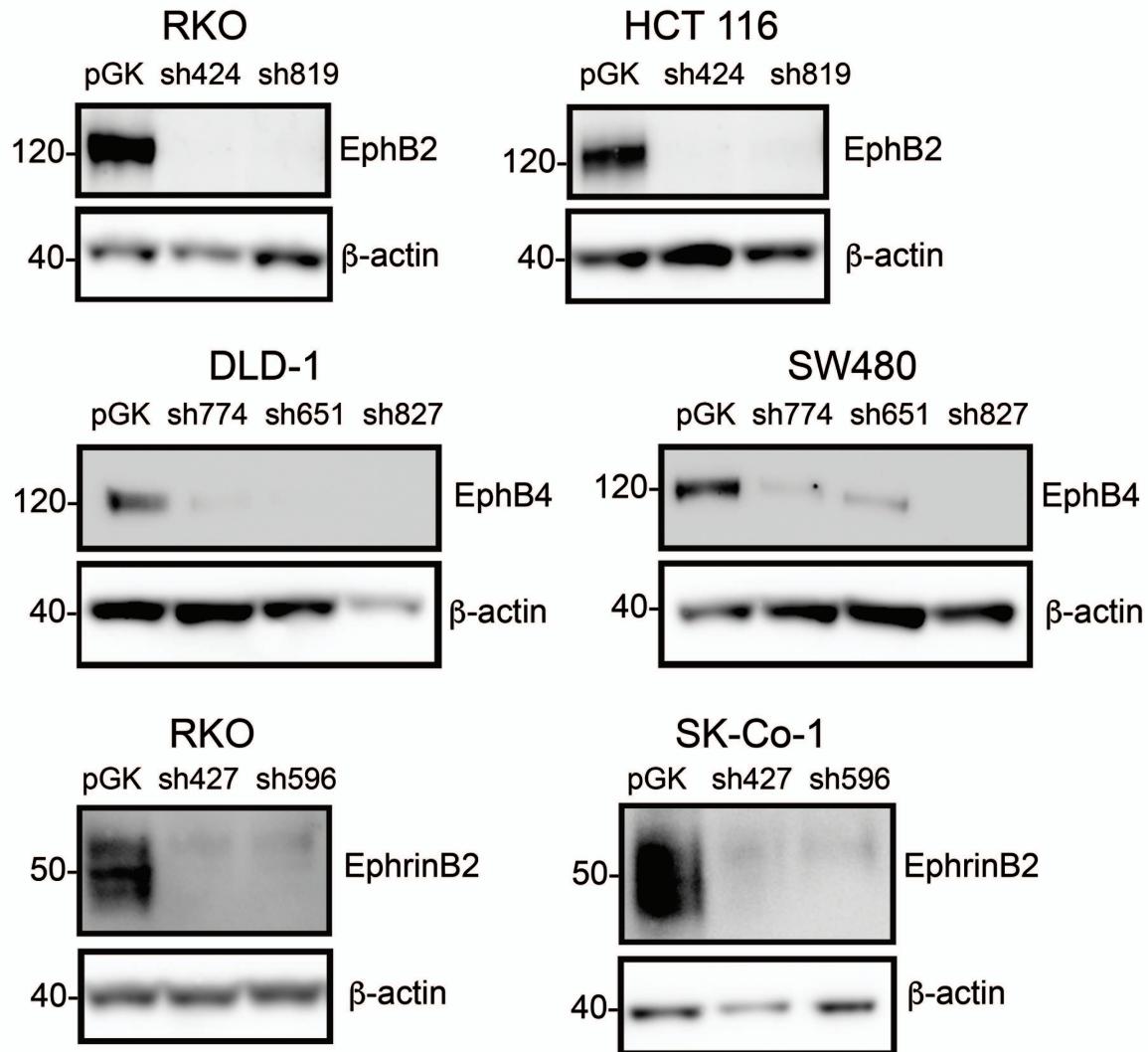
A, mono, monoclonal; poly, polyclonal; B, immunofluorescence; C IP, immunoprecipitation



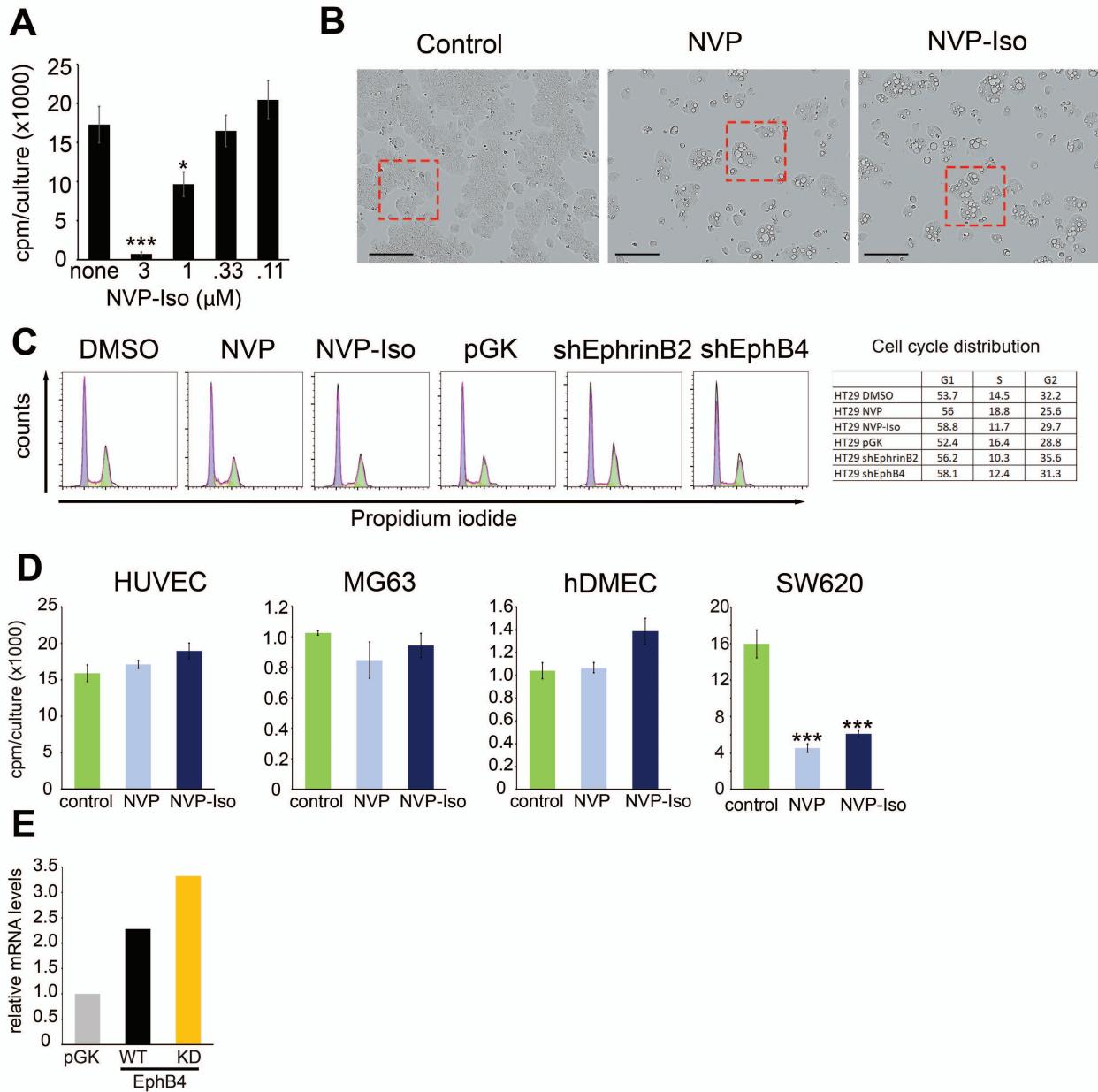
**Fig. S1.** EphB1 protein detection and EphrinB2 silencing by shRNA. (A) Specific EphB1 detection by immunoblotting in the indicated cell lines; GAPDH is used as a control. (B) Effects of EphrinB2 shRNAs and controls (empty vector and non-targeting shRNA) on relative levels of EphrinB2 mRNA expression by qPCR (top bar graph) and relative EphrinB2 protein levels detected by immunoblotting and band quantitation in HT-29 cells (bottom bar graph). Results at 72 hours after lentiviral infection.



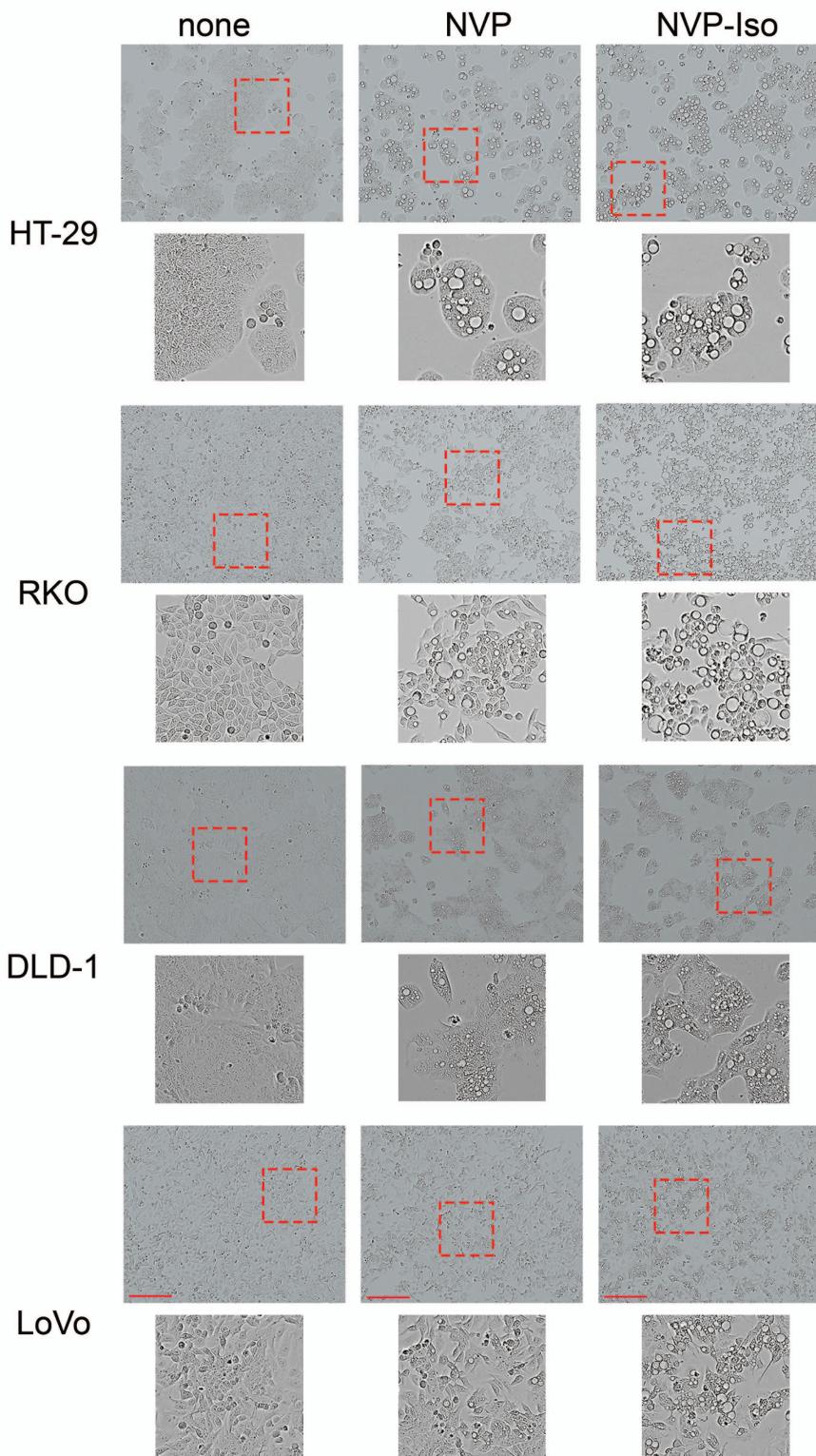
**Fig. S2.** EphB4 and EphB2 detection and silencing in colorectal carcinoma cells. (A) EphB4 protein levels are specifically reduced in HT-29 cells by sh774, sh851 and sh827; immunoblotting results; β-actin reblotting used as a control. (B) Reduced HT-29 cell viability 72 hours after EphB4 silencing with sh774 and EphrinB2 silencing with sh427. Representative flow cytometry profiles showing reduced HT-29 cell viability (percent live cells in the lower right quadrant) after EphB4 silencing. (C) Specific immunohistochemical detection of EphB4 (green) and EphrinB2 (red) in HT-29 cells. Nuclei are stained with DAPI (blue). Representative confocal images; scale bar 20μm (top), 33μm (bottom). (D) EphB2 protein levels are specifically reduced in SW620 cells 72 hours after infection with sh424, sh426, sh423 and sh819; immunoblotting results.



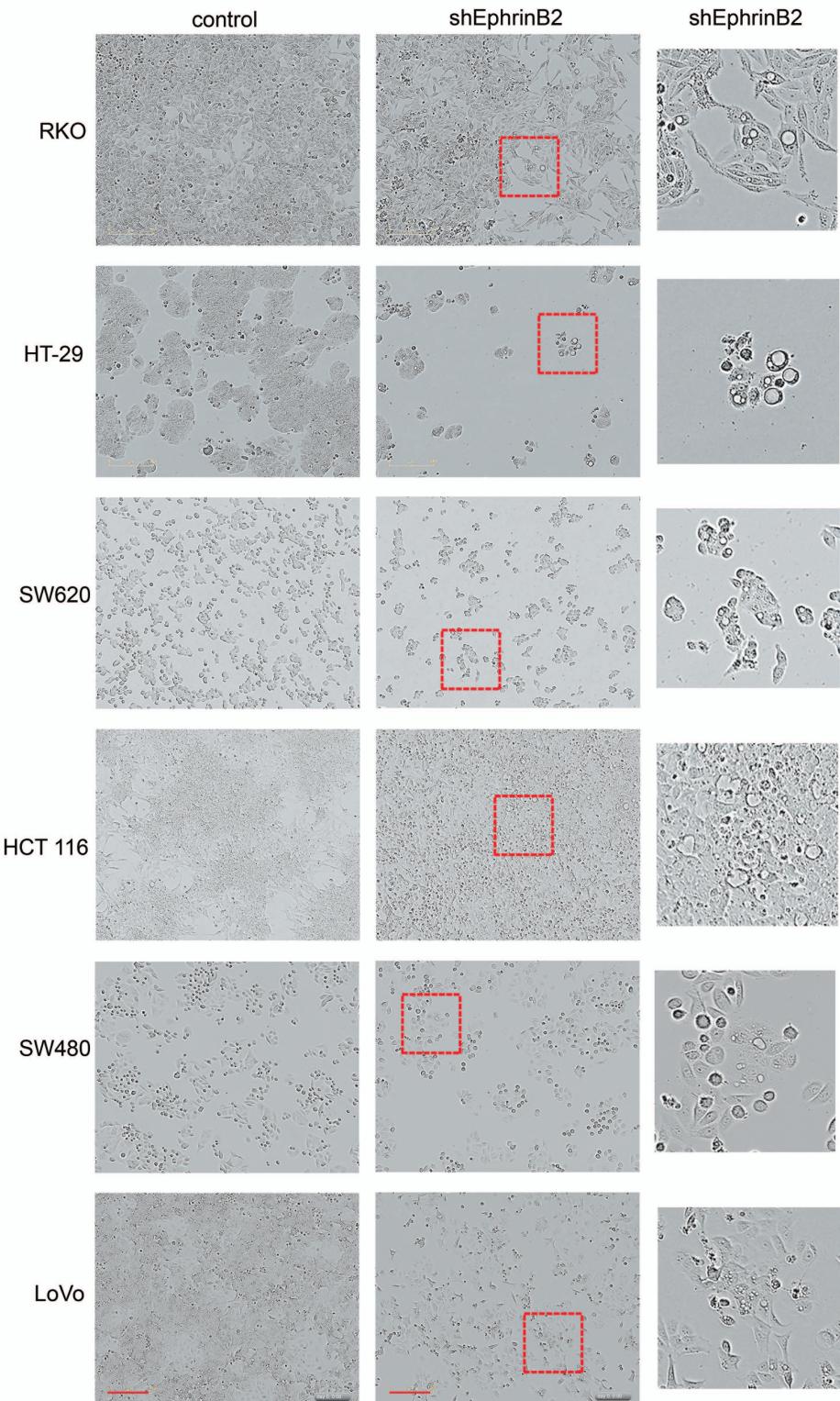
**Fig. S3.** EphrinB2, EphB2 and EphB4 silencing in colorectal carcinoma cell lines. Reduced EphrinB2, EphB2 and EphB4 protein levels in selected colorectal carcinoma cell lines 72 hours after shRNA infection. Representative immunoblotting results; β-actin reblotting used as a control.



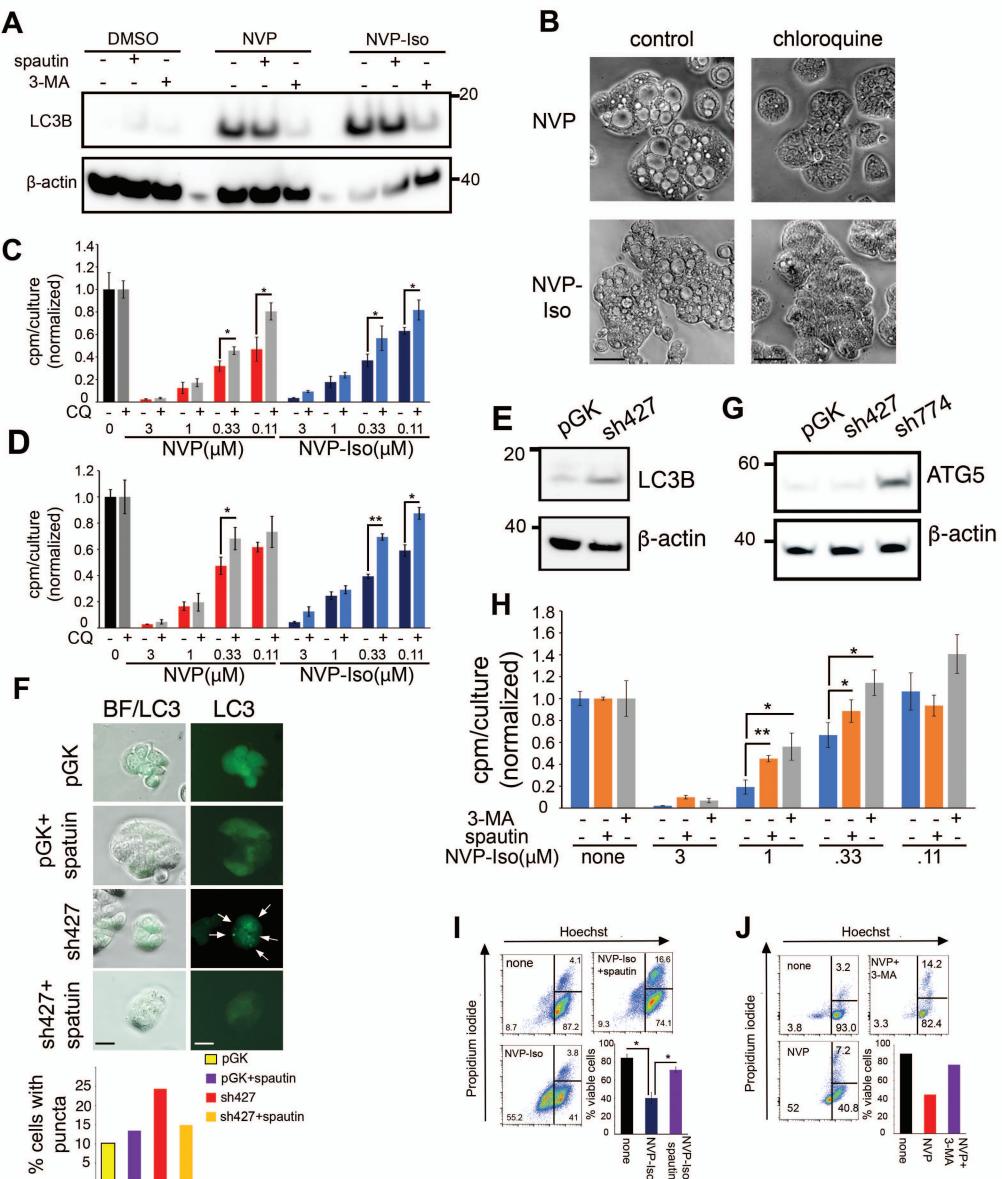
**Fig. S4.** (A), NVP-Iso dose-dependently inhibits HT-29 cell proliferation. Results are expressed as mean cpm/triplicate cultures. (B), Representative bright-field images showing reduced cell confluence in 3-day cultures supplemented with NVP or NVP-Iso (1 $\mu$ M). Boxed areas are magnified in main figure 4e; scale bars: 200 $\mu$ m. (C), Cell cycle analysis in HT-29 cells treated with NVP or NVP-Iso (1 $\mu$ M) or transduced with pGK (control), shEphrinB2 (sh427), or shEphB4 (sh774) for 72hrs. Cells were fixed, permeabilized and stained with propidium iodide. (D), Proliferation of HUVEC, MG63, hDMEC and SW620 cells after incubation with NVP or NVP-Iso (1 $\mu$ M) for 72hrs. Representative results of proliferation expressed as mean cpm/culture  $\pm$ SD (triplicate cultures). (E), Relative mRNA levels of EphB4 in HT-29 cells transduced with pGK control vector, WT EphB4 or kinase deficient (KD) mutant EphB4 (72hrs) by qRT-PCR. Statistical significance of difference calculated by two-tailed Student's t-test. \*P<0.05; \*\*\*P≤0.001.



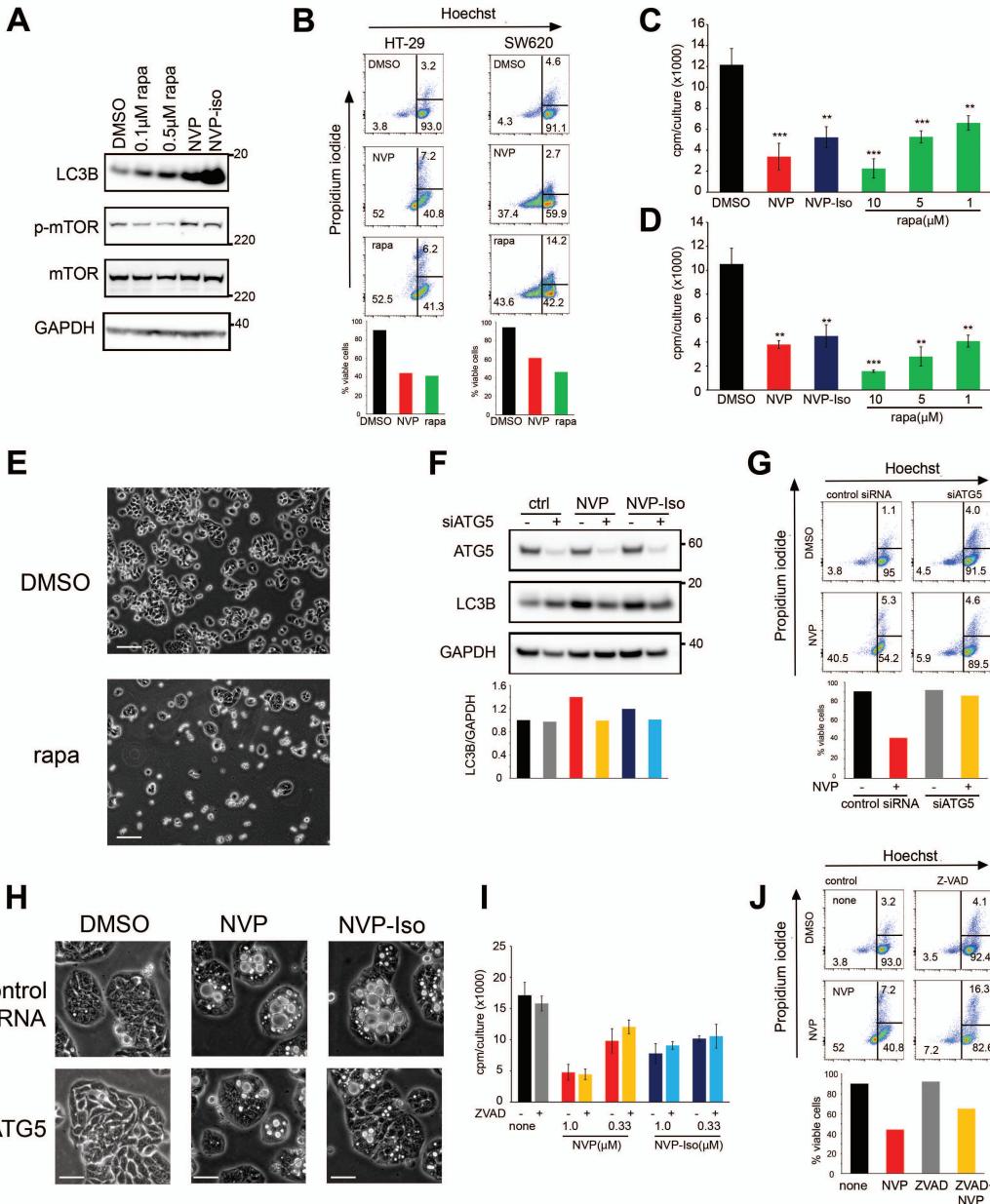
**Fig. S5.** Morphological change in colorectal carcinoma cell lines after incubation with NVP or NVP-Iso. Representative bright field Incucyte images of colorectal carcinoma cell lines incubated with 1 $\mu$ M NVP, 1 $\mu$ M NVP-Iso or none (control DMSO) for 48 hours. The areas limited by the red boxes are magnified immediately below. Scale bar: 200  $\mu$ m.



**Fig. S6.** Morphological change in colorectal carcinoma cell lines after EphrinB2 silencing. Representative bright field Incucyte images of colorectal carcinoma cell lines after EphrinB2 silencing with sh427 or infection with pGK control vector. Images obtained 48-120 hours after transduction. The areas limited by the red boxes are magnified on the right. Scale bar: 200  $\mu$ m.



**Fig. S7.** Autophagy markers in colorectal carcinoma cells. (A) NVP and NVP-Iso (1 μM) induce LC3B accumulation in SW620 cells after 72 hours; pre-treatment (4 hours) with spautin (5 μM) or 3-MA (5 mM) reduces this LC3B accumulation. (B) Chloroquine (10 μM) reduces autophagic vacuoles in HT-29 cells treated with NVP or NVP-Iso (1 μM); 72-hour culture; scale bars: 25 μm. (C,D) Chloroquine (CQ; 10 μM) enhances proliferation in HT-29 (C) and SW620 (D) colorectal cancer cells treated with NVP and NVP-Iso (0.11-3 μM); 72-hour culture; mean cpm/culture ± SD (triplicate cultures). (E) EphrinB2 silencing (sh427) induces LC3B accumulation in HT-29 cells. (F) EphrinB2 silencing induces autophagosomes in HT-29 cells; spautin (5 μM) reduces this accumulation. Fluorescent “puncta” in LC3-expressing HT-29 cells; bright-field/fluorescent (left) and fluorescent (right) imaging; 20x objective. Bar graph: mean percent cells with ≥ 2 puncta/cell identified in 5 fields (400-500 total cells counted) in each experimental condition, scale bars: 10 μm. (G) EphB4 silencing (sh774) induces the accumulation of the autophagy marker ATG5 in HT-29 cells. No ATG5 is induced after EphrinB2 silencing (sh427). (H) Pretreatment with spautin (5 μM; 4 hours) and 3-MA (10 mM) augments HT-29 cell proliferation inhibited by NVP-Iso (0.11-3 μM) at 72 hours; mean cpm/culture ± SD (triplicate cultures). (I) Spautin (5 μM) and (J) 3-MA (5 mM) reduce HT-29 cell death induced by NVP-Iso (0.5 μM) after 72 hours; representative profiles and quantitative results (bar graphs) of 3 independent experiments (I) or single (J) experiment (mean % ± SD). Statistical significance of group differences by two-tailed Student’s t-test. \*P<0.05; \*\*P≤0.01.



**Fig. S8.** Autophagy in colorectal carcinoma cells. (A) Rapamycin (rapa; 72-hour incubation) induces LC3B accumulation in HT-29 cells; immunoblots probed with specific antibodies to phospho(p)-mTOR (S2448), total m-TOR, LC3 and GAPDH. (B) Rapa (10 μM) and NVP (1 μM) induce similar cell death in HT-29 and SW620 cells after 72 hours; flow cytometry profiles (top) and quantitative results (bar graphs). (C, D) Rapa dose-dependently reduces cell proliferation in HT-29 (C) and SW620 (D) cells after 72 hours; mean cpm/culture ± SD (triplicate cultures). (E) Rapa (10 μM, 72-hour incubation) induces cell death in HT-29 cells; representative bright field images; scale bars 50 μm. (F) Effect of ATG5 silencing (24 hours prior to drug addition) on LC3B content in HT-29 cells incubated for 72 hours with medium only or medium with NVP and NVP-Iso (1 μM); immunoblots (top) and band quantification (bar graph). (G) Effect of ATG5 silencing (24 hours prior to drug addition) on HT-29 cell death induced by NVP (1 μM) after 72 hours; flow cytometry profiles (top) and quantification (bar graph). (H) ATG5 silencing in HT-29 cells mitigates the autophagic morphology of HT-29 cells treated with NVP or NVP-Iso (1 μM); scale bars: 12.5 μm. (I) Z-VAD-FMK (ZVAD; 0.5 μM) minimally affects the proliferation of HT-29 cells incubated for 72 hours with NVP or NVP-Iso (0.33 or 1 μM); mean cpm/culture ± SD (triplicate cultures). Statistical significance of group differences by two-tailed Student's t-test. \*\*P≤0.01, \*\*\*P≤0.001.