Supplementary Materials (Methods, References, Figures)

Supplementary Methods

Tumor Microarray

Patient-derived primary colon tumors and their associated serially-passaged xenografts were serially sectioned and processed via heat-induced epitope retrieval in 10 mM sodium citrate buffer. Sections were stained for KIT using a titered polyclonal rabbit anti-human cKit antibody (cat# A4502, Dako), and KITLG using a monoclonal rabbit anti-human SCF antibody (cat #2093, Cell Signaling Technology). Staining was scored as Uninterpretable (due to extensive necrosis or absence of sufficient tumor cells), or None, Weak, or Strong (Fig. 1A and B, 4A and B). Tumor cells were clearly distinguishable from infiltrating KIT+ mast cells based on nuclear:cytoplasmic ratio and other pathological features. Images were captured on a Leica DMI4000B microscope (Leica Microsystems Inc.) with a QImaging Retiga2000R CCD camera (QImaging), processed with ImagePro 6.3 software (MediaCybernetics), and post-processed with Adobe Photoshop CS3.

Tissue Culture

All human colon cancer cell lines and organoids derived from colon cancer xenografts were grown in advanced Dulbecco's modified Eagle medium/F12 (Invitrogen) with 10 mM Hepes, 1x Glutamax (Life Technologies), 10% heat-inactivated fetal bovine serum, 120 µg/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin-B. Cell lines used include DLD1, LS174T, COLO320DM, HT29, SW620, CACO2, and HCT116 (ATCC). Organoids from UM-COLON#8 and POP77 human colon cancer xenografts were derived by mechanically and enzymatically dissociating tumors into single cells in media with 100 units/mL DNAse and 200 units/mL type III

Collagenase (Worthington). FACS-sorted cells were resuspended in growth factor reduced Matrigel (BD) and plated in 48-well plates with 150 µL Matrigel per well. Matrigel was polymerized at 37°C for 10 minutes and overlaid with 300 µL media per well. All cultures were grown in humidified tissue culture incubators at 37°C in 5% CO_2 and 20% O_2 and monitored daily under a microscope. For growth curves, cells were trypsinized and counted using a hemocytometer and excluding dead cells using Trypan Blue staining. Organoid volumes were calculated according to the formula $V = 0.4 \times a \times b^2$, where V represents the volume, a is the organoid's largest diameter, and b is the organoid's smallest diameter, as used previously[1].

shRNA Lentiviral Transduction

shRNA oligonucleotides containing KIT- or KITLG-targeting sequences (Table S1) were designed using pSicoOligomaker 1.5 (T. Jacks Lab) and Basic Local Alignment Search Tool (NCBI) against the human genome to minimize off-target effects. Five oligonucleotides each were screened for KIT and KITLG knockdown, and the best two were selected based on degree of knockdown by gene expression and/or flow cytometry. A modified pSico-Pgk-GFP vector (plasmid# 12093, Addgene) containing the shRNA sequences preceded by the EF1α promoter, along with lentiviral packaging vectors Δ8.9 and VSVG, were transfected into 293T fibroblasts using FuGENE (Promega)[2]. Media with lentiviral particles was collected, filtered, concentrated, and titered on 293T cells. For colon cancer cell lines, cells were grown in 2D and incubated with lentivirus for 24 hours. For xenografts, tumors were dissociated into single-cells and maintained in suspension culture with lentivirus for 24 hours and then FACS-sorted for GFP+ cells prior to injection.

Xenograft Formation

Colon cancer xenografts were derived from dissociated cells or tumor chunks. For dissociated cells, cells were suspended in a 1:1 mixture of Dulbecco's modified Eagle medium/F12 and Matrigel, and injected subcutaneously into the flanks of NSG mice. Tumor chunks were mixed with Matrigel and surgically implanted subcutaneously into the flanks of NSG mice.

Flow Cytometry

Methods for flow cytometry have been previously described[3]. Briefly, cells in single-cell suspension were stained in the dark on ice with titered fluorescently-conjugated antibodies: Kit-PE (cat# 340529, BD Biosciences), Kit-PE-Cy7 (cat# 339195, BD Biosciences), CD44-PE-Cy7 (cat# 560533, BD Biosciences), CD44-APC (cat# 559942, BD Biosciences), EPCAM-Alexa488 (clone 9C4, BioLegend), H2KD-Pacific Blue (clone 30-F11, BioLegend), and MKI67-PerCP-Cy5.5 (cat# 561284, BD Biosciences). EdU staining was performed with the Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Life Technologies) and BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Bioscences). Flow cytometry was performed with a 100 µM nozzle on a BD FACSAria II using FACSDiva software. Debris and doublets were excluded by sequential gating on forward scatter area vs. side scatter area, followed by forward scatter width vs. forward scatter height, followed by side scatter height vs. side width area. Dead cells were gated out with DAPI. Other gates were drawn with fluorescence minus one (FMO) or isotype controls. Purity was checked on all sorted populations and was 95% or higher. Data analysis was performed with FlowJo v10.

Gene Expression Analysis

FACS-sorted cells were resuspended in Trizol (Invitrogen) and RNA was extracted with the PureLink RNA Micro Kit (Invitrogen). Complementary DNA was generated using the Superscript3 Kit (Invitrogen) with random hexamers, oligo dT, or gene-specific primers; then, qRT-PCR (either

with or without preceding preamplification with gene-specific primers) was conducted on an ABI7900HT Thermocycler using TaqMan assays (Applied Biosystems) for genes of interest (Table S2). Samples were loaded in triplicate, and fold changes were calculated using ΔΔCt, normalizing to glyceraldehyde-3-phosphate dehydrogenase or beta-actin.

Single-Cell Gene Expression

Double -sorted FACS-isolated single cells with purity > 95% were sorted into individual wells of 96-well plates containing 5 µL lysis buffer (CellsDirect qRT-PCR mix; Invitrogen) and 2 U (0.1 µL) SuperaseIn (Invitrogen) and processed as described[3,4]. For data analysis, single cell gene-expression results were normalized gene-by-gene, by mean-centering and dividing by 3 times the standard deviation of expressing cells. Unsupervised hierarchical clustering was performed on both cells and genes, with correlation distance metric and complete linkage[4]. *P*-values for differential gene expression were calculated using the Wilcoxon rank-sum test.

Bioluminescence Imaging

Colon cancer cells/xenografts engineered to constitutively express luciferase (LUC2) by lentiviral transduction were injected into the flanks of NSG mice[5]. To visualize bioluminescence, mice received intraperitoneal injections of luciferin (Biosynth), and were imaged on an IVIS Spectrum (Caliper Life Sciences) instrument and quantified using Living Image 4.0 Software (Caliper Life Sciences). Total flux (photons/second) was recorded for each tumor.

Immunofluorescence Imaging

As described previously[2], tissue was formalin-fixed, cryoprotected in 30% sucrose, embedded in OCT, and sectioned at −20°C. Sections were permeabilized with PBS + 0.1% Triton X-100 (PBS-T),

blocked with 5% normal goat serum in PBS-T for 30 minutes at 25°C, and stained with antibodies in block for 2 to 4 hours at 25°C. After washing in PBS-T, slides were incubated for 2 to 12 hours in minimally cross-reactive fluorescently conjugated secondary antibodies (Jackson ImmunoResearch) at 1:400 washed in PBS-T, and mounted in ProLong Gold+Dapi (Molecular Probes). Antibodies used include mouse anti-human Ki67 (Clone MIB-1, Dako) and rabbit anti-human CD117 (cat# A4502, Dako) primary antibodies, with donkey anti-mouse Cy5 (cat# 715-175-150, Jackson ImmunoResearch) and goat anti-rabbit A568 (cat# SAB4600084, Sigma) secondary antibodies. Images were captured on a Zeiss LSM510Meta confocal microscope (Zeiss Microscopy), and post-processed with Adobe Photoshop CS3.

Imatinib Experiments

Imatinib mesylate (LC Laboratories) was dissolved in phosphate buffered saline (PBS) at the specified concentrations for each experiment. For *in vitro* studies, dissolved imatinib was added to cell media, with PBS serving as the control. For *in vivo* studies, imatinib at a dose of 50 mg/kg/day was administered to mice via intraperitoneal injections.

Statistical Analysis

Values represent mean, standard deviation, or standard error of mean as indicated. Differences between groups were determined using the two-tailed Student *t*-test. For Fig. 5A, one-way ANOVA was performed with the Tukey's post-hoc test. For Fig. 5D, the dependent variable was transformed with the natural log and linear regression was performed. For Fig. 6C, L-Calc (Stemcell Technologies) was used for limiting dilution analysis. All tests were carried out with a significance cutoff of $p < 0.05$. Analysis was performed with GraphPad Prism 5.

References

1. **Attoub S**, Rivat C, Rodrigues S, et al. The c-kit tyrosine kinase inhibitor STI571 for colorectal cancer therapy. Cancer Res 2002;62:4879-4883.

2. **Ventura A, Meissner A**, Dillon CP, et al. Cre-lox-regulated conditional RNA interference from transgenes. Proc Natl Acad Sci U S A 2004;101:10380-10385.

3. **Rothenberg ME**, Nusse Y, Kalisky T, et al. Identification of a cKit(+) colonic crypt base secretory cell that supports Lgr5(+) stem cells in mice. Gastroenterology 2012;142:1195-1205.e6.

4. **Dalerba P**, **Kalisky T**, **Sahoo D**, et al. Single-cell dissection of transcriptional heterogeneity in human colon tumors. Nat Biotechnol 2011;29:1120-1127.

5. **Willingham SB, Volkmer JP**, Gentles AJ, et al. The CD47-signal regulatory protein alpha (SIRPa) interaction is a therapeutic target for human solid tumors. Proc Natl Acad Sci U S A 2012;109:6662-6667.

Author names in bold designate shared co-first authors.

Supplementary Figures

Oligo pairs	Oligo sequences (5'→3')
shKit3 Sense	TGTAGATTAAGAGCCATATATTCAAGAGATATATGGCTCTTAATCTACTTTTTTC
shKit3 Antisense	TCGAGAAAAAAGTAGATTAAGAGCCATATATCTCTTGAATATATGGCTCTTAATCTACA
shKit4 Sense	TGTATGTGTGTAGACAAATATTCAAGAGATATTTGTCTACACACATACTTTTTTC
shKit4 Antisense	TCGAGAAAAAAGTATGTGTGTAGACAAATATCTCTTGAATATTTGTCTACACACATACA
shKitlg1 Sense	TGCAGGAATCGTGTGACTAATTCAAGAGATTAGTCACACGATTCCTGCTTTTTTC
shKitlg1 Antisense	TCGAGAAAAAAGCAGGAATCGTGTGACTAATCTCTTGAATTAGTCACACGATTCCTGCA
shKitlg2 Sense	TGGAATCGTGTGACTAATAATTCAAGAGATTATTAGTCACACGATTCCTTTTTTC
shKitlg2 Antisense	TCGAGAAAAAAGGAATCGTGTGACTAATAATCTCTTGAATTATTAGTCACACGATTCCA

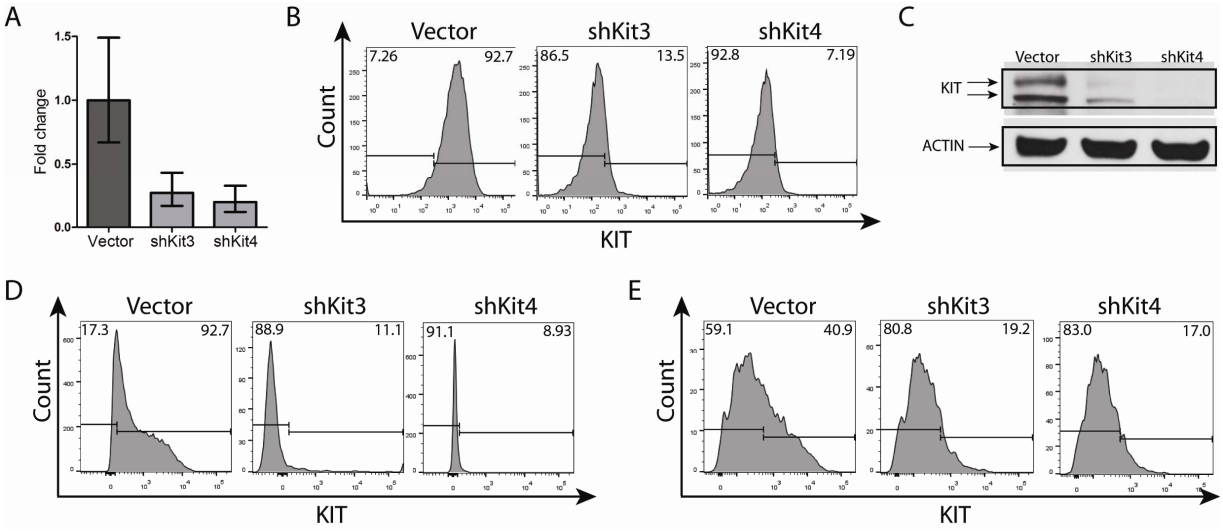
Supplementary Table S1. Oligonucleotides used in the generation of KIT and KITLG knockdown shRNA.

TaqMan assays	
ALCAM	Hs00233455_m1
ASCL2	AIMRUO9
AXIN2	Hs00610344_m1
BIRC5	Hs00153353_m1
BMI-assay1	Hs00180411_m1
BMI-assay2	Hs00995519_g1
CD44	Hs00153304_m1
CDKN1A	Hs00355782_m1
CEACAM-assay1	Hs00266109_m1
CEACAM-assay2	Hs00989784_m1
CTFR	Hs01565537_m1
DLL1-assay1	Hs01011325_g1
DLL1-assay2	Hs00194509_m1
DLL4-assay1	Hs01117332_g1
DLL4-assay2	Hs00184092_m1
DPP4	Hs00175210_m1
KIT-assay1	Hs00922210_m1
KIT-assay2	Hs00174029_m1
KITLG	Hs00241497_m1
KRT20	Hs00300643_m1
LGR5-assay1	Hs00969421_m1
LGR5-assay2	Hs00969423_m1
LRIG1	Hs00394267_m1
MKI67	Hs00267195_m1
MYC	Hs00153408_m1
OLFM4	Hs00197437_m1
PTPRO	Hs00243097_m1
RNF43	Hs00993304_m1
SPINK4	Hs01018780_m1
TFF3	Hs00173625_m1
TOP2A	Hs01032137_m1

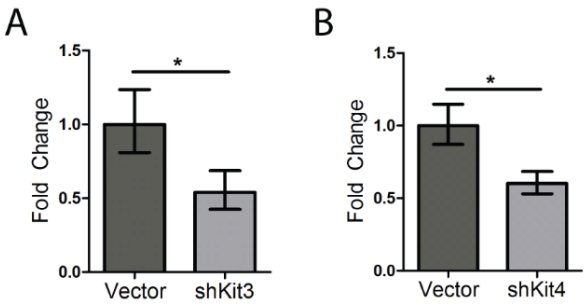
Supplementary Table S2. List of TaqMan assays used.

Primer pairs	Primer sequences (5'→3')
Exon 9 Sense	GTATGCCACATCCCAAGTGT
Exon 9 Antisense	CATGACTGATATGGTAGACA
Exon 11 Sense	CCAGAGTGCTCTAATGACTG
Exon 11 Antisense	GGAAGCCACTGGAGTTCCTT
Exon 13 Sense	GACATCAGTTTGCCAGTTGT
Exon 13 Antisense	TGTTTTGATAACCTGACAGAC
Exon 17 Sense	GCAACACTATAGTATTAAAAAG
Exon 17 Antisense	CCTTTGCAGGACTGTCAAGCA

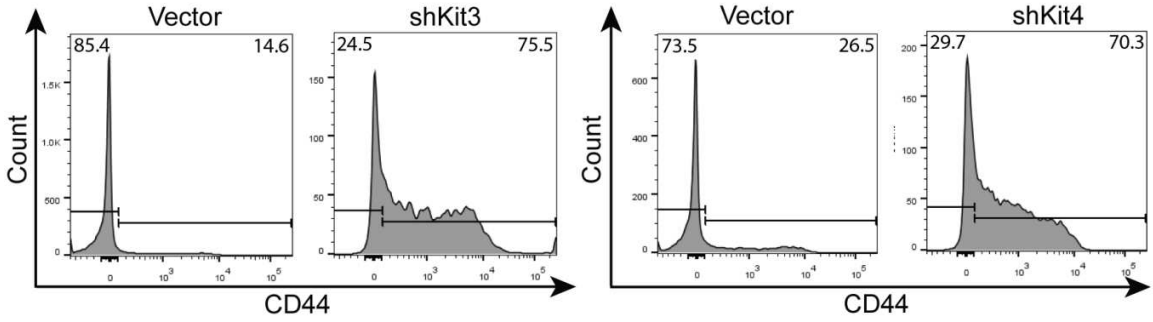
Supplementary Table S3. Primer pairs used in sequencing selected KIT exons in human colon cancer cell lines and xenografts.



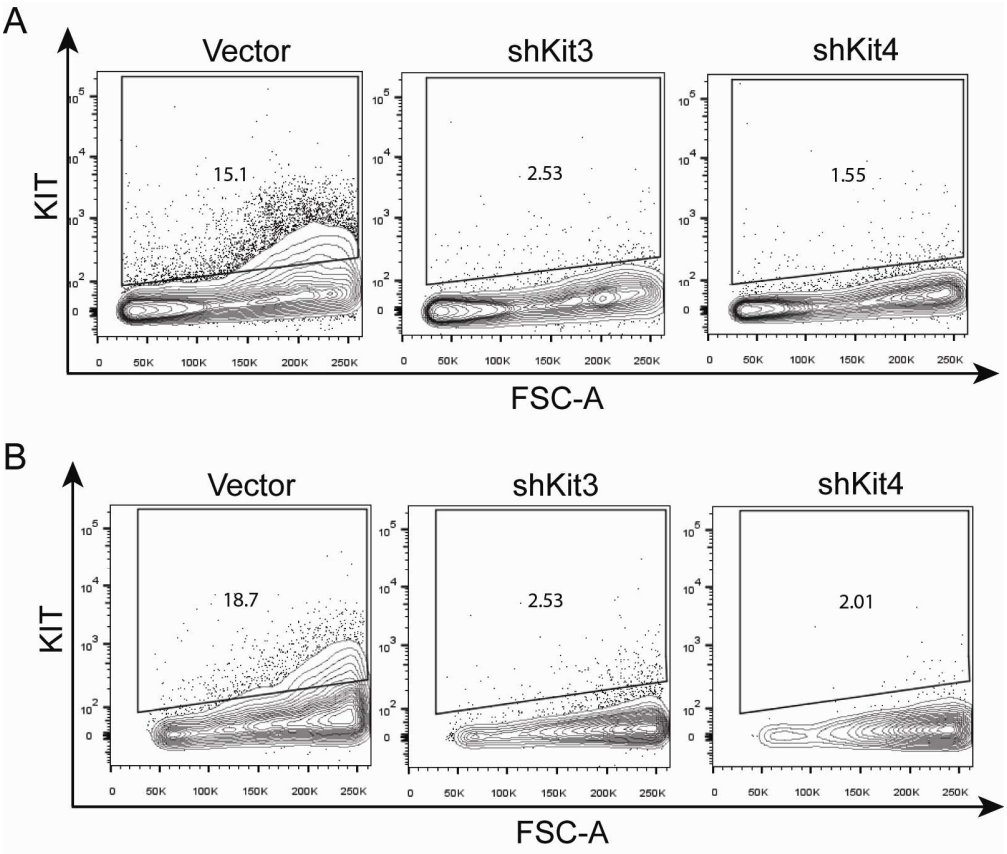
Supplementary Figure S1. KIT shRNA successfully knocks down KIT expression in colon cancer cell lines. A) KIT mRNA levels in DLD1 cells after transduction with two independent KIT shRNA (shKit3 and shKit4) or the empty vector control (mean ± 95% CI shown). Gene expression was determined by qRT-PCR. B) Flow cytometry histograms of DLD1 KIT protein levels after shRNA transduction. C) Western blot showing KIT protein levels in DLD1 cells after shRNA transduction. D and E) Flow cytometric histograms of KIT protein levels in LS174T cells (D) and COLO320 cells (E) after shRNA transduction.



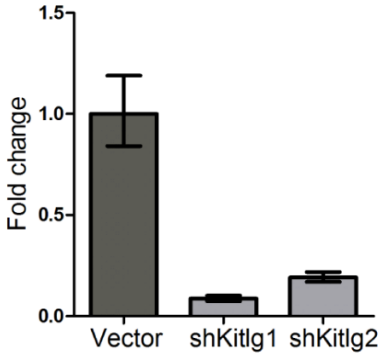
Supplementary Figure S2. KIT mRNA levels remain low in KIT knockdown DLD1 tumors after passage *in vivo*. A) KIT knockdown by shKit3 (representative sample of $n = 4$ tumors; mean ± 95% CI shown). B) KIT knockdown by shKit4 (representative sample of $n = 4$ tumors; mean ± 95% CI shown). Gene expression was determined by qRT-PCR. *, $P < 0.05$ in Student *t* test.



Supplementary Figure S3. CD44 expression is increased in KIT knockdown DLD1 xenografts compared to vector control tumors (representative image of *n* = 4 tumors/group shown).



Supplementary Figure S4. KIT shRNA successfully knocks down KIT expression in POP77 and UM-COLON#8 organoids. A and B) FACS plots for POP77 organoids (A) or UM-COLON#8 organoids (B) transduced with KIT shRNA or the empty vector control.



Supplementary Figure S5. KITLG shRNA successfully knocks down KITLG expression. mRNA levels of KITLG in DLD1 cells after transduction with two independent KITLG shRNA (shKitlg1 and shKitlg2) or the empty vector control (mean ± 95% CI shown). Gene expression was determined by qRT-PCR.