SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Sequence data

Name	Sequence
GFP siRNA target	CGCTGACCCTGAAGTTCAT
CBP siRNA target	CGCCGACAACTAAGGAGTT
p300 siRNA target	TCTACTCTCAAATCCGGCG
EphB4 siRNA target	GGTGAATGTCAAGACGCTG
EphB4 siRNAΔ target	AGTTAATATCAAGACGCTG
EphrinB2 siRNA target	GCAGACAGATGCACTATTA
EphB2 siRNA	HP Validated siRNA SI02224796 for gene EPHB2 from QIAGEN (Hilden, Germany)
TRAIL siRNA	HP Validated siRNA SI1027400 for gene TNFSF10 from QIAGEN (Hilden, Germany)
shRNA amplification primers	F – GCAGACAGATGCACTATTA R – GCAGACAGATGCACTATTA
mEphB4 amplification primers	F – agttAAGCTTGTGAGGCGGCGCCATGG (HindIII) R - attgCAATTGTCAGAACTGCTGGGCTGG (MfeI, ligate with EcoRI on vector)
Cytoplasmic tail-deleted hEphB4 amplification primers	F – GACCTTAGTACCATGTCCGAT (with 5'-phos) R – CAGTGTCCAGCACATGAAGTC
eGFP amplification primers	F – ATGGTGAGCAAGGGCGAGGA (with 5'-phos) R - attatgcggccgcTTACTTGTACAGCTCGTCCATG (NotI)
EphB4 shRNA expression vector insert	TGCTGTTGACAGTGAGCGGGTGAATGTCAAGACGCT GTTTAGTGAAGCCACAGATGTAAACAGCGTCTTGACA TTCACCTGCCTACTGCCTCGGA

Antibody data

Antibody against	Clone	Source	Application	Concentration
EphB4	V131	Vasgene Therapeutics, Los Angeles, CA	IF	1.5μg/ml
EphB4	V265	Vasgene Therapeutics, Los Angeles, CA	IB	1μg/ml
EphB4	V47	Vasgene Therapeutics, Los Angeles, CA	IP	2μg/ml
EphB2	AF-467	R&D Systems, Minneapolis, MN	IB/IF	1μg/ml
EphB3	AP7624a	Abgent (San Diego, CA).	IB	2μg/ml
β-actin	A1978	Sigma-Aldrich, St. Louis, MO	IB	0.5μg/ml
APC	FE9	Calbiochem, San Diego, CA	IB	1μg/ml
P300	RW128	Upstate, Lake Placid, NY	IB/IP	2μg/ml
CBP	AC238	Chemicon, Tamecula, CA	IB/IP	2μg/ml
β-catenin	5H10	Upstate, Lake Placid, NY	IB	2μg/ml
Phospho-tyrosine	4G10	Upstate, Lake Placid, NY	IB	1μg/ml
EphrinB2	P20	Santa Cruz Biotechnology, Santa Cruz, CA	IB	1μg/ml
Ki-67	MIB-1	DAKO, Carpinteria, CA	IF	1:50 dilution
CD31	M20	Santa Cruz Biotechnology, Santa Cruz, CA	IB	1μg/ml
Murine EphB4	MAB446	R&D Systems, Minneapolis, MN	IB	2μg/ml
TRAIL	B35-1	BD Pharmingen, San Diego, CA	IB	1μg/ml
Human-Fc	-	Jackson Immunoresearch, West Grove, PA	*In vitro*	100ng/ml
All secondary antibodies		Santa Cruz Biotechnology, Santa Cruz, CA	IB/IF/ FACS	

Transfection, sorting and invasion assays

Transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. For sorting transfected cells, the MACSelect 4.1 (Miltenyi-Biotec, Auburn, CA) transfected cell selection kit was used as per manufacturer's instructions. To study invasion, cells were transferred into 8μ Matrigel-precoated inserts. The inserts were placed in

companion wells containing 700μl RPMI supplemented with 5 % FBS and 100ng/ml EGF as chemoattractant. Following 12 hours incubation, the inserts were removed and non-invading cells on the upper surface scraped with a cotton swab. The cells on the lower surface of the membrane were stained with Giemsa.

Expression vectors

mEphB4 full length cDNA was amplified from pCMV/mEphB4. This was then subcloned into pcDNA3.1/myc-His A vector with HindIII and EcoRI digestion. For construction of EphB4-eGFP mutant protein expression vector, we amplified the pEF6-hEphB4-FL plasmid with a deletion of the C-terminal 398-amino acid cytoplasmic tail of EphB4 and digested with NotI. Subsequently, the eGFP sequence from the eGFP expression vector (Invitrogen, Carlsbad, CA) was amplified, digested with NotI, and sub-cloned into the NotI-digested vector. EphB3 expression vector was obtained from PlasmID (Cambridge, MA) and subcloned into pCDNA3.1. Full length APC expression vector was a kind gift from Bert Vogelstein (Baltimore, MD).

Statistical analysis

All values are expressed as mean ± SEM. Statistical analysis was performed using Statistical Analysis Software (SAS 9.2). Spearman's coefficient was used to assess correlation. Regression analyses were used to study correlation

between tumor stage and grade and protein expression levels. ANOVA was used to compare multiple proportions and students' t-test for pair-wise comparisons. P value < 0.05 was considered significant.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental figure S1 10ng of the extracellular domain of various EphB receptors was immobilized on ELISA wells and detected with anti-EphB4 #V131 antibody. Antibody (Ab) binding was detected using HRP-coated secondary antibody. Binding to EphrinB2-alkaline phosphatase (AP) was used as positive control (A). Sections of primary tumor and metastatic deposit were stained with anti-EphB4 #V131 (Ab) antibody native or pre-incubated at 4ºC for 1h with blocking antigenic peptide (peptide) (B). Serial dilutions of soluble monomeric form of extracellular domain of EphB4 (sEphB4) were detected by immunoblotting using anti-EphB4 #v265 antibody to ascertain antibody sensitivity (C). 20ng of extracellular domain of various Eph receptors were immunoblotted and probed using anti-EphB4 #v265 antibody to ascertain antibody specificity (D). h-human protein, m-murine protein, r-rat protein.

Supplemental figure S2 Representative examples of immunostaining for EphB4 (A, top rows, and B) and EphB2 (A, bottom rows) in tissue arrays comprising serial 7μ sections of multiple colorectal tumors (tumor) and matched normal mucosa (normal). Corresponding immunoblots of tumor and normal tissue are

shown in B. Similar examples of immunostaining for EphB4 and EphB2 in matched colon cancer metastases to liver and adjacent normal liver (C). Expression of EphB4 and EphB2 was studied (D) in a colon cancer specimen, adjacent normal colon, and metastasis-bearing lymph node by immunostaining and immunoblotting. Total mRNA was extracted from tumor and matched normal tissue. EphB4 mRNA levels were assessed by qPCR, normalized to β-actin mRNA levels and expressed as fold change in tumor compared to normal tissue (E). Colorectal primary tumor and liver metastases were immunostained for EphB4 and β-catenin (F).

Supplemental figure S3 FACS analysis (A) was used to study membrane expression of EphB4 (top right panel), its ligand EphrinB2 (bottom left panel) or both (bottom right panel) in HT29 cells. HT29 cells were serum starved overnight (serum-free) and stimulated for 20 min with various doses of clustered EphrinB2/Fc or Fc fragment alone. EphB4 was immunoprecipitated from 100μg whole cell lysates and phosphorylation status analyzed by anti-phosphotyrosine antibody immunoblotting (B top panel, top row). A duplicate membrane was probed for EphB4 to document immunoprecipitation efficiency (B top panel, bottom row). The experiment was repeated using 1μg/ml clustered EphrinB2/Fc or Fc alone for various time-periods shown (B bottom panel). HT29 cells were grown in the presence or absence of serum and following knockdown of

EphrinB2 using specific siRNA. EphB4 phosphorylation status was assessed as above (C).

Supplemental figure S4 HT29 cells were transiently transfected with EphB4-specific siRNA and EphB4 levels assessed by immunoblotting at various time-points following transfection (A). HT29 cells were transiently transfected with Lipofectamine alone (Control), 50nM of mutated EphB4 siRNA (EphB4 siRNAΔ), or EphB4-specific siRNA (EphB4 siRNA). Surface expression of EphB4 was analyzed by FACS 36h later (B). 293T cells (293T-parent) were transfected with expression vector for full length human EphB4 (293T-hEphB4) or murine EphB4 (293T-mEphB4). Cells were transiently transfected with various doses of EphB4-specific siRNA (EphB4 siRNA), and EphB4 and β-actin levels in 20μg cell lysates analyzed by immunoblotting (C). HT29 cells (HT29-parent) were transfected with expression vector for murine EphB4 (HT29-mEphB4) along with a truncated CD4 receptor that was used to sort transfected cells. 1X 10^4 parent and transfected and sorted cells were transfected with varying doses of EphB4-specific siRNA. Cell number was assessed by MTT assay at 72 hours and expressed as percentage of absorbance relative to untreated cells (D). 1X10^4 SW620 cells were plated in a 48-well plate and transfected with various doses of EphB4-specific siRNA (EphB4 siRNA) or mutated EphB4 siRNA (EphB4 siRNAΔ). Cell number was assessed by MTT assay at 72 hours (E). Data is expressed as mean ± SEM. HT29 (F, left panel) and COLO-205 (F, right panel) cells were

transiently transfected with 100nM EphB4-, EphB2- and EphrinB2-specific siRNA and levels of various proteins assessed 48h later by immunoblotting.

Supplemental figure S5 Flow diagram (A) describing the generation of FF4 cells, a single clone of cells engineered to stably produce EphB4-specific siRNA under tetracycline control (Tet-off). FF4 cells were plated in a multi-well plate and cultured for five days in the presence of varying doses of doxycycline and total cellular RNA was extracted. RT-PCR was performed using primers directed against generated shRNA (B).

Supplemental figure S6 Ten- to twelve-week old, male Balb/C athymic mice (n=5 per group, experiment repeated twice) were injected with $1X10^6$ HT29-mock cells in the right flank and $1 X 10^6$ FF4 cells in the left flank. One half of the mice were fed doxycycline (Tetracycline +). $1 X 10^6$ HT29 cells (HT29-parent) were implanted in separate mice as control. Animals were sacrificed four weeks later (A) and tumors harvested (B). Bar in extreme right panel in B represents 1cm.

Supplemental figure S7 Confluent cultures of HT29 cells were scraped with a plastic Pasteur pipette to produce 3mm wide breaks in the monolayer. The ability of cells to migrate and close the wound following transfection with 50nM EphB4-specific (EphB4-siRNA) or mutant siRNA (EphB4-siRNAΔ) was assessed over 12hr. Representative photomicrographs are shown (A, left panel). Cell-free area

was averaged over five random high power fields at the various time points and represented as ratio of area at time t=0 (A, right panel). HT29 cells were treated with lipofectamine alone (control), 50nM EphB4-specific (EphB4 siRNA) or mutant siRNA (EphB4 siRNAΔ) and 0.5 x 10^5 cells were transferred into 8μ Matrigel-precoated inserts. Following 12 hours incubation, cells invading the inserts in response to 100ng/ml EGF were stained with Giemsa. Representative photomicrographs are shown (B, left panel). Cell number was averaged over five random high-power fields (B, right panel). All data is expressed as mean ± SEM.

Supplemental figure S8 HT29 cells were transiently transfected with various doses of EphB4-specific siRNA (EphB4 siRNA) or mutated siRNA (EphB4 siRNAΔ). Apoptosis was analyzed by ELISA for cytoplasmic nucleosomes using whole cell lysates (A). Caspase-8 and caspase-9 activation was assayed colorimetrically and expressed as percent activity compared to lipofectamine-treated cells (B). Data is expressed as mean ± SEM. HT29 cells were transfected with 100nm EphB4-specific siRNA (EphB4 siRNA) or mutated siRNA (EphB4 siRNAΔ) and levels of TRAIL and related proteins were assessed by immunoblotting 48h later (C).

Supplemental figure S9 FF4 cells were transfected with TRAIL-specific siRNA (TRAIL-siRNA) or siRNA targeting GFP (GFP-siRNA). TRAIL knockdown was confirmed by immunoblotting of protein samples extracted two days following

transfection (top panel). Transfected cells were then cultured for five days in the absence of doxycycline. Cell number was assessed by MTT assay and expressed as percentage of absorbance relative to untreated cells (bottom panel).

Supplemental figure S10 In order to delineate the role of signaling by EphB4, a mutant protein with the cytoplasmic tail of EphB4 replaced by GFP was generated (A). The mutant EphB4-eGFP protein was anticipated to allow reverse signaling without forward signaling (B). 293 cells were stably transfected with full length EphB4 (293-EphB4), eGFP (293-eGFP) or mutant EphB4 (293-EphB4-eGFP). EphB4 expression was analyzed by immunoblotting 20μg protein lysates from each cell line (C). Cells were evaluated by fluorescent microscopy to delineate cytoplasmic versus membrane fluorescence (D, bottom panel). Cells were stained with PE-labeled anti-EphB4 antibody and evaluated by FACS with green fluorescence along X-axis and red on Y-axis (D, top panel). Parent 293 cells and transfected cells were serum starved overnight and stimulated for 20 min with 2μg/ml of clustered EphrinB2/Fc. EphB4 was immunoprecipitated from 100μg whole cell lysates and phosphorylation status analyzed by anti-phosphotyrosine antibody immunoblotting (E top panel). A duplicate membrane was probed for EphB4 to document immunoprecipitation efficiency. Equal numbers of cells from the four transfections were mixed with solution containing equal amount of alkaline phosphatase-tagged EphrinB2. Cells were pelleted and

AP activity tested in the pellet (E, bottom panel). PM-plasma membrane, EC-extracellular, IC-intracelular, G-globular domain, C-cysteine-rich domain, F-fibronectin III-repeat domain, WT-wild type, AP-alkaline phosphatase

Supplemental figure S11 SW480 cells were transiently transfected with various doses of EphB4-specific siRNA (EphB4 siRNA). Transfected cells were exposed to various doses of TRAIL for 16 hours and cell survival assessed by MTT assay (A). SW480 cells were co-transfected with full length EphB4 expression vector (EphB4-FL), vector expressing EphB4 in which the intra-cellular domain was replaced with GFP (EphB4-eGFP) or null vector (Empty vector) along with truncated CD4 receptor to allow for sorting of transfected cells. $1X10^4$ sorted cells were treated overnight with varying doses of TRAIL and cell number assessed by MTT assay (B). Data is expressed as mean $\pm$ SEM.