Supplemental Figure Legends

Figure S1

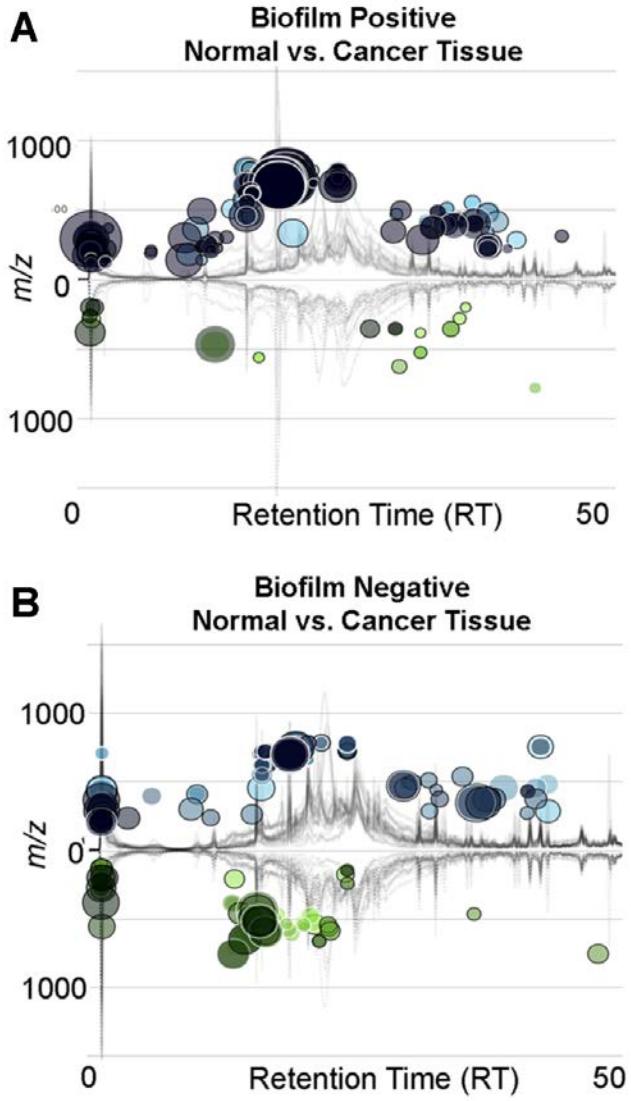

Figure S2

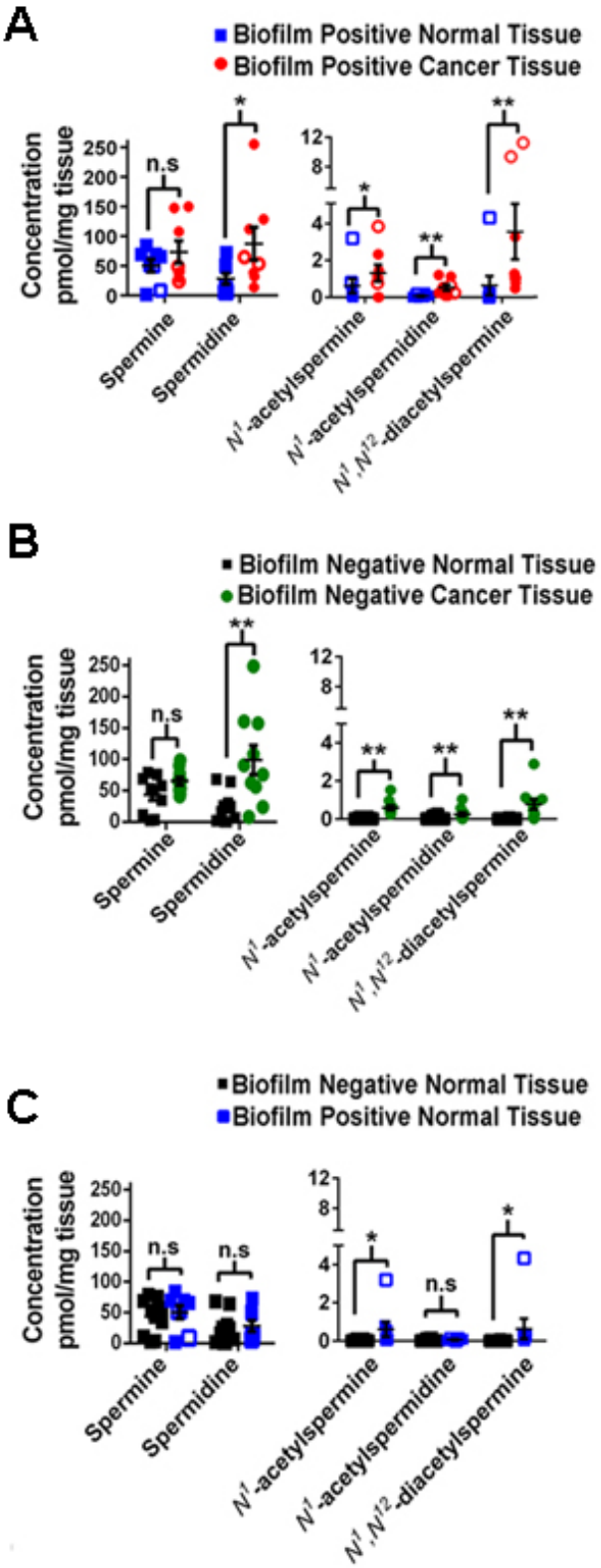
A
Biofilm Positive Normal Tissue
Biofilm Positive Cancer Tissue
Concentration pmol/mg tissue
n.s
*
**
Spermine
Spermidine
N1-acetylspermine
N1-acetylspermidine
N1,N12-diacetylspermine
B
Biofilm Negative Normal Tissue
Biofilm Negative Cancer Tissue
C
Biofilm Negative Normal Tissue
Biofilm Positive Normal Tissue

Figure S3

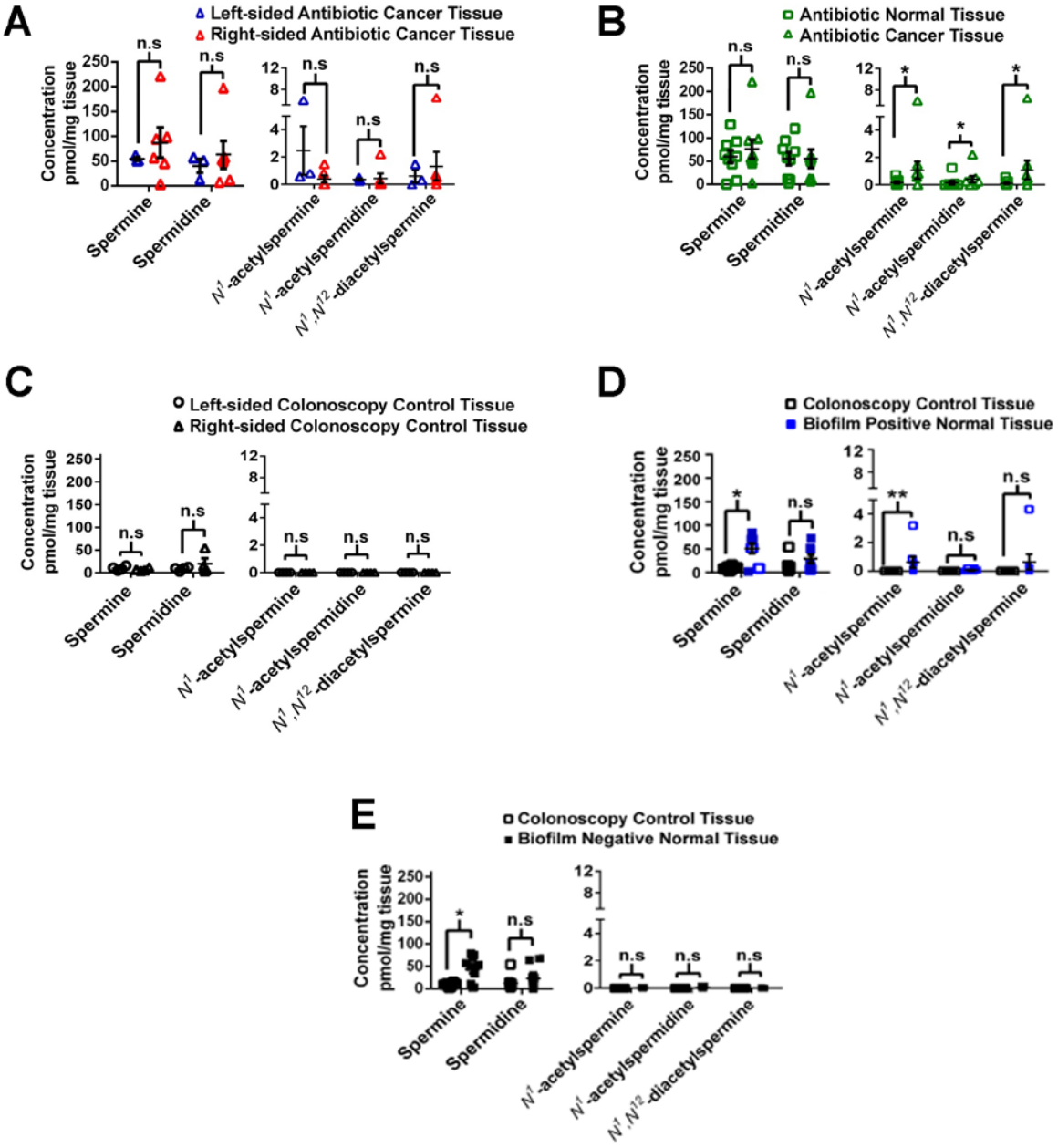
A
Left-sided Antibiotic Cancer Tissue
Right-sided Antibiotic Cancer Tissue
Concentration pmol/mg tissue
n.s
Spermine
Spermidine
N1-acetylspermine
N1-acetylspermidine
N1,N12-diacetylspermine
B
Antibiotic Normal Tissue
Antibiotic Cancer Tissue
*
C
Left-sided Colonoscopy Control Tissue
Right-sided Colonoscopy Control Tissue
D
Colonoscopy Control Tissue
Biofilm Positive Normal Tissue
**
E
Colonoscopy Control Tissue
Biofilm Negative Normal Tissue

Figure Legends

Figure S1. Untargeted metabolomics of biofilm positive and negative colon cancer and normal tissue pairs from colon cancer patients, Related to Figure 1. Cloud plot generated by XCMS Online. (A) Biofilm positive normal tissues compared to their paired biofilm positive cancer tissues (*n*=7 tissue pairs examined) and, (B) Biofilm negative normal tissues compared to their paired biofilm negative cancer tissues (*n*=11 tissue pairs examined).

Figure S2. Biofilm effects on metabolites in colon tissues, Related to Figure 2. Targeted metabolomics, concentrations of polyamines and polyamine metabolites in: (A) Paired biofilm positive normal and cancer tissues (*n*=8). Empty symbols indicate left-sided tissues. (B) Paired normal and cancer tissues lacking biofilms (*n*=10). (C) Unpaired normal tissues from cancer hosts with (*n*=9) or without biofilms (*n*=10). Empty symbols indicate left-sided tissues. Two-tailed paired Mann-Whitney, unpaired Wilcoxon test. Error bars are SEM, *p<0.05, **p<0.01, n.s = not significant.

Figure S3: Further comparison of antibiotic-treated cancer tissues and normal colonoscopy biopsies from healthy individuals, Related to Figure 4. Absolute concentrations of polyamines and polyamine metabolites in (A) left- and right-sided cancers (*n*=9, three left-sided, six right-sided) from antibiotic-treated colon cancer patients. (B) surgically-resected normal and cancers from antibiotic-treated colon cancer patients (*n*=9 tissue pairs). (C) left- and right-sided normal colonoscopy biopsies from healthy individuals (*n*=4 right and left colon biopsy pairs from four patients). (D) colonoscopy controls (*n*=8) and biofilm positive normal tissues (empty squares are left-sided tissues) (*n*=8). (E) colonoscopy controls (*n*=8) and biofilm negative normal tissues

(*n*=10). Two-tailed Wilcoxon matched-pairs signed rank test. Error bars are SEM, *p<0.05, **p<0.01, ***p<0.001, n.s = not significant.

Supplemental Table

Table S1. Metabolites upregulated in biofilm positive cancer samples compared to biofilm negative samples. Identified by RPLC-MS-based metabolomics, Related to Figure 1.

Fold change	*m/z*	Retention Time (min)	Identification through tandem MS	Replicated in 2nd sample set? Yes/No
6.6	468.3011	30.5	Phosphoethanolamine (17:0/0:0)*	Y
4.6	536.3622	36.2	Phosphocholine (19:1/0:0)*	N
3.1	482.3163	32.0	Lysophosphoethanolamine (0:0/18:0) *	N
2.7	494.3161	30.9	Phosphoethanolamine (19:1/0:0)*	Y
2.6	508.3313	32.9	Phosphoethanolamine (20:1/0:0)*	N
2.5	536.3623	36.6	Phosphocholine (19:1/0:0)*	N
2.5	550.3776	37.7	Phosphocholine (20:1/0:0)*	N
2.3	548.3622	35.5	Phosphocholine (20:2/0:0)*	N
4.9	438.2907	33.6	Phosphoethanolamine (P-18:0/0:0)*	N
4.8	372.3048	34.0	Myristoylcarnitine	N
4.0	344.2738	30.1	Lauroylcarnitine	N
2.8	414.3509	40.1	Heptadecanoylcarnitine	N
3.0	400.3357	37.9	Palmitoylcarnitine	N
2.5	424.3354	36.4	Linoleyl/Linoelaidylcarnitine	N
2.5	426.3509	39.0	Eladic/Vaccenylcarnitine	N
2.4	232.1504	3.4	Isobutrylcarnitine	N
2.1	428.3663	42.5	Stearoylcarnitine	Y
11.3	344.2559	37.5	Unknown/fatty acid backbone	N
10.9	401.2762	40.9	Fatty acid fragment	N
8.2	370.2715	38.5	Unknown/ fatty acid backbone	N
5.2	331.1997	20.6	Unknown	N
2.1	137.0434	11.7	Hypoxanthine	N

*exact double bond location unknown

Supplemental Experimental Procedures

Materials. Spermine, spermidine, N^1-acetylspermine and N^8-acetylspermidine were purchased from Sigma Aldrich (St. Louis, MO). N^1-acetylspermidine was obtained from Frank J. Gonzalez, National Cancer Institute, Bethesda, MD. N^1, N^{12}-diacetylspermine dihydrochloride and [U-^{15}N]-N^1, N^{12}-diacetylspermine dihydrochloride were obtained from Masao Kawakita, Tokyo Metropolitan Institute of Medical Science, Toyko, Japan, respectively. All other chemicals were of the highest chemical grade and purchased from Sigma Aldrich.

Sample collection at the Johns Hopkins Hospital. Colon cancers and paired histologically normal tissues were collected from patients undergoing surgery. Tumor and normal tissues not required for pathologic diagnosis were preserved in Carnoy's fixative or snap frozen in liquid nitrogen for analysis. Patients who received pre-operative radiation, chemotherapy or had a personal history of CRC were excluded. Pre-operative intravenous antibiotics were administered in all cases (cefotetan or clindamycin/gentimycin). A subset of patients received oral antibiotics (neomycin and erythromycin) the day prior to surgery. Healthy control patients undergoing screening colonoscopy were recruited and signed informed consent. All patients underwent a standard mechanical bowel preparation**.** Mucosal biopsies from grossly normal colon were taken from the ascending (right) and descending (left) colon during the colonoscopy. Mucosal biopsies were rapidly preserved in Carnoy's fixative or snap frozen in liquid nitrogen for analysis. Patients who had a personal history of CRC, inflammatory bowel disease or were treated with antibiotics within the past three months were excluded. This study was approved by the Johns Hopkins Institutional Review Board.

Sample collection at Karolinska University Hospital. Colon cancers and paired histologically normal tissues were collected from patients undergoing surgery. Tumor and normal tissues not required for pathologic diagnosis were snap frozen in liquid nitrogen for analysis. Patient exclusion criteria were as for the Johns Hopkins patients. Only two of the patients from the Karolinska University Hospital were treated with preoperative bowel preparation. Prophylactic per-oral sulfamethoxazole/trimethoprim and metronidazole was given preoperatively. This study was approved by the regional ethical board at the Karolinska Institutet, Stockholm.

Fluorescent in situ hybridization (FISH) analysis. The universal bacterial probe, EUB338 (5'GCTGCCTCCCGTAGGAGT3'), and nonsense probe NON338 (5'ACTCCTACGGGAGGCAGC), were synthesized and conjugated at the 5' end to Cy3 (Eub338) or Alexa488 (NON338) (Invitrogen Life Technologies). Universal probe was applied to 5 μm thick Carnoy's-fixed paraffin-embedded tissue sections. The nonsense probe, NON338, was also applied to test for nonspecific binding. Successive sections were stained with Periodic acid Schiff (PAS) to confirm mucus presence and preservation.

Slides were de-waxed following standard procedures and subjected to 10 minutes of 10 mg/ml lysozyme in Tris buffer, followed by three rinses in Tris buffer. Eub338 oligonucleotide probe was applied to slides at a concentration of 2 pmol/ul in prewarmed hybridization buffer (900 mM NaCl, 20 mM Tris pH 7.5, 0.01% SDS, 20% formamide). Slides were incubated at 46°C in a humid chamber for 2 hours, and washed at 48°C for 15 minutes in wash buffer (215 mM NaCl, 20 mM Tris pH 7.5, 5 mM EDTA). Coverslips were mounted using ProLong Gold antifade reagent (Life Technologies) and slides were imaged using a Nikon E800 and imaged NIS elements software.

Untargeted metabolomics. For each sample, 10 mg was weighed and added to 1.5 mL centrifuge tubes containing 600 μl ice cold acetone. Samples were vortexed for 30s, snap frozen in liquid nitrogen for 1 min, thawed for 3 min and sonicated for 15 min at 50^{o}C. Freeze-thaw cycles were repeated two more times before storing samples at -20^{o}C for 1 h. Samples were centrifuged at 13,000 rpm for 15 min and the supernatant transferred to a new 1.5 mL centrifuge tube for storage at -20^{o}C. The pellet was resuspended in 400 μL ice cold methanol/water/formic acid (86.5/12.5/1.0 v/v/v), vortexed for 30s and sonicated for 15 min at 50^{o}C. The pellet samples were stored at -20^{o}C for 1 h followed by centrifugation (13,000 rpm for 15 min). The supernatant was pooled with the supernatant collected earlier and dried down in a Speedvac for 4h. The samples were resuspended in 100 μl acetonitrile/water/isopropanol (50/40/10 v/v), sonicated for 5 min at 50^{o}C and stored at 4^{o}C for 1h. The samples were finally centrifuged at 13,000 rpm for 15 min and the supernatants transferred to glass HPLC vials for LC/MS analysis. These methods recover both hydrophobic and hydrophilic metabolites from the samples.

Samples were then randomized and analyzed by high performance liquid chromatography-electrospray ionization quadrupole time-of-flight mass spectrometry (HPLC-ESI-QTOFMS). Samples were analyzed by reversed-phase LC (RPLC) and HILIC analysis as previously described (Ivanisevic et al., 2013). The data were processed using XCMS Online (Tautenhahn et al., 2012). It reports integrated areas of each detected peak in individual samples and calculates the Welch's t test for two sample groups. For this study paired and unpaired non-parametric tests were carried out (Wilcoxon Rank Sum and Mann-Whitney). Features were listed in a feature list table and as an interactive cloud plot, containing their integrated intensities (extracted ion chromatographic peak areas) observed fold changes across the two sample groups, and *p*-values for each sample. Integration of METLIN to XCMS Online allowed for putative identification of

metabolites. Identifications were then made by comparing retention time and tandem MS fragmentation patterns to the sample and a standard compound. **Tandem MS experiments** were carried out with the collision energy set to 20 eV and caused the fragmentation of the metabolites into a number of fragments specific for the metabolite. This fragmentation pattern combined with the retention time comparison to a standard allows for accurate identification. The full dataset is available as a public share on XCMS Online.

Targeted metabolomics of polyamines. Samples (8 μL) were injected onto a Scherzo SM-C18 column 150 x 0.5 mm 3μm (Imtakt, Philadelphia, PA) using an Agilent Technologies series 1200 HPLC with a gradient mobile phase of 5 mM ammonium acetate (solution A) and 50 mM acetate and acetonitrile (50/50 v/v) (solution B) at a flow rate of 20 μL/min: 2% B for 5 min, to 17% B at 11 min, to 98% B at 13.5 min, held for 5 min at 98% B then re-equilibration at 2% B. Targeted analysis for quantitation of the polyamines were measured by using the above column conditions and selected reaction monitoring triple quadrupole mass spectrometry (Agilent 6410 QqQ-MS). The following quantifier and qualifier transitions were used for each metabolite: spermine: 203.2 -> 112.1, 203.2 -> 129.1; spermidine: 146.2 -> 112.1, 146.2 -> 72.1; N^1-acetylspermine: 245.2 -> 100.1, 245.2 -> 112.1; N^1-acetylspermidine: 188.2 -> 100.1, 188.2 -> 72.1; N^8-acetylspermidine: 188.2 -> 114.1, 188.2 -> 72.1; N^1, N^{12}-diacetylspermine: 287.2 -> 100.1, 287.2 -> 171.1. The fragmentor voltage and collision energies were as follows: spermine: 110 V, 6 V (quantifier), 18 V (qualifier); spermidine: 107 V, 10 V (quantifier), 14 V (qualifier); N^1-acetylspermine: 119 V, 18 V (quantifier), 18 V (qualifier); N^1-acetylspermidine: 107 V, 14 V (quantifier), 18 V (qualifier); N^8-acetylspermidine: 119 V, 14 V (quantifier), 22 V (qualifier); N^1, N^{12}-diacetylspermine: 113 V, 14 V (quantifier), 22 V (qualifier). Concentrations were expressed as mean ± S.E.M after two-tailed paired and unpaired non-parametric tests were carried out

(Wilcoxon Rank Sum and Mann-Whitney *U* test) using GraphPad Prism v 6.00 (GraphPad Software, Inc, San Diego, CA). Comparisons with $p < 0.05$ were statistically significant and noted on each graph.

NIMS analysis. P-type silicon wafers, 500 to 550 μm thick with 0.01 to 0.02 Ω cm resistivity (Silicon Quest International, Santa Clara, CA) were cut into 33 mm^2 pieces. The wafers were soaked in Piranha solution (sulfuric acid and hydrogen peroxide (2:1)) overnight, washed thoroughly with nanopure water and then dried using nitrogen gas. Etching was carried out by clamping the wafer in a Teflon chamber. Gold foil was used for the anode and a platinum loop as the cathode; a 25% ethanolic hydrogen fluoride solution was then added to the chamber. A BIO-RAD PowerPack1000 (Hercules, CA, USA) was connected and run at a constant-current mode (300 mA) for 30 minutes. The etched wafers were washed in methanol and evaporated to dryness using nitrogen gas. Bis(heptadecafluoro-1,1,2,2-tetrahydrodecyl)tetramethyldisiloxane (Gelest, Morrisville, PA, USA) (100 μL) was applied to the surface of the chip and allowed to sit at room temperature for 1 h before using nitrogen gas to remove excess from the surface. Tissue-Tek® Optimal Cutting Temperature (OCT) medium (Sakura Finetek, Torrance, CA) -embedded cancers were cut to 1-2 μm slices using a microtome and placed on top of the NIMS surface. A consecutive slice (5 μm) was taken, applied to a Superfrost Plus microscope slide (Fisher Scientific, Signal Hill, CA) and stored at $^{-}80^{o}C$ for histology. The NIMS chip was transferred to a room temperature vacuum for drying. Tissues were visibly dry within one minute.

Hematoxylin and eosin staining. Microscope slides containing frozen tissue sections were fixed in 95% then 70% ice cold ethanol for 2 min each before fully hydrated in water for 5 min. The slides were then placed in Gill's hematoxylin (Sigma Aldrich) for 2 min, washed in water for 5

min, placed in Scott's solution (2.0 g sodium bicarbonate, 20 g magnesium sulfate in 1 liter of water (Sigma Aldrich) for 3 min, and finally washed in water for 5 min. The slides were then dipped three times in eosin (Sigma Aldrich), washed and dehydrated in 70%, 95% and then 100% ice cold ethanol for 1 min each. Slides were washed in safe-clear II (Fisher Scientific) for 5 min before mounting with Permount (Fisher Scientific).

SSAT immunohistochemical staining. Immunohistochemistry was performed on tissue fixed in 10% formalin and paraffin-embedded following standard procedures. Sections were de-paraffinized and rehydrated through a xylene, ethanol-water gradient. Antigen unmasking was performed by steaming in high temperature target retrieval solution (Target Retrieval Solution, Dako, Carpinteria, CA) for 45 minutes. Endogenous peroxidase activity was quenched by incubation with peroxidase block for 5 minutes at room temperature. Slides were incubated with SSAT (1:6000) overnight at 4°C. A horseradish peroxidase-labeled polymer (PowerVision, Leica Microsystems, Buffalo Grove, IL) was applied for 30 minutes at room temperature. Signal detection was performed using 3, 3'-diamino-benzidine as the chromagen. Slides were counterstained with hematoxylin, dehydrated and mounted with glass coverslips using Permount (Fisher Scientific). HCT116 cells served as an assay positive control.

Global isotope metabolomics. HT-29 cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). They were seeded at a density of 5 million cells per 100 mm plate in complete growth medium consisting of McCoys 5A medium (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated Fetal Bovine Serum, 100 I.U./ml penicillin and 100 μg/ml streptomycin. At 70% confluence the cells were dosed with 0 μM, 10 μM, 50 μM or 100 μM $^{14}N^{1}$, $^{14}N^{12}$-diacetylspermine or $^{15}N^{1}$, $^{15}N^{12}$-diacetylspermine in McCoys 5A medium in triplicate for 24 h. The cells were harvested by removing the media,

washing twice in PBS and adding 0.5 ml HPLC water on dry ice. The cells were scraped and washed off the plate with additional 1 ml HPLC water and collected into glass high recovery vials (Agilent Technologies, Santa Clara, CA). The samples were sonicated for 15 min, frozen in liquid nitrogen and left to thaw. This was repeated two more times. The samples were divided into three vials and 2 ml methanol added, they were vortexed for 30s and stored overnight at -20^{o}C. After storage the samples were centrifuged for 15 min at 13,000 rpm at 4^{o}C. The supernatant was collected and lyophilized in a speedvac. The samples were resuspended in 150 µl acetonitrile/water (50/50 v/v), sonicated for 10 min, centrifuged for 15 min at 13,000 rpm at 4^{o}C and transferred to autosampler vials ready for analysis. Three technical replicates were acquired for each sample by HPLC-ESI-QTOFMS as for the untargeted metabolomics method described above. The data was processed using isoXCMS as previously described (Huang et al., 2014). Parameters were as follows: isotopeMassDiff =0.99703, RTwindow=10, ppm=25, massOfLabeledAtom=14.003, noiseCutoff=8000, alpha=0.05

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