Therapeutic potential of FLANC, a novel primate-specific long non-coding RNA in colorectal cancer

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METHODS

Patient samples

Four different patient cohorts were studied with the staging performed by Dukes' classification: cohorts A and B for the evaluation of FLANC expression in CRC tissue compared with matched normal colon, and two larger CRC cohorts C and D for testing the prognostic value of FLANC. The cohort A included 31 Croatian patients (14 men and 17 women) with mean age of 64.6 years (range between 41 and 82 years) diagnosed in 2002 and 2003. Samples were obtained from Croatian Human Tumor Bank [1]. Samples of resected CRC and normal adjacent mucosa (>15 cm from the tumor) were collected during a routine surgery, snap-frozen in liquid nitrogen and stored at -80°C until further use. Patients did not receive preoperative radiotherapy and chemotherapy. The cohort B included 25 colon samples and 25 adjacent normal mucosae collected between 2003 and 2008 at the Department of Experimental and Diagnostic Medicine, University of Ferrara, Italy, as previously described [2]. The screening cohort C (n = 170 CRC samples) was obtained from the Baylor Research Institute in Dallas, Texas, US (**online supplementary table 1** and **2**). For this cohort, the surgical samples were collected from the Department of Gastrointestinal and Pediatric Surgery at Mie University Graduate School of Medicine, Japan, between 2005 and 2011. The samples were preserved immediately after surgical resection in RNA later (QIAGEN, Chatsworth, CA) and stored at -80 °C until RNA extraction. All CRC patients who underwent surgery were followed up for tumor recurrence at regular intervals for up to five years with physical examination and tumor marker assays (CEA, CA19-9) performed every 1-3 months and computed tomography performed every 6 months. Patients treated with radiotherapy or chemotherapy before surgery were excluded from this study. Patients with stage III/IV disease received 5-fluorouracil-based chemotherapy, whereas no adjuvant chemotherapy was given to stage I/II patients. The validation cohort D, was obtained from the Department of Comprehensive Cancer Care, Masaryk Memorial Cancer Institute, Czech Republic (n=126) (**online supplementary table 1** and **3)**. The patients' clinico-pathological data were collected from medical records at the same institutions. All cases were reviewed based on pathology reports and histological slides. The Tumor Node Metastasis (TNM) staging system from the American Joint Committee on Cancer was used for the classification of pathological tumor staging of CRC. Patients were treated by standard surgical procedures and received adjuvant treatment when appropriate (stage II with risk factors or stage III). If advanced disease was found at the time of diagnosis, the patients received medical treatment according to a physician-choice following the recommendation

of appropriate national guidelines. Written informed consent was obtained from each patient for these four cohorts, and the studies were approved by the institutional review boards of all the involved institutions.

In vivo treatment of colorectal cancer metastases

Male athymic nude mice were purchased from Taconic Farms (Hudson, NY). All animals were 6-8 weeks old at the time of injection. Metastatic liver models of colorectal cancer were developed as described previously [3]. For all animal experiments, HCT116 cells (labelled with a stable expression luciferase gene) were harvested using trypsin-EDTA, neutralized with FBS-containing medium, washed, and re-suspended in appropriate cell numbers in Hanks balanced salt solution (Gibco, Carlsbad, CA) prior to intra-splenic injection. For all therapeutic experiments, the dose of FLANC siRNA was 200 µg/kg, as described previously [4]. The oligos were incorporated into neutral 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) nanoliposomes. These were administered via intravenous injection twice weekly beginning two weeks after tumor cell injection and continued for 4 weeks. Mice in any group were sacrificed when they became moribund by the cervical dislocation method. They were subjected to necropsy after death and tumors were harvested. Tumor weight and number and location of tumor nodules were recorded. Liver samples with metastatic tumor nodules were either, formalin fixed and paraffin embedded, or frozen in optimal cutting temperature (OCT) medium, to prepare slides for microscopy. The animals were cared for according to the guidelines of the American Association for Accreditation of Laboratory Animal Care and the US Public Health Service policy on Humane Care and Use of Laboratory Animals. All mouse studies were approved and supervised by MD Anderson Cancer Center (MDACC) IACUC.

In situ hybridization

The tissue sections were first digested with 5 µg/mL proteinase K for 5 min at room temperature and then loaded onto a Ventana Discovery Ultra system (Ventana Medical Systems, Inc, Tucson, AZ, USA) for *in situ* hybridization (ISH). The tissue slides were incubated with double-DIG labeled custom LNA probe for FLANC (5DigN/ATAGACACGTGCTTTGTTTAGT/3Dig_N/Exiqon) for 2 hours at 55 °C. The digoxigenins were detected with a polyclonal anti-DIG antibody and Alkaline Phosphatase conjugated second antibody (Ventana) using NBT-BCIP as the substrate. The double-DIG labeled control U6 snRNA probe was also from Exiqon.

Tissue microarray and image analysis

The tissue microarray was purchased from US Biomax, Inc (Cat # BC05002a). To quantify the levels of FLANC by *in situ* hybridization of tissue microarray, images of each tissue core were automatically captured using a PerkinElmer Caliper Vectra 2 microscope and then analyzed using inForm 2.0 image analysis software (Perkin Elmer, Inc., Waltham, MA, USA). In particular, the quantification of FLANC was automatically calculated as mean intensity measured within the tumor tissue (adenocarcinoma and metastatic), normal tissue, benign/polyp tissue, and colitis tissue. Non-epithelial tissue (e.g. stromal tissue) was excluded from the analysis. We excluded individual TMA cores, when they did not have enough tissue (epithelial versus non-epithelial tissue) for inForm 2.0 image analysis. Both image acquisition and analysis were performed at the North Campus Flow Cytometry and Cellular Imaging Core Facility at The University of Texas MD Anderson Cancer Center (UT-MDACC, Co-director: Jared K. Burks, Department of Leukemia) as previously described [5 2].

RNA extraction and qRT-PCR

Total RNA from both tissues and cell lines was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and DNase-digestion (TURBO DNA-free, Thermo Fisher Scientific) according to manufacturers' instructions. RNA from nuclear and cytoplasmic compartment was isolated using Ambion's Protein and RNA Isolation System, PARIS™ Kit (ThermoFisher Scientific). Total complementary DNA (cDNAs) was reverse transcribed using SuperScript III cDNA kit (Invitrogen) with random hexamers, according to the manufacturer's protocol. For the sense-specific cDNA synthesis, either the forward or reverse primer of FLANC was used instead of random hexamers]. For the quantification of FLANC, either specific primers (Fw: 5-GCAGGCAGATCACCTGAAAT-3´ Rv: 5´- AGAGTCACTGTCGCCCAAAC-3´) or customized TaqMan Gene Expression Assay (FLANC: ABI assay ID: AIVI4Y6, housekeeping gene U6 snRNA assay ID: 001973) probe were used. We carried out qRT-PCR and then products were loaded on 3% agarose gels. Only primers that generated amplicons as single clear bands and good melting curves were selected and products were confirmed by sequencing. U6 snRNA or GAPDH were employed as endogenous controls if not otherwise stated. The 2–ΔCt method was used to calculate the relative amount of each transcript compared with expression of endogenous control (U6 and GAPDH). If expression values for the RNA of interest were not obtained after 35 cycles of amplification in two successive experiments in duplicate wells, then the specific values were considered not detectable.

Cell Lines and Cell culture

All cell lines were purchased from ATCC and cells were cultured in the recommended media of the supplier at 37°C in 5% CO_2 if not otherwise specified. All cell lines used in this study were validated by STR DNA fingerprinting using the AmpF/STR Identifiler kit, according to manufacturer instructions (Applied Biosystems). The STR profiles were compared with known ATCC fingerprints (ATCC.org), to the Cell Line Integrated Molecular Authentication database version 0.1.200808 (http:// bioinformatics.istge.it/clima/), and to the MD Anderson fingerprint database (**online supplementary table 4**)

Cloning

We used the GeneRacer kit (Invitrogen) to carry out the rapid amplification of cDNA ends (RACE) method for FLANC. The kit was used in accordance with the manufacturers' protocols. We obtained cDNA from DNase treated total RNA from HCT116 cell (2 µg). The 5′- and 3′-RACE products were cloned into pCR4-TOPO (Invitrogen) and transformed into E. coli TOP10 cells. Cloned RACE products were fully sequenced in both directions.

***In vitro* translation assay**

The *in vitro* translation assay was performed using TnT® T7 Quick Starter Bundle Chemiluminescent (CatNo. L1210, Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, reaction components including TNT® T7 Quick Master Mix, Methionine, plasmid DNA template (pcDNA3.1 empty vector or luciferase T7 positive control vector or pcDNA-FLANC vector) and Transcend™ Biotin-LysyltRNA were incubated at 30 °C for 90 min. Once the 50 µL translation reaction was complete, 1 µL aliquot was added into 15 µL of SDS sample buffer, heated at 90–100 °C for 2 min, loaded on an SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane using a semi-dry system. The Transcend™ Non-Radioactive Translation Detection Systems (Cat No. L50811, from Promega, Madison, WI, USA) was used for the detection of proteins synthesized *in vitro* according to the manufacturer's instructions. Additionally, the luciferase activity in the positive control was verified with a luciferase assay measured with a microplate luminometer.

***In silico* analysis of open reading frame and genomic evolution**

Open reading frame (ORF) analysis was performed using the CLC Genomic Workbench 8.5.4. Analysis was performed in the sense

direction of the gene, including analysis of ORFs staring with AUG (Sup Figure 3B) as well as for ORFs with any combination of three bases that is not a stop-codon as a start-codon (data not shown).

Genomic conservation was analyzed by downloading Multiz Alignments of 100 Vertebrates from the UCSC Genome Browser (GRCh38/hg38 Assembly, chr22:46,492,389-46,493,270). Sequence identity was determined using the CLC Genomic Workbench 8.5.4. A phylogenetic tree was generated (Algorithm = UPGMA, Distance measure = Jukes-Cantor, Bootstrap = 100 Replicates), and identity was calculated by summarizing the distance from Homo sapiens.

Proteomics identification of small peptides

The HCT116 empty and HCT116 FLANC cells were lysed with 30mM Tris (pH 8.0), 4M urea, 3% OG, 3% isopropanol and approximately 2mg amount of proteins were extracted. The lysates were reduced with TCEP, alkylated with acrylamide and passed through Amicon Ultra centrifugal filter 30,000 NMWL (EMD Millipore) and the peptides were separated using an off-line 1100 series HPLC system (Shimadzu, Kyoto, Japan) with reversed phase column (4.6 mm ID × 150 mm length, Column Technology Inc, Fremont, CA, USA). Peptides were fractionated with a 41 min elution program at a flow rate of 2.1 ml/min: 0–2% B (0-5 min), 2–35% B (5-30 min), 35-50% B (30-33 min), 50-95% B (33-35 min), 95% B (35-37 min), 95-2% B (37-37.5 min), 2% B (37.5-41 min). Mobile phase A: 5% acetonitrile, 95% H_2O, 0.1% trifluoroacetic acid (TFA); Mobile phase B: 5% H2O, 95% acetonitrile, 0.1% TFA. The fractionated peptides were collected in 96 consecutive fractions and pooled into 7 fractions, lyophilized and subsequently re-suspended in 0.1% Formic acid/2% acetonitrile.

The peptides were separated by reversed-phase chromatography using an EASY*nano* HPLC system (Thermo Scientific) coupled online with an Orbitrp ELITE mass spectrometer (Thermo Scientific). Mass spectrometer parameters were spray voltage 2.5 kV, capillary temperature 280 °C, FT resolution 60,000, FT target value 3×10^6, HCD target value $1x10^5$, 1 FT microscan with 30 ms injection time, and 1 HCD microscan with 100 ms injection time. Mass spectra were acquired in a data-dependent mode with the *m*/*z* range of 300-4,000. The full mass spectrum (MS scan) was acquired by the FT and tandem mass spectrum (MS/MS scan) was acquired by the HCD with a 40% normalized collision energy. Acquisition of each full mass spectrum was followed by the ten acquisition of MS/MS spectra without charge state exclusion.

The acquired LC-MS/MS data was processed by the PEAKS Studio (Bioinformatics Solutions Inc). The search parameters of the

PEAKS allowing 10 ppm error for parent mass, 0.02Da error for MS2, and methionine (Met) oxidation (15.99491@M) as a variable modification and acrylamide (71.03714@C) as a fixed modification with no enzyme digestion. Data was primarily searched against the Uniprot Human database 2017 which consists of 92,947 protein accessions and further added de novo peak assignment. The identified peptides were filtered with False Discovery Rate (FDR) ≤1%.

Transfection and siRNA experiments

We designed siRNAs against FLANC using the Dharmacon algorithm (Dharmacon siDESIGN http://www.dharmacon.com/sidesign/). Each of four highest-ranking siRNA sequences for FLANC were tested in our experiments. These siRNAs were re-suspended in 1X siRNA buffer (Dharmacon, LaFayette CO, USA) to a stock concentration of 50 µM. The performance was assessed at 48 to 96 hours intervals post-transfection by qRT-PCR. The cells were transfected with the respective siRNA at the final concentrations of 50 nM by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For the final experiments, we selected the two siRNAs with the highest knock-down efficacy (FLANC siRNA#1 sense sequence: CGGCCGAAACCCAGGAGGA, FLANC siRNA#2 sense sequence: GGACAGAAAUUACCUCUUU). As control, we used a scrambled control of non-targeting siRNAs (Dharmacon), as positive control we used the Allstar cell death control (Qiagen, Hilden, Germany).

FLANC shRNAs and overexpressing stable clones establishment

FLANC shRNA stable clones were constructed using two different shRNAs which were cloned into a pGreen Puro lentivector (SI506A-1). The constructed vectors and empty lentivectors were transfected into HEK293 cells. Media was collected in days 1 and 2 after transfection. This virus supernatant was filtered through 0.45-femto/m pores and transferred to HCT116 cells. Two to five days after transduction, strong green-fluorescence (GFP) signal indicated positive cells which were additionally selected in puromycin-containing media (2 µg/mL), and the expression level of FLANC was tested by qRT-PCR after 3 to 4 passages.

For construction of lentiviral overexpressing vector, the mono-exonic human FLANC was PCR-amplified by Pfu Ultra II Fusion HS DNA Polymerase (Stratagene, Agilent Technologies) from commercial Human Genomic DNA and subcloned into the XbaI and NotI sites of pCDH-CMV-MCSEF1-puro lentiviral vector. Following infection, the positive transfected cells were selected by puromycin-containing (2 µg/mL) media for one to two weeks. For the purpose of *in vivo* imaging by using luciferase-labelled cells, HCT116 cells were seeded in 12-

well plates 24 hours prior to viral infection and incubated overnight in 2 ml of complete growth medium containing 10% FBS and 1% antibiotics. On the day of transfection, the medium was removed and 2 ml of complete growth medium containing 8 µg/ml polybrene (Santa Cruz Biotechnology, Santa Cruz, CA) and 5 µl of ViralPlus Transduction Enhancer (ABM, Richmond, BC, Canada) were added. Then, cells were infected by adding a Lentiviral Dual Reporter Imaging Construct (CMV-RFP-T2A-Luciferase, Biocat. Heidelberg, Germany) and grown up for 2-3 weeks before the *in vivo* experiment was initiated.

Cellular growth, colony forming, soft agar and tumor sphere formation assays

To test the influence of FLANC on cellular growth, we measured the short-term effects (up to 120 hours at 24 hours interval) by applying cell counting kit 8 CCK, (Dojindo Molecular Technical, Inc.). Cells were seeded at a density of 3000 to 5000 cells per well in a 96-well plate in six technical replicates. We used transient transfection protocols with siRNAs [50µM] according to the manufacturer protocols (Lipofectamin 2000, Invitrogen). Stable lentiviral transduced FLANC shRNA or overexpression cells were also seeded in 96-well plates. Cells were incubated from 24-120 hours and every 24 hours the CCK8 proliferation reagent was added in the wells according to the manufacturer's recommendations. Colorimetric changes were measured using a SpectraMax Plus (Molecular Devices, Germany) at a wavelength of 450 nm with a reference wavelength at 620 nm. Three independent biological replicates were performed each. To confirm the cellular growth changes by a second independent method, a clonogenic assay (i.e. colony formation assay) was performed in 6-well plates. Transient or stable transfected cells were trypsinized 24 hours after transfection and seeding. After trypsinization, cells were counted and seeded for colony formation assay in 6-well plates at 100-500 cells/well, depending on the cell line. Cells were cultured at 37°C and 5 % CO2 and after 10-21 days, cells were fixed as well as stained with 0.01 % (w/v) crystal violet (Sigma-Aldrich) in 20 % methanol and PBS. The number of colonies was counted and each experiment was carried out in biological and technical triplicates.

The efficiency of colony formation under anchorage-independent growth conditions (soft agar assay) was determined by plating 2,500 cells in complete growth medium containing 0.35 % low gelling temperature agarose (Sigma-Aldrich) over 2 ml of growth medium containing 0.5 % agar (Sigma-Aldrich) in a 35 mm dish (6-well plate). Cells were cultured at 37°C and 5 % CO2 for up to 4 weeks. Colonies were stained with 0.005 % crystal violet (Sigma-Aldrich) in 20 % methanol and the number of colonies was counted.

To assess the effect of FLANC expression on the self-renewal capacity (tumor sphere formation), we performed a spheroid growth model as previously described [6]. In detail, the adherent growing CRC cell lines were dissociated into single cells using trypsin/EDTA and 2,000 single cells per well were seeded in ultra-low attachment 6-well plates (Corning, NY, USA) using serum-free MEBM (Lonza, Basel, Switzerland) medium (SFM). SFM was supplemented with 1xB27 supplement (Gibco), 20 ng/ml human epidermal growth factor EGF (Peprotech, Hamburg, Germany), 10 ng/ml human basic fibroblast growth factor FGF (Peprotech), 20 IU/ml Heparin (Baxter, Vienna, Austria) and 1% antibiotic/antimycotic solution (Sigma-Aldrich). Tumor spheres were observed and counted under a microscope 10 days later. Three independent experiments per cell line with each three technical replicates were performed.

EdU Cell Proliferation Assay

HCT116 stably overexpressing FLANC and empty vector control cells were plated at 1×10^6 cells/dish in 60mm cell culture dishes. After 48 hours cells were labeled with 20 μM 5-ethynyl-2′-deoxyuridine (EdU) for 2 hours, then stained using a Click-iT™ EdU Alexa Fluor™ 488 Flow Cytometry Assay Kit (Invitrogen, C10425), according to the manufacturer's instruction. EdU positive cells were analyzed by flow cytometry.

Migration assay

Cell migration assays were performed according to the protocol described previously [2]. After 48 hours of transient siRNA FLANC knock-down, cells were re-suspended in serum free media (50000 cells/insert) and seeded onto a 0.1% gelatin-coated inserts. After 24 hours, cells that migrated to the bottom of the wells were fixed and stained with HEMA 3™ (Fisher Scientific, MA, USA) and counted by microscope. For each well, ten random fields were counted and the average number of cells was determined. The experiments were performed in triplicate.

Apoptosis assay by imaging flow cytometry

For multiparametric apoptosis evaluation, FLANC siRNA transfected HCT116 cells resuspended in McCoy 5.5A media were stained with 5 different dyes, following a protocol developed by the South Campus Flow Cytometry and Cell Sorting Facility at MD Anderson Cancer Center. Briefly, cells were labeled with 2mM cell event caspase 3/7 green detection reagent (Invitrogen) for 30 min at 37°C to measure

caspase 3 and 7 activation, following manufacturer's protocol. Cells were then washed and resuspended in Annexin binding buffer (BD Pharmingen). Annexin V Alexa Fluor 594 (AnV AF594) (Invitrogen) was added, and cells were incubated 25 min at room temperature to quantify phosphatidylserine inversion. Upon incubation, cells were washed with cold PBS and resuspended in binding buffer. Cells were then treated with 50nM TMRE (Invitrogen) for mitochondrial membrane depolarization; Sytox blue (Invitrogen) for membrane permeability, and 1µM Draq5 (eBiosciences) for chromatin condensation. Cells were incubated for 5 min before data acquisition. For apoptosis evaluation, untreated and 1µM staurosporine-treated cells were used as negative and positive controls, respectively. Imaging flow cytometry (IFC) was performed using an ASSIST-calibrated ImageStreamX MarkII system (Amnis Corporation), equipped with a 60x objective, 2 CCD cameras and 5 lasers. Data were acquired by using INSPIRE software (Amnis Corporation). An acquisition region R1 was created based on aspect ratio and area of the bright field (BF) channel (Ch 1) and 10.000 cells were recorded. Cell event caspase reagent was excited with a 488nm laser and emission detected at a 505-560nm band (Ch 2) using a multi-spectral decomposition unit; a 561nm laser was used for TMRE (Ch 3, 560-595 band) and AnV Alexa Fluor 594 (Ch 4, 595-640 band), a 405nm laser and 430-505 band for Sytox Blue (Ch 7) and a 642nm laser and 642-745nm band for Draq5 (Ch 11). SCC signal was detected with a 785nm laser and 745-780 band (Ch 12), and bright field illumination provided by a white light lamp and detected on Ch 1 and Ch 9. IDEAS software (Amnis Corporation) was used for analysis. Gating on the high root mean square values of BF images was used to identify focused images, gating of focused cells with intermediate BF area and BF aspect ratio close to 1 was used to identify single cells and gating on Raw Max Pixel in Ch 7 to remove saturated events. Single color controls were used to correct for spectral overlap between fluorochromes. Further gating was performed to determine percentage of negative and positive cells for Cell Event (Caspase), TMRE, AnV AF594 and Sytox Blue, based on fluorescence intensity. Data are expressed as percentage of cells. The percentage of cells displaying chromatin condensation was calculated based on Draq 5 intensity and width. False positives were identified and subtracted based on bright detail intensity and X, Y delta centroid for Ch4, as well as TMRE positivity.

Apoptosis assay by conventional flow cytometry (CFC)

FLANC siRNA transfected HCT116 cells were resuspended in McCoy 5.5A media and were stained following the protocol described before. CFC was performed on a CS&T-calibrated LSR Fortessa X-20 (Becton Dickinson), equipped with 5 lasers and 18 filter sets. Data were acquired with FACSDiva software (Becton Dickinson) and 10.000 events were recorded. Cell event caspase reagent was excited with

a 488nm laser and its emission detected with a 530/30 band pass filter. A 561nm laser was used to excite TMRE and AnV Alexa Fluor 594 and emissions were collected at 586/15 and 610/20, respectively. A 405nm laser and a 450/50 filter were used for Sytox Blue and a 642nm laser and a 670/30nm filter for Draq5. FlowJo software (FLOWJO, LLC) was used for data analysis. Cells were gated based on forward (FSC) and side scatter (SSC) and on area and height of the FSC pulse for single cell discrimination. Further gating was performed to determine percentage of cells negative and positive for TMRE, Caspase, AnV AF594 and Sytox Blue, based on fluorescence intensity. Single-color controls were used to correct for spectral overlap, and untreated or 1uM staurosporine-treated cells were used as controls. Data were presented as percentage of cells.

Apoptosis evaluation by caspase 3/7 assays

Caspase-Glo 3/7 assay (Promega, Madison, WI, USA) was applied to measure the activity of caspases 3 and 7 according to the manufacturer's instructions. For transient transfection experiments, 5000-10.000 cells were seeded in 96-well plates and transfected using the reverse transfection protocol (Lipofectamin 2000, Qiagen) in five technical replicates. Caspases 3 and 7 activities were measured 24-72 hours after transient transfection. Lentiviral stable-transduced cells were also seeded in 96-well plates and apoptosis was measured after 48 hours. After adding the substrate, luminescence was measured using a luminometer.

Protein coding gene expression by 44K Agilent array and data analyses

Agilent 44K two color arrays of the FLANC overexpression and two FLANK knock-down stable clones versus the corresponding empty vector control clones were performed in duplicate. The analysis was performed in R using functions of LIMMA library. Probe intensities were background corrected, log2 transformed, loess normalized within arrays and quantile-normalized between arrays. Finally, replicates spots were averaged. A linear model was then fitted to each gene and empirical Bayes methods were used to obtain the statistics. Genes were considered statistically significant if their p-value was less than 0.005 and the absolute value of the fold change was at least 1.5. Pathway analysis for the significantly differentially regulated genes in the comparisons of interest was done using the web-based tool Enrichr (http://amp.pharm.mssm.edu/Enrichr/). The threshold for significance was set up to a p-value, obtained from the Fisher exact test, of less than 0.05. Significantly enriched pathways of differentially expressed genes were represented in R. The array data was submitted to GEO and the accession numbers are GSE127785 and GSE127786.

Reverse Phase Protein Array (RPPA)

A set of 436 antibodies were used, as described in [7]. Cell lysate from HCT-116 Empty and FLANC OE were serially diluted two-fold for 5 dilutions (undiluted, 1:2, 1:4, 1:8; 1:16) and arrayed on nitrocellulose-coated slides in an 11x11 format to produce sample spots. Sample spots were probed with antibodies by a tyramide-based signal amplification approach and visualized by DAB colorimetric reaction to produce stained slides. Stained slides were scanned on a Huron TissueScope scanner to produce 16-bit tiff images. Sample spots in tiff images were identified and their densities quantified by Array-Pro Analyzer. Relative protein levels for each sample were determined by interpolating each dilution curve produced from the densities of the 5-dilution sample spots using a "standard curve" (SuperCurve) for each antibody. Relative protein levels are designated as log2 values. Normalized log-data was obtained from the MD Anderson RPPA core and processed in R (version 3.5.1). First, a logarithmic transformation using base 2 (log2(x+1)) was applied. Differential expression between Empty and FLANC OE for the cell line HCT116 was determined by a p-value, obtained from the moderated t-statistic from LIMMA package, of less than 0.05. Pathway analysis for the significantly differentially regulated proteins in the comparison of interest was done using the web-based tool Enrichr (http://amp.pharm.mssm.edu/Enrichr/). The threshold for significance was set up to a p-value, obtained from the Fisher exact test, of less than 0.05. The most significantly enriched pathways of differentially expressed genes were represented in R.

Liposomal nanoparticle preparation

FLANC siRNA for *in vivo* intra-tumoral delivery was incorporated into DOPC liposomes, as previously described [4]. DOPC and FLANC siRNA were mixed in the presence of excess tertiary butanol at a ratio of 1:10 (w/w). Tween-20 was added to the mixture in a ratio of 1:19. The mixture was subjected to vortexing, to freezing in an acetone/dry ice bath, and to lyophilization. Before *in vivo* administration, this preparation was hydrated with phosphate-buffered saline solution (PBS) at room temperature at a concentration of 200 µg of siRNA/kg per injection.

Animal models and tissue processing experiments

For subcutaneous xenografts and intra-spleen injection liver metastases models, male athymic nude mice were purchased from the NCI, Frederick Cancer Research and Development Center (Frederick, MD, USA). These animals were cared for according to guidelines by the

American Association for Accreditation of Laboratory Animal Care and the U.S. Laboratory Animals. All mouse studies were approved and supervised by the UT-MDACC Institutional Animal Care and Use Committee (IACUC). All animals used were aged six to eight weeks at the time of injection. For all the animal experiments, cells were trypsinized, washed, and re-suspended in Hanks' balanced salt solution (HBSS; Gibco) before injection. For subcutaneous and intra-splenic cancer models, 1×10^6 HTC116 cells in 50 µL HBSS per mouse were injected under sterile conditions. The mice were anesthetized under isofluorane for splenic isolation and cell line injection (day 1), as well as the following day after injection (day 2) to perform splenectomy[3]. For the subcutaneous local tumor growth model, tumor growth was monitored every seven days by caliper measurements, and animals were sacrificed before tumors reached a diameter of 10mm. Tumors were harvested for histological analyses and tumor volumes were calculated by the equation V(mm3) = (width)2 x length/2. For the intra-spleen injection model, liver metastases continued until mice in any group became moribund (approximately four to six weeks). Weekly imaging was performed using the Xenogen IVIS spectrum system within 12 min following injection of D-Luciferin (150 mg/mL). Living image 4.1 software was used to determine the regions of interest (ROI) and average photon radiance (p/s/cm^2/sr) was measured for each mouse. For all the experiments, once mice became moribund in any group, they were all sacrificed, necropsied, and livers were harvested. The number of liver metastases and location of tumor nodules within the abdominal cavity were recorded. Tumor tissue was either fixed in formalin for paraffin embedding, frozen in optimal cutting temperature (OCT) media to prepare frozen slides, or snap-frozen for lysate preparation. Cell proliferation (Ki-67), apoptosis (TUNEL assay), and angiogenesis (CD31) were assessed as previously described[4]. For VEGFA, slides were incubated with primary antibody anti-VEGFA (Rabbit monoclonal, Cat.No. ab52917, 1:100), overnight at 4 ºC. After primary antibody was washed with PBS, the appropriate amount of horseradish peroxidase-conjugated secondary antibody was added and visualized with 3,3'-diaminobenzidine chromogen and counterstained with Gill's hematoxylin #3. Ki-67, TUNEL, CD31 and VEGFA positive cells were counted in three random fields per slide and five slides per group were analyzed at 200x magnification.

Animal Model Mathematical descriptions

We modeled the tumor response to drug treatment based on a logistic ordinary differential equation (ODE) model:

$$\frac{dT}{dt} = (r - \alpha_i) \cdot (1 - \frac{T}{K}) \cdot T,$$

where T is the tumor volume, r is the tumor growth rate, α_i is the tumor inhibition rate due to drug treatment i (this parameter is 0 in the absence of treatment), and K is a constant that represents the carrying capacity of the tumor system. Note that K varies across different tumor systems and different *in vivo* animal models, but its value is not important in interpreting our model results. Hence, we set K to 2090 mm^3 according to the literature [8].

***In vivo* toxicology assessment of siRNA FLANC**

Males C57BL/6J mice purchased from MD Anderson Cancer Center Charles Rivers with a weight of 25-30g were randomized into the same three groups as above (n=10). Liposomal nanoparticles were injected into the respective mice via tail vein injection at a concentration of 200µg/Kg of body weight. Body weight was measured before and after treatment. After 72 hours of treatment, mice were euthanized by exsanguination following IACUC approved protocol. Blood samples and tissues (fixed and embedded in paraffin) were collected at necropsy for further analyses. Blood samples were processed for blood chemistry, and plasma cytokines levels analyses. Plasma cytokine levels were measured using the MILLIPLEX MAP Mouse Cytokine Magnetic Bead Panel (MCYTOMAG-70K). The analysis includes: interferon gamma (IFNγ), interleukin 4 (IL-4), IL-6, IL-10, IL-12(p40), IL-13, keratinocyte-derived cytokine (KC), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), tumor necrosis factor alpha (TNF-α) and vascular endothelial growth factor (VEGF). Paraffin-embedded tissue sections were stained with hematoxylin and eosin (H&E) stain for routine histopathology.

Tube formation assay

Human immortalized umbilical endothelial cells (EC-RF24), were grown in Minimum Essential Medium Eagle (MEM, Corning Cellgro, #10–010-CV) supplemented with 10% FBS, 1% MEM vitamins, 1% sodium pyruvate, 1% l-glutamine, and 1% nonessential amino acids. EC-RF24 cells were seeded in 6-well tissue culture plates (2×10^5 per well). Next day, cells were transfected with FLANC siRNA#1, FLANC siRNA#2 and control siRNA at 25 nM concentrations for 48 hours. The cells were next trypsinized and plated in growth factor-reduced Matrigel matrix coated 15-well µ-angiogenesis slides (ibidi, Germany) (10,000 cells/well), which were pre-incubated for 10 minutes at 37°C. Eight hours after seeding, the capillary-like structures were photographed using an Olympus IX81 inverted microscope and the number of tubes per image was quantified using Image J.

Immunoblotting

Immunoblotting was performed using lysates from whole-cell pellets samples in radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Protein was quantified by Bradford assay (Bio-Rad), equal concentration of proteins (20 ug) were loaded on 4-20% acrylamide CriterionTM TGXTM precast gels (Bio-Rad) for Western Blot analysis. Resolved proteins were semi-dry transferred to nitrocellulose membranes, which were then probed overnight with the following anti-human primary antibodies: VEGFA (Abcam, Cat. No. 51745; rabbit polyclonal, 1:1000), STAT3 (Cell Signaling, Cat. No. 9139S; mouse monoclonal, 1:3,000), phosphorylated STAT3 at Tyr 705 (Cell Signaling, Cat. No. 9145S; rabbit monoclonal, 1:1,000), β-actin (Sigma, Cat. No. A1978; mouse monoclonal, 1:10,000). Membranes were then incubated with appropriate HRP-conjugated secondary antibody, followed by incubation with SuperSignal West Femto substrate (ThermoFisher Scientific), and chemoluminescent signal detected with autoradiographic paper.

Cycloheximide chase assay

Protein half-life studies were performed as previously described [9], HCT116 empty control vector of HCT116 FLANC overexpression clones were cultured in six-well plates for 24 hours and then cultured with cycloheximide (CHX, 100μg/ml), which blocks the translation of mRNA. After culturing with cycloheximide for 0, 4, 8, and 12 hours, the cells were collected and lysed with RIPA lysis buffer for protein extraction. The degradation of STAT3 and pSTAT3 at Tyr 705 was then detected by western blotting analysis.

Statistical Analysis

Statistical analysis was performed using Medcalc version 12.3.0 (Broekstraat 52, 9030; Mariakerke, Belgium) or SPSS workpackage version 18. Differences between groups were estimated by the χ^2 test and Kruskal-Wallis test, where appropriate. Overall survival (OS) was measured from the date of histological confirmed diagnosis of colorectal cancer until the date of death resulting from any cause, or last known follow-up for patients still alive. For time-to-event analyses, survival estimates were calculated using the Kaplan-Meier analysis, and groups were compared with the log-rank test. Receiver operating characteristic (ROC) curves with Youden's Index correction[10] were used for determining optimal FLANC expression cutoff thresholds for analyzing OS. Uni- and multivariate Cox's proportional hazards models were used to estimate hazard ratios (HRs) of death. Forced-entry regression was used to include all multiple variables in order to analyze

whether each of the predictors affected the outcome after adjusting for known confounders. All p-values were 2-sided, and those less than 0.05 were considered statistically significant.

Supplementary Table 1. Summary and comparison of clinico-pathological characteristics of the colorectal cancer patients in the screening (cohort C, n = 170) and validation cohort (cohort D, n = 126).

		Screening cohort (n=170)	**Validation cohort (n=126)**
		No (%)	No (%)
Age	median	68	68
	minimum	34	18
	maximum	92	88
Gender	female	72 (42.4)	49 (38.9)
	male	98 (57.6)	77 (61.1)
Location	colon	100 (58.8)	51 (40.5)
	rectum	70 (41.2)	79 (59.5)
Grading	differentiated	153 (90)	101 (80.2)
	undifferentiated	17 (10)	23 (18.3)*
pT stage	pT1	23 (13.5)	2 (1.6)
	pT2	26 (15.2)	36 (28.5)
	pT3	102 (60)	78 (61.9)
	pT4	19 (11.2)	10 (7.9)
Vessel involvement	negative	97 (57.1)	n.a.
	positive	73 (42.9)	n.a.
Lymphatic vessel involvement	negative	42 (24.7)	n.a.
	positive	128 (75.3)	n.a.
Lymph node metastasis	N0	90 (52.9)	80 (63.5)
	N1	80 (47.1)	45 (35.7)**
Distant metastasis	M0	131 (77.1)	95 (75.4)
	M1	39 (22.9)	31 (24.6)
TNM stage	Stage I	35 (20.6)	32 (25.4)
	Stage II	51 (30)	42 (33.3)
	Stage III	45 (26.5)	21 (16.7)
	Stage IV	39 (22.9)	31 (24.6)

*2 cases missing information for grading
**1 case missing information for lymph node involvement
n.a. not available

Supplementary Table 2. Clinico-pathological variables and FLANC expression in colorectal cancer patients of cohort C (n = 170).

Variable		FLANC expression		*p*
		high (n=56)	low (n=114)	
Gender	Male	34	64	0.688
	Female	22	50	
Age (y)	<68 (median)	33	56	0.298
	≧68	23	58	
Location	Colon	31	69	0.633
	Rectum	25	45	
Histological type	Differentiated	50	103	0.957
	Undifferentiated	6	11	
Pathological T category	pT1/2	7	42	**0.002***
	pT3/4	49	72	
Vessel involvement	+	32	41	**0.014***
	-	24	73	
Lymphatic vessel involvement	+	47	81	0.101
	-	9	33	
Lymph node metastasis	N0	24	66	0.092
	N1	32	48	
Distant metastasis	M0	42	89	0.799
	M1	14	25	
TNM stage	stage I	5	30	0.06
	stage II	19	32	
	stage III	18	27	
	stage IV	14	25	
		#: Pearson's chi-square test		***** ***p*<0.05**

Supplementary Table 3. Clinico-pathological variables and FLANC expression in colorectal cancer patients of cohort D (n = 126).

Variable		FLANC expression		*p*
		high (n=85)	low (n=41)	
Gender	Male	54	23	0.423
	Female	31	18	
Age (y)	<68 (median)	39	22	0.413
	≧68	46	19	
Location	Colon	31	20	0.187
	Rectum	54	21	
Histological type	Differentiated	68	33	0.846
	Undifferentiated	15	8	
Pathological T category	pT1/2	20	18	**0.020***
	pT3/4	65	23	
Vessel involvement	n.a.			
Lymphatic vessel involvement	n.a.			
Lymph node metastasis	N0	53	27	0.576
	N1	32	13	
Distant metastasis	M0	63	32	0.631
	M1	22	9	
TNM stage	stage I	17	15	0.252
	stage II	31	11	
	stage III	15	6	
	stage IV	22	9	
n.a. not available information		#: Pearson's chi-square test		***** ***p*<0.05**

Supplementary Table 4. Colon cancer cell lines used in this study (characteristics according to reference [11]).

Cell Line	FLANC	CMS	MSI status	CIN	CIMP	KRAS	BRAF	PIK3CA	TP53	Origin
HCT116	High	4	MSI	-	+	G13D	WT	H1047R	WT	CRC adult, male
SW480	High	4	MSS	+	-	G12V	WT	WT	R273H;P309S	Dukes' type B, colorectal adenocarcinoma; 50 years, Caucasian
RKO	Low	4	MSI	-	+	WT	V600E	H1047R	WT	Colon carcinoma
HT-29	Low	3	MSS	+	+	WT	V600E	P449T	R273H	CR adenocarcinoma, 44 years, female, Caucasian
DLD-1	High	1	MSI	-	+	G13D	WT	E545K;D549N	WT	Dukes' type C, colorectal adenocarcinoma; adult male
Colo 205	High	1	MSS	-	+	WT	V600E	WT	Y107fs;Y103fs	Colon; derived from *metastatic site*: ascites; Dukes' type D, colorectal adenocarcinoma; 70 years male Caucasian
LS174T	Low	3	MSI	-	-	G12D	WT	H104R	WT	Dukes' type B, colorectal adenocarcinoma; 58 years female, Caucasian
Colo 320	High	4	MSS	+	-	WT	WT	WT	R248W	Neuroendocrine origin (amine precursor uptake and decarboxylation), Dukes' type C, colorectal adenocarcinoma; 55 years female, Caucasian

Supplementary Table 5. RPPA dataset of protein expression on FLANC overexpressor clones.

FLANC up-regulated proteins				FLANC down-regulated proteins			
Name	**Antibody name**	**p-value**	**FCH**	**Name**	**Antibody name**	**p-value**	**FCH**
MMP14	MMP14-R-V	<0.0001	1.26	**RPS6**	S6_pS240_S244-R-V	<0.0001	-1.27
MYH11	MYH11-R-C	<0.0001	1.15	**RPS6**	S6_pS235_S236-R-V	<0.0001	-1.25
NDRG1	NDRG1_pT346-R-V	<0.0001	1.13	**PARP1**	PARP-R-V	0.00014	-1.06
PAR	PAR-R-C	0.000164	1.14	**HSBP1**	HSP27_pS82-R-V	0.00018	-1.09
CLDN7	Claudin-7-R-V	0.000353	1.06	**NRG1**	Heregulin-R-V	0.00066	-1.08
NDRG1	NDRG1_pT346-R-V	0.000353	1.13	**RB1**	Rb_pS807_S811-R-V	0.00068	-1.07
PDGFRB	PDGFRB-R-V	0.000364	1.09	**DNMT1**	DNMT1-R-V	0.00075	-1.07
MMP14	MMP14-R-V	0.000364	1.26	**CAV1**	Caveolin-1-R-V	0.00091	-1.06
PRKAA1/2	AMPKa_pT172-R-C	0.000643	1.06	**PRKAR1A**	PKA-a-R-V	0.00099	-1.08
PAR	PAR-R-C	0.000643	1.14	**DUSP6**	DUSP6-R-C	0.00124	-1.05
AXL	Axl-R-V	0.000700	1.06	**RRM2**	RRM2-R-C	0.00142	-1.06
HSPA5	BiP-GRP78-M-C	0.001726	1.04	**COX4I1**	Cox-IV-R-V	0.00176	-1.14
L1CAM	CD171-M-V	0.004287	1.06	**BRD4**	BRD4-R-V	0.00181	-1.05
ERRFI1	MIG6-M-V	0.004287	1.07	**RPA2**	RPA32_pS4_S8-R-C	0.00183	-1.05
ITGB1	CD29-M-V	0.006357	1.04	**PAICS**	PAICS-R-C	0.00252	-1.05
SDHB	Complex-II-Subunit-M-V	0.007469	1.04	**FASN**	FASN-R-V	0.00256	-1.04
ERRFI1	MIG6-M-V	0.011299	1.07	**AKT1/2/3**	Akt_pT308-R-V	0.00316	-1.09
MYH11	MYH11-R-C	0.011299	1.15	**XRCC1**	XRCC1-R-C	0.00329	-1.05
CD276	B7-H3-R-C	0.011577	1.05	**SRC**	Src_pY527-R-V	0.00444	-1.04
PYGM	PYGM-M-C	0.012151	1.03	**MAPK1/MAPK3**	MAPK_pT202_Y204-R-V	0.00575	-1.04
EPHA2	EphA2-R-V	0.014726	1.03	**RAD51**	Rad51-R-C	0.00592	-1.08
ERBB2	HER2-M-V	0.016726	1.05	**EIF4G1**	eIF4G-R-C	0.00720	-1.05
KDR	VEGFR-2-R-V	0.019144	1.06	**TRIP13**	TRIP13-R-V	0.00800	-1.04
PDGFRB	PDGFRB-R-V	0.019144	1.09	**AKT1/2/3**	Akt_pS473-R-V	0.00821	-1.12
MT-CO1	MTCO1-M-V	0.022813	1.05	**ARID1A**	ARID1A-R-C	0.01044	-1.06
TGM2	Transglutaminase-M-V	0.024219	1.04	**PLK1**	PLK1-R-C	0.01065	-1.04
SERPINE1	PAI-1-M-V	0.026564	1.04	**RPS6KA1/2/3**	RSK-R-C	0.01343	-1.04
CDH1	E-Cadherin-R-V	0.029345	1.04	**TP53**	p53-R-C	0.01378	-1.06
ANXA1	Annexin-I-M-V	0.033405	1.03	**MSI2**	MSI2-R-C	0.01428	-1.04
CD4	CD4-R-V	0.035884	1.03	**CDK1/2/3**	CDK1_pT14-R-C	0.01891	-1.04
FGF2	FGF-basic-R-C	0.036697	1.03	**EEF2K**	eEF2K-R-V	0.02479	-1.03
HSBP1	HSP27-M-C	0.040428	1.03	**BAX**	Bax-R-V	0.02676	-1.03
GSK3A/GSK3B	GSK-3a-b-M-V	0.040967	1.02	**RBM15**	RBM15-R-V	0.02923	-1.03

AKT1S1	PRAS40-M-C	0.047670	1.03	**NOTCH1**	Notch1-cleaved-R-V	0.02955	-1.03
				BABAM1	MERIT40_pS29-R-V	0.03355	-1.03
				MSH6	MSH6-R-C	0.03625	-1.04
				NOTCH1	Notch1-R-V	0.03715	-1.02
				CHEK1	Chk1-M-C	0.03866	-1.02
				BBC3	Puma-R-C	0.03977	-1.03
				IGFBP2	IGFBP2-R-V	0.04148	-1.03
				AKT2	Akt2-R-V	0.04742	-1.03
				GYS1	Gys_pS641-R-V	0.04766	-1.02
				ATG5	Atg5-R-C	0.04970	-1.05

Supplementary Figure 1. (A) Genomic location of FLANC. FLANC resides in the antisense direction within the first intron of the protein coding gene CELSR1 located at 22q13.31. The pyknon sequence (marked in red in the FLANC gene) is present in one of the two Alu elements located within the FLANC gene; **(B)** Sense-antisense transcription. **(C)** FLANC expression in a panel of eight colorectal cancer cell lines. Five of them showed higher expression than a pool of normal colon samples, while HT-29 cell lines exhibited almost undetectable levels of FLANC. (**D**) FLANC expression in healthy human organs and peripheral blood mononuclear cells. Overall, FLANC showed a very low expression in healthy tissues, with the highest in lung and testis. (Student's t-test: *$p < 0.05$).

Supplementary Figure 2. (A) Nucleotide sequence of the 873 bp FLANC clone; **(B)** Genomic evolution of FLANC. Cross species sequence identity analysis only identifies regions of FLANC within the class Mammalia. Further, sequence aliments suggest that two ALU elements were inserted within the gene in primates.

Supplementary Figure 3. (A) *In vitro* transcription-translation assay showing lack of protein synthesis by FLANC; **(B)** Open reading frame (ORF) *in silico* analysis of FLANC. Only ORFs less than 100 amino acids were identified in ORF analysis of FLANC.

Supplementary Figure 4. *In Situ* Hybridization (ISH) and hematoxylin and eosin (H&E) staining of colon tissues microarray. ISH to assess the FLANC expression levels was performed in normal, adenocarcinoma, metastatic, benign, and colitis tissues (from top to bottom). H&E staining was done to show tissue morphology. Serial images were captured with increasing magnification (5X, left; 20X, center; 60X, right). Each box shows the corresponding tissue area magnification (from left to right). The ISH and H&E staining were performed on serial sections and each image corresponds to the same area for comparison (ISH and H&E).

Supplementary Figure 5. (A) Biochemical separation of nuclear and cytoplasmic fractions in DLD-1, HCT116 and SW480 shows FLANC expression both in the nucleus as well as in the cytoplasm. GAPDH was used as a positive control of the cytosol fraction and U6 as a positive control of the nuclear fraction. **(B)** H&E and ISH staining of colon tissue showing that FLANC is localized both in the nucleus and in the cytoplasm.

Supplementary Figure 6. (A-B) Knock-down with FLANC-directed siRNAs reduced the levels of FLANC, but not **(C-D)** the levels of the host gene *CELSR1*, as shown with two independent housekeeping genes U6 and GAPDH in HCT116 cells. (Student's t-test: ns = not statistic, *p < 0.05).

Supplementary Figure 7. (A-B) Knock-down of FLANC in HT-29 cells, which have only very low endogenous FLANC levels, did not show any significant difference in cellular growth in CCK-8 assay, nor in clonogenic assay. **(C-D)** Tumor sphere formation assay resulted in a significantly lower numbers of tumor spheres when FLANC was reduced by siRNA knock-down in HCT116 and DLD-1**. (E-F)** Cellular *in vitro* transwell migration was significantly reduced when FLANC levels were decreased by siRNA treatment in two colorectal cancer cell lines (HCT116 and SW480). (Student's t-test: ns = not statistic; *p < 0.05; ***p < 0.001).

Supplementary Figure 8. Representative pictures of the scatter plots after fluorescence activated cell sorting using a multiparametric staining protocol. **(A)** Scrambled control siRNA treated HCT116 cells, **(B)** FLANC-1 siRNA treated cells, and **(C)** FLANC-2 siRNA treated cells.

Supplementary Figure 9. (A) Schematic explanation of the apoptosis markers used in the high-resolution single cell apoptosis assay. **(B)** Representative pictures of a non-apoptotic cell (**left panel**) and a cell undergoing apoptosis (**right panel**).

Supplementary Figure 10. Representative pictures of the scatter plots generated by the image flow cytometry. **(A)** Scrambled control siRNA treated HCT116 cells, **(B)** FLANC-1 siRNA treated cells, and **(C)** FLANC-2 siRNA treated cells.

Supplementary Figure 11. (A) Generation of stable shRNA knock-down FLANC HCT116 cells and their respective reduced FLANC expression levels. **(B)** The shRNA FLANC knock-down clones showed decreased cellular growth in CCK-8 assay, **(C)** decreased numbers of colonies in clonogenic assay, corroborated by **(D)** increased apoptotic activity. (Student's t-test: *p < 0.05; ***p < 0.001).

Supplementary Figure 12. (A) Lenti-viral transduction of the FLANC transcript leads to stable overexpression of this transcript in HCT116 cells. **(B)** Significantly higher cellular growth of FLANC overexpressing HCT116 cells was confirmed by CCK-8 and **(C)** EdU assay. **(D)** Additionally, the FLANC overexpressing HCT116 cells showed a significantly higher number of colonies and a **(E)** decreased apoptosis activity. **(F)** Generation of FLANC overexpressing HT-29 cell line, which shows

very low endogenous expression levels, **(G)** resulted in a significantly higher rate of cellular growth. (Student's t-test; *p < 0.05; ***p < 0.001; ****p < 0.0001).

Supplementary Figure 13. (A) Mice injected with HCT116 FLANC overexpression cells showed larger tumors than the empty vector control. **(B)** Representative images of TUNEL assay (marks apoptotic bodies) in empty vector control and FLANC overexpression mouse tumors **(C)** The corresponding graphic of TUNEL assay suggests that overexpression of FLANC does not increase apoptosis, compared to empty vector control. Data are presented as means ± s.d. (Student's t-test: ns = not statistic).

Supplementary Figure 14. (A) The effect of FLANC overexpression at protein level by RPPA. By comparing the protein level of HCT116 FLANC overexpression versus HCT116 empty vector control, 28 proteins were found significantly overexpressed in FLANC overexpression versus control, and 43 downregulated (see also **online Supplementary Table 5**). **(B)** FLANC up-regulated proteins grouped into pathways, top 20 stimulated pathways. **(C)** FLANC down-regulated proteins grouped into pathways, top 20 inhibited pathways. **(D)** The network regulated by top 5 inhibited and top 5 activated pathways, according to the highest p-value, with the corresponding proteins. Top 5 activated pathways at the protein level by FLANC are: PI-3K cascade: FGFR1/2/3/4, PI3K-Akt signaling pathway, Axon guidance, Cell adhesion molecules and Leptin signaling pathway. Top 5 inhibited pathways are: Integrated Cancer Pathway, Progesterone-mediated oocyte maturation, Signaling Pathways in Glioblastoma, mTOR signaling pathway and DNA Damage Response.

Supplementary Figure 15. **(A)** In order to determine which are the pathways regulated by FLANC at transcriptional level and protein level we performed a Venn diagram, intersecting the opposite pathways inhibited in the array analysis of FLANC overexpression versus empty control in HCT116 with the pathways activated in the array analysis of FLANC shRNA versus shRNA control. We obtained 25 pathways regulated at the transcriptional level. Next, we intersected these 25 pathways with the pathways inhibited in the RPPA analysis of FLANC overexpression versus empty control in HCT116. **(B)** A total of 11 pathways are regulated at the transcriptional and protein level by FLANC.

Supplementary Figure 16. Knock-down of FLANC confirmed *in vivo* by *in situ* hybridization in liver metastases of FLANC siRNA-targeted mice.

Supplementary Figure 17. Effect of *in vivo* single dose treatments on blood chemistry and plasma cytokines levels. (A) Bar graphs of mean levels of measured aspartate aminotransferase (AST), blood urea nitrogen content (BUN), and lactic dehydrogenase (LDH; to assess overall tissue damage) in treated and control mice. Error bars represent standard deviations. Statistical comparisons were performed by two-tailed *t*-test between the two groups (n=5) **(B)** Bar graphs of mean plasma levels of measured cytokines: interferon gamma (IFNγ), interleukin 4 (IL-4), IL-6, IL-10, IL-12(p40), IL-13, keratinocyte-derived cytokine (KC), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), tumor necrosis factor alpha (TNF-α) and vascular endothelial growth factor (VEGF). Error bars represent standard deviations. Statistical comparisons were performed by two-tailed one-way Anova between the groups (n≥5).

Supplementary Figure 18. Effect of *in vivo* single dose treatments on organs histopathology. Representative organs (e.g., brain, heart, kidney, liver, lung, spleen) histology section stained with hematoxylin and eosin (H&E) from mice untreated and treated with control siRNA, FLANC-siRNA, at a concentration of 200µg/Kg of body weight for 72 hours. Images were taken at 200X magnification (20X lens). Scale bars represent 50µm.

Supplementary References

1. Spaventi R, Pecur L, Pavelic K, et al. Human tumour bank in Croatia: a possible model for a small bank as part of the future European tumour bank network. Eur J Cancer 1994;30A:419.
2. Rigoutsos I, Lee SK, Nam SY, et al. N-BLR, a primate-specific non-coding transcript leads to colorectal cancer invasion and migration. Genome Biol 2017;18:98.
3. Lu J, Ye X, Fan F, et al. Endothelial cells promote the colorectal cancer stem cell phenotype through a soluble form of Jagged-1. Cancer Cell 2013;23:171-85.
4. Nishimura M, Jung EJ, Shah MY, et al. Therapeutic synergy between microRNA and siRNA in ovarian cancer treatment. Cancer Discov 2013;3:1302-15.
5. Ling H, Spizzo R, Atlasi Y, et al. CCAT2, a novel noncoding RNA mapping to 8q24, underlies metastatic progression and chromosomal instability in colon cancer. Genome Res 2013;23:1446-61.
6. Schwarzenbacher D, Stiegelbauer V, Deutsch A, et al. Low spinophilin expression enhances aggressive biological behavior of breast cancer. Oncotarget 2015;6:11191-202.
7. Lu Y, Ling S, Hegde AM, et al. Using reverse-phase protein arrays as pharmacodynamic assays for functional proteomics, biomarker discovery, and drug development in cancer. Semin Oncol 2016;43:476-83.
8. Chen W, Chen R, Li J, et al. Pharmacokinetic/Pharmacodynamic Modeling of Schedule-Dependent Interaction between Docetaxel and Cabozantinib in Human Prostate Cancer Xenograft Models. J Pharmacol Exp Ther 2018;364:13-25.
9. Zhou P. Determining protein half-lives. Methods Mol Biol 2004;284:67-77.
10. Ruopp MD, Perkins NJ, Whitcomb BW, et al. Youden Index and optimal cut-point estimated from observations affected by a lower limit of detection. Biom J 2008;50:419-30.

11. Berg KCG, Eide PW, Eilertsen IA, et al. Multi-omics of 34 colorectal cancer cell lines - a resource for biomedical studies. Mol Cancer 2017;16:116.