SUPPLEMENTARY METHODS

A. In vitro CRC cell maintenance

All the CRC cell lines (DLD1, HCT116, SW620, SW480, LS174T, T84, HT29) were purchased through the ATCC and grew in complete media as recommended by ATCC.

Every 2 weeks, cells are mycoplasma tested using MycoAlert detection kit (Lonza). The cells are used between passages 2 and 4. Briefly, the cells were washed twice in warm PBS and dissociated into single cell suspension with warm trypsin-EDTA (Dutscher P10-023100) (except the HCT116 and HT29 cells, dissociated with Accutase (Sigma A6964)). The dissociation was stopped by adding warm complete medium, and a sample was incubated for 1 minute at room temperature with 1 µg/mL propidium iodide (Sigma P4170) before assaying the viable cell concentration by flow cytometry with a C6 Accuri flow cytometer (Becton-Dickinson). For cell counting, the cellular debris were excluded by FSC-A (<1/10th the size of a cell) and PI staining (PI negative). In the remaining events, the PI positive cells were considered as dead. Among the viable cells, aggregation of the cells was evaluated by FSC-H x FSC-W, and single cells, doublets and triplets were analyzed counted.

PG-overexpressing HCT116 cells were engineered by Vectalys (Toulouse, France), from the HCT116 cells bought at the ATCC and infected by custom-made lentiviral expression vectors containing GAST cDNA or by the empty vector. These cells were named HCT116-PG and HCT116-WT respectively.

B. Migration and invasion assays

We used Boyden chamber, with Transwell Fluoroblok (Corning 351152) and Transwell Fluoroblok BioCoat (Corning 354165).

100,000 untreated HCT116 cells were seeded per chamber in warm complete medium (4 to 8 replicates per group) and left to adhere overnight (16h) in a 37°C 5% CO_2 humidified incubator. The next day, the plates were moved to a 5% CO_2 1% O_2 humidified incubator-hood (Sci-Tive-U,

Ruskinn-Awel), washed with warm oxygen-depleted PBS and treated by anti-PG antibodies diluted in oxygen-depleted serum-free medium. The treatment medium was renewed after 24h. After 48h, the medium was replaced by a chemoattractant gradient (oxygen-depleted serum-free medium in the upper chamber and oxygen-depleted complete medium in the lower chamber). HCT116 were left to migrate/invade for 8h.

in vivo treated subcutaneous xenograft- were directly seeded in the Boyden chambers with a chemoattractant gradient but no further anti-PG treatment (4 to 8 replicates per group). *In vivo* treated cells result from pooling and sorting equal number of viable cells from 6 to 8 tumors, such as described for *in vivo* ELDA. The plates were then incubated in a 5% CO_2 1% O_2 humidified incubator-hood (Sci-Tive-U, Ruskinn-Awel). 50,000 HT29 cells per chamber, and 80,000 T84 cells per chamber were left to migrate/invade for 48h with a renewal of the gradient with warm oxygen-depleted media at mid-point.

For all the migration and invasion assays with HCT116 and invasion assay with T84, automatic Hoechst 33342 fluorescence reading was performed using a FluoroSkan Infinite F200 Pro (TECAN) fluorescence plate reader. For all the migration assays with T84 and HT29, the viable cell-specific Calcein AM staining was preferred.

The data were analyzed after substracting the background measured in wells with cells that did not receive the chemoattractant gradient.

C. GAST depletion by siRNA

siRNA transfection was performed with Lipofectamine RNAi max (Invitrogen 13772-075) according to the manufacturer's instructions. Briefly, 250 µL of transfection mix (1% Lipofectamine RNAi max with 60 nM siRNA for T84 and LS174T cells respectively, in OptiMEM (Gibco)) was settled per well in a 6-well plate, and 125,000 cells in 1.25 mL per well of complete medium were homogenously seeded on top. The plates were slowly swirled twice and

then incubated in a 37°C 5% CO_2 humidified incubator. After 8h, the medium was removed and replaced with 1.5 mL per well of fresh complete medium.

D. Wnt signaling reporter assay after siRNA or antibody treatment

Renilla plasmidic vector (pRL-TK) was purchased from Promega (E2241) and TOP-Flash and FOP-Flash from Millipore (21-270 and 21-269 respectively).

For PG neutralization experiments, 25,000 T84 cells were seeded ULA T25 flasks in 2.5 mL of antibiotics-free M11 medium. The cells were then transfected by Lipofectamine LTX plus (Invitrogen 15338-100) according to the manufacturer's instructions, by adding 250 µL of transfection mix (7.5 µL of Lipofectamine per 2.5 µg of vector in a final volume of 250 µL OptiMEM (Gibco)) per flask. After 8h at 37°C and 5% CO2, 2.5 mL of antibody-containing (10 µg/mL) fresh M11 medium were added to each flask. 48h after the transfection, 100 µL of 250 µg/mL antibodies were added to the plates to renew the treatment. Four to six replicates were done for each experiment. 96h after transfection, the cells and the growing colonospheres were centrifuged and washed with cold PBS before the transcriptional activity of the reporter vectors was assayed with Dual Luciferase Assay kit (Promega E1910) according to the manufacturer's instructions, with a LuminoSkan (TECAN) using the Ascent software.

For GAST siRNA experiments, LS174T or T84 cells were transfected by siRNA in T25 instead of 6-well plates (see above paragraph) in 5 mL complete medium and a surface-adjusted scale-up of the reagents (19.5 µL of Lipofectamine per 6.5 µg of vector in a final volume of 650 µL OptiMEM (Gibco)). The cells were then transfected by Fugene HD (Promega E2313) according to the manufacturer's instructions, by adding 500 µL of transfection mix (18 µL of transfection reagent per 6 µg of vector in a final volume of 500 µL OptiMEM (Gibco)) per flask. 72h later, the cells were washed with cold PBS, before assaying the luciferase activity as described for the PG neutralization experiments.

E. Tissue cryo-grinding for subsequent mRNA or protein analysis

Lysis of tumor samples stored in liquid nitrogen was achieved by pulverization with a cryo-grinder (6770 Freezer/Mill). Briefly, the frozen samples were settled in a pre-cooled grinding tube, and then pulverized by a cycle of 1 minute at 10 impacts per second (with 1 minute for pre- and post-cooling steps of the machine). The powder of each sample was then separated in pre-weighed pre-cooled tubes, and quickly weighed before being stored at -80°C for later RNA or protein analysis.

F. RNA extraction from cells and tissues, reverse-transcription and qPCR

mRNA extraction was done with RNeasy kits (Qiagen 74126) according to the manufacturer's instructions. Suspension cells were centrifuged and washed in cold PBS before resuspending in the RLT lysis buffer, whereas adherent cells were washed with cold PBS and the lysis step was performed directly in the wells. 250 µL RLT/ß-mercaptoethanol lysis buffer was used if cell number was under $5x10^6$ cells, 600 µL if the cell number was above this threshold. Cryo-grinded tissues were weighed and 10 mg of powder was resuspended in 600 µL lysis buffer. At this stage, the lysates were stored at -80°C. If one sample was stored frozen, all the other samples of the experiment were frozen too.

The thawed lysates were centrifuged on Qiashredders columns (Qiagen 79656), before continuing the manufacturer's protocol, including the recommended DNase I step. The mRNA were collected in ultra-pure water, and the mRNA concentration and purity was assayed with a NanoDrop 2000 (Thermo Scientific). mRNA could be stored at -80°C at this stage.

Retrotranscription was performed on 1 µg mRNA with the Primescript RT Mastermix (Takara) according to the manufacturer's instructions on a MasterCycler ep Gradient (Eppendorf) machine. cDNA could be stored at -20°C at this stage.

Finally, quantitative PCR was performed on 2 µL of cDNA with Sybr Premix ExTaq kit (Takara) according to the manufacturer's instructions in white skirted Twin.Tec real-time PCR

plates (Eppendorf 951022015) sealed with adhesive film (Dutscher 035713) on a Realplex (Eppendorf) machine. The program was as follow: de-hybridization of duplexes (95°C 30 sec), amplification (45 cycles of 95°C 5 sec and 60°C 30 sec), final elongation (95°C 15 sec and 60°C 1 min), melting curve (20 min progressive heating until 95°C).

G. Protein extraction, immunoprecipitation and blotting

Protein extraction was performed with an in-house RIPA lysis buffer -50 mM Tris (TRIZMA Base, Sigma T1503) / 150 mM NaCl (Sigma 31434) / 1% Igepal CA-630 (Sigma I3021) / 0.25% sodium deoxycholic acid / pH 7.5- supplemented by Halt Protease Phosphatase Inhibitor Cocktail (Thermo Scientific 78442) according to the manufacturer's instructions.

Cryo-grinded tissues and were directly resuspended in cold RIPA buffer, whereas adherent cells were washed with cold PBS before adding the cold RIPA lysis buffer, scraping and transferring in pre-cooled tubes. As for cell culture supernatant, the samples were concentrated on a Concentrator 20 mL/9K (Pierce 89885A) at 4°C until the liquid almost vanished, then re-centifuged with 10 mL of cold 150 mM NaCL until about 1 mL was left, and the other components of the RIPA lysis buffer were then added.

The samples were then incubated for 45 minutes at 4°C with 10 seconds of vortexing every 10 minutes, before being centrifuged (16 500 g) at 4°C for 45 minutes. After the centrifugation step, the supernatant was transferred in a new pre-cooled tube, and protein concentration was measured by Micro-Lowry assay (BioRad 500-0113/0114/0115) according to the manufacturer's instructions.

For immunoprecipitation, the sample were diluted in cold RIPA lysis buffer without the inhibitor cocktail to adjust their concentrations (1 mg of cell lysate in 600 µL or 600 µL of cell culture supernatant), and were pre-cleared on 50 µL agarose-G protein beads (Santa Cruz SC-2002, pre-washed twice in RIPA lysis buffer without the inhibitor cocktail) on a wheel at 4°C during 30 minutes. After centrifugation, the pre-cleared samples were transferred in a new cold tube with 4

µg of the immunoprecipitating antibody, and left on the wheel at 4°C overnight (16h). On the next day, the samples are transferred in a new tube containing 50 µL agarose-G protein beads (Santa Cruz SC-2002, pre-washed twice in RIPA lysis buffer without the inhibitor cocktail), and incubated for 3 more hours on the wheel at 4°C. The beads are then washed three times by a cycle of centrifugation and resuspension in cold RIPA lysis buffer without inhibitor cocktail.

The elution of progastrin was performed by adding 25 µL of 2X Laemmli buffer (BioRad 1610737) on the beads before heating, centrifugation and recovering before a denaturing step (95°C 5 min).

For direct SDS-PAGE, the samples were diluted in cold RIPA lysis buffer to adjust their concentrations, and 4X Laemmli buffer was added with a final 1X dilution before the sample were subjected to denaturation (95°C during 5 min).

SDS-PAGE was performed in mini Protean Tetra Cell or in Protean II XL (Biorad) depending on the size of the gels (pre-cast mini-TGX or home-made maxi-gels). After electrophoretic migration, the gels were either stained with Page Blue Protein Staining Solution (Thermo Scientific 24620) according to the manufacturer's instructions, or transferred on nitrocellulose for Western Blots, or PVDF membrane for Amido black (Sigma A8181) staining, with a Trans-Blot Turbo machine and membrane kits (BioRad). After the transfer on nitrocellulose membranes, the proteins were stained and fixed on the membrane by a Ponceau red (Sigma 141194) staining with 2% acetic acid. The samples to compare were always separated on the same SDS-PAGE gel.

Western blotting was done with the antibodies diluted in PBS / 0.1% Tween 20 / 5% non-fat milk, and the membranes were washed by PBS / 0.1% Tween 20. The revelation was performed with ECLplus kits (Pierce 32132), and the luminescence measures were acquired by a G-BOX (Syngene) and quantitated with the GeneTools software (Syngene).

H. Sandwich ELISA

Capture antibody was incubated overnight (16h) at 4°C in MAxiSORP Nunc-Immuno 96-well Microplates (Nunc 055221). The wells were emptied and washed thrice with PBS / 0.1% Tween 20 before adding the blocking buffer (PBS/0.1% Tween 20/0.1% BSA) and incubating 2h at 22°C. The wells were emptied and washed thrice with PBS/0.1% Tween 20 again. The plasma from patients and standard are added to the wells and incubated for 2h at 22°C. The wells were emptied and washed thrice with PBS/0.1% Tween 20 again, and the HRP-coupled secondary antibodies - diluted in the blocking buffer- were added before incubating 1h at 22°C. The pre-heated revelation solution (TMB (Sigma T0440) 22°C) was added after 3 more washing steps, and incubated 30 min at 22°C without light and stopped by adding 4N H_2SO_4, before OD 450 nm measurement.

I. Post-translational modifications

To study sulfation, the cells were cultivated 60h with 50 mM $NaClO_3$ (Sigma 244147) or NaCl prior to cell lysis or supernatant recovery. To study phosphorylation of progastrin, the immunoprecipitate from supernatant or whole cell lysates were treated or not by 20U λ-phosphatase (Santa Cruz 200312) at 30°C for 1h.

J. Flow cytometry and FACS

In addition to the cell counting and sorting described in the previous paragraphs, flow cytometry was also used to measure CRC stem cell markers, and assay the apoptosis/necrosis with AnnexinV/propidium iodide staining.

The apoptosis/necrosis assay was performed with the AnnexinV kit (BD Pharmingen 556570) according to the manufacturer's instructions.

ALDH1 activity was measured with the ALDEfluor kit (StemCell Technologies 01700) according to the manufacturer's instructions. Extracellular staining of CD44 was done according to the instructions of the ALDEfluor kit for labeling with antibodies post-ALDH activity assay protocol. After labeling with anti-CD44 antibodies and APC-coupled secondary antibodies, 1

µg/mL propidium iodide was also added 1 minute prior to data acquisition on C6 Accuri (Becton-Dickinson). The ALDH/CD44 staining was only analyzed in live (PI-negative large events) single (separated from aggregated cells by FSC-H x FSC-W) cells.

K. Antibodies and primers

The sequences of the qPCR primers (Sigma) were: hGAST-F (5'-CAT GAG AAG TAT GAC AAC AGC CT-3'), hGAST-R (5'-AGT CCT TCC ACG ATA CCA AAG T-3'), hGAPDH-F (5'-CCT GGA GCT ACC CTG GCT GGA-3'), hGAPDH-R (5'-CTT GGA CGG GTC TGC CAC GA-3').

The anti-PG antibodies were generated according to patent WO/2011/045080 and were used as follow: N- or C- terminal sequence of human progastrin (hPG) as antigenic sequence that correspond to residues 1 to 14 of hPG (SWKPRSQQPDAPLG) and 55 to 80 of hPG (QGPWLEEEEEAYGWMDFGRRSAEDEN) respectively.

Commercially available primary antibodies were as follow: anti-survivin (Cell Signaling Technologies 2802S), anti-actin (Sigma A4700), APC-coupled anti-CD44 (BD Pharmingen 559942), APC-coupled isotype IgG2bK (BD Pharmingen 555745),

Commercially available secondary antibodies were as follow: HRP-coupled goat anti-mouse (Thermo scientific 31439), HRP-coupled goat anti-human (Thermo Scientific 31413), Alexa488-coupled goat anti-human (Molecular Probes A11013),

L. Statistical analyses

The Prism software (GraphPad) has been used to statistically analyze the results of most of our experiments. Normal distribution of the data was assessed with normality tests to determine if Gaussian approximation had to be used. In the end, a vast majority of the results was analyzed with parametric hypothesis, with the noteworthy exception of the ELDA that requires the specific use of the ELDA Webtool bioinf.wehi.edu.au/software/elda/ because of the Poisson distribution of the stem cells (Hu et al., 2009).

When more than one parameter was assayed (e.g. tumor growth follow-up in time depending on the treatment protocol), two-way ANOVA was used. The survival of the mouse cohorts was also analyzed with a log-rank (Mantel-Cox) test.

All tests were two-tailed, and p-values under 0.05 were considered significant. P-values are indicated as follow: ns = $p>0.05$; * = $0.01<p\leq0.05$; ** = $0.001<p\leq0.01$; *** = $p\leq0.001$.