GeLC-MS/MS

In-depth proteomics analysis was performed using 1D gel electrophoresis followed by nano-liquid chromatography coupled to tandem mass spectrometry (GeLC-MS/MS). In brief, 15 µl of proximal fluid protein samples were separated by SDS-PAGE gel-electrophoresis using a 10% acrylamide gel. The gel was fixed in 50% ethanol containing 3% phosphoric acid, stained with Coomassie R-250, washed in MilliQ water, and stored at 4°C until processing for in-gel digestion. The gel lanes corresponding to proximal fluids from three normal colons and three colon tumors were cut in 10 bands. Each band was processed for in-gel digestion, by mixing gel cubes with 400 µl of 50 mM ammonium bicarbonate followed by several cycles of wash/dehydration steps in 50 mM ammonium bicarbonate. Subsequently, cysteine bonds were reduced with 10 mM dithiotreitol for 1 h at 56°C and alkylated with 50 mM iodoacetamide for 45 min at room temperature in the dark. After two wash/dehydration cycles the bands were dried for 10 minutes in a vacuum centrifuge and incubated overnight with 6.25 ng/µl trypsin at 25°C. Peptides were extracted once in 1% formic acid and subsequently twice in 5% formic acid in 50% acetonitrile. The final volume was reduced to 100 µl in a vacuum centrifuge prior to LC-MS/MS analysis. Peptides were separated by an Ultimate 3000 nanoLC system (Dionex LC-Packings, Amsterdam, The Netherlands) equipped with a 20 cm × 75 µm ID fused silica column custom packed with 3 µm 100 Å ReproSil Pur C18 aqua (Dr Maisch GMBH, Ammerbuch-Entringen, Germany). After injection, peptides were trapped at 30 µl/min on a 5 mm × 300 µm ID Pepmap C18 cartridge (Dionex LC-Packings, Amsterdam, The Netherlands) at 2% buffer B (buffer A: 0.05% formic acid in MQ; buffer B: 80% acetonitrile + 0.05% formic acid in MQ) and separated at 300 nl/min in a 10–40% buffer B gradient in 60 min. Eluting peptides were ionized at 1.7 kV in a Nanomate Triversa Chip-based nanospray source using a Triversa LC coupler (Advion, Ithaca, NJ). Intact peptide mass spectra and fragmentation spectra were acquired on a LTQ-FT hybrid mass spectrometer (Thermo Fisher, Bremen, Germany). Intact masses were measured at
resolution 50,000 in the ICR cell using a target value of $1 \times 10^6$ charges. In parallel, following an FT pre-scan, the top 5 peptide signals (charge-states 2+ and higher) were submitted to MS/MS in the linear ion trap (3 amu isolation width, 30 ms activation, 35% normalized activation energy, Q value of 0.25 and a threshold of 5000 counts). Dynamic exclusion was applied with a repeat count of 1 and an exclusion time of 30 seconds.

Collection of CRC cell line secretome microvesicles and soluble proteins

Cells from the HT29 CRC cell line were cultured in three T-175 flasks until 70% confluency, washed four times with serum-free medium and once with PBS. Cells were incubated with 13 ml serum-free medium per flask at 37°C for 18 hours. The conditioned media were collected and centrifuged gently (500g at room temperature for 10 minutes) to remove dead cells. The supernatant was collected and centrifuged again to remove cell debris (2,000g at 4°C for 15 minutes). Once more, the supernatant was collected and centrifuged harder (20,000g at 4°C for 45 minutes) to remove also small cell debris. Next, the supernatant was transferred to a 38.5 ml Thinwall Ultra-Clear™ tube (Beckman Coulter, Woerden, The Netherlands) and centrifuged at high speed (100,000g at 4°C for 90 minutes). The supernatant was collected and represented the soluble (S) fraction of the HT29 cell line secretome. The pellet was resuspended in 2 ml ice cold PBS upon which the tube was filled with PBS and centrifuged again (100,000g at 4°C for 90 minutes). The supernatant was discarded while the pellet representing the microvesicle (MV) fraction was collected in sample buffer, heated at 99°C for 5 minutes, and stored at -20°C until further use. The S-fraction of the HT29 secretome was concentrated using a 3kDa Amicon filter (Millipore, Amsterdam, The Netherlands), mixed with sample buffer, heated at 99°C for 5 minutes, and stored at -20°C until further use. In parallel, the HT29 cells were washed five times with PBS to generate cell lysates (CL-fraction). The MV-, S-, and CL-fractions were used for in-depth GeLC-MS/MS proteomics.