**Supplementary Material & Methods**

**Isolation and expansion of colonospheres**

The isolation and expansion of colorectal cancer colonospheres was performed as described (1). In short, the obtained tissue fragments were washed extensively with PBS and were mechanically dissociated using scalpels and vigorous trituration to yield small fragments (<1mm3) and single cells. Enzymatic digestion was performed using thermolysin 0.05% (Sigma, Type X) in DMEM/F12 containing 5 mM Hepes (Gibco) for 2hr at 37ºC. The suspension was then filtered through a 40-µm-pore size nylon cell strainer (BD Falcon) to separate the tissue fragments from the single cells. The single cell suspension was cultured in advanced DMEM/F12 (Gibco) supplemented with 0,6% glucose (BDH Lab. Supplies), 2mM L-glutamine (Biowhittaker), 9.6 µg/ml putrescin (Sigma), 6.3 ng/ml progesterone (Sigma), 5.2 ng/ml sodium selenite (Sigma), 25 µg/ml insulin (Sigma), 100 µg/ml apotransferrin (Sigma), 5 mM hepes (Gibco), 0,005 µg/ml trace element A (Cellgro), 0,01µg/ml trace element B (Cellgro), 0,01 µg/ml trace element C (Cellgro), 100 μM β-mercapto ethanol (Merck), 10 ml antibiotic-antimycotic (Gibco), 4 µg/ml gentamicine (Invitrogen), 0.002% lipid mixture (Sigma) and 5 µg/ml glutathione (Roche). This Advanced DMEM/F12 medium plus supplements is defined as Stem Cell Medium. Growth factor FGF (10 ng/ml, Abcam) were added to the cell culture medium freshly each week. All cell culture was carried out in non-tissue culture treated flasks (BD Falcon) at 37° C in a 5% CO2 humidified incubator.

HT-29 cells (human colon adenocarcinoma cells; German Collection of Microorganisms and Cell Cultures) were grown as colonospheres, under non-adherent conditions in Stem Cell Medium at 37°C in a 5% CO2 humidified incubator.

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| **Cultures used for GPx2 knockdown** |
| **Case** | **Tumor site** | **Tumor type** | **Tumor stage** | **Differentiation** |
| CRC29 | Right colon | Adenocarcinoma | T3N0Mx | Poor |
| L145 | Liver segment VII | Adenocarcinoma | metastasis | Moderate  |
| HT29  | Colon | Adenocarcinoma | Not known | Mesenchymal subtype (2) |
| **Cultures used for GPx2 overexpression** |
| **Case** | **Tumor site** | **Tumor type** | **Tumor stage** | **Differentiation** |
| CRC29 | Right colon | Adenocarcinoma | T3N0Mx | Poor |
| L145 | Liver segment VII | Adenocarcinoma | metastasis | Moderate  |
| L167 | Liver segment II-IV | Adenocarcinoma | metastasis | Moderate |
| L169 | Liver segment IV | Adenocarcinoma | metastasis | Well |

**Isolation of single cells from xenografts and colonospheres**

Tumor xenografts were mechanically dissociated to yield small fragments that were further dissociated using dispase II (Roche)/ collagenase XI (Sigma; 30 min 37C). The cells were washed once with PBS and were subsequently incubated with TrypLE express (Invitrogen) including 2,000 U/ml DNase (Sigma; 30 min 37C). To obtain single cell suspensions from colonospheres, they were incubated with Accumax (Innovative Cell Technologies) plus 2 U/ml DNAse1 (Sigma) for 10 minutes in a rotary incubator at 37°C. Both types of cell suspensions were then filtered through a 40-μm-pore size nylon cell strainer (BD Falcon) to obtain single cells.

**Tumor formation**

Single cell populations (GPx2 knockdown, GPx2 over-expressing cells and controls) were diluted to 10.000 live spheroid-derived cells, mixed with BD Matrigel (BD Biosciences) at a 1:1 ratio (total volume 100 μl) and injected subcutaneous into the flanks of 6 weeks old BALB/cnu/nu mice. The same procedure was followed after FACS sorting experiments in which GPx2-GFP high and low cell

populations were mixed with Matrigel (BD Biosciences) at 1000 cells/50μl and injected into

the flanks of BALB/cnu/nu mice.

**Liver metastasis model**

Pathogen-free, 10–11-week-old female SCID (severe combined immunodeficiency) mice were purchased from Taconic (Ejby, Denmark). Animals were quarantined for 2 weeks. HT29 cells expressing YFP were harvested by brief trypsinization. Colorectal liver metastases were induced as previously described ([3](#_ENREF_1)). In brief, single cell suspensions were prepared in phosphate-buffered saline to a final concentration of 7.5 x 104 cells/100 µl. Cells were injected into the parenchyma of the spleen of 125-day-old SCID mice using a 27-gauge needle. The injection site on the spleen was pressed with a cotton stick to ensure hemostasis. The peritoneum and skin were closed in a single layer with surgical thread.

**Tumor analysis**

After six weeks mice were sacrificed and tumor load in the liver was assessed in all liver lobes. Tumor load was scored as hepatic replacement area (HRA), that is, the percentage of liver tissue that had been replaced by tumor tissue, exactly as described before ([3](#_ENREF_1)). In brief, on hematoxylin and eosin-stained sections, at least 100 fields were selected using an interactive video overlay system, including an automated microscope (Q-Prodit; Leica Microsystems) at a x40 magnification. Using a four-point grid overlay, the ratio of tumor cells versus normal hepatocytes was determined for each field. Tumor load (HRA) was expressed as the average area ratio of all fields.

**Antibodies and reagents**

The antibodies used in this study are rabbit anti-human GPx2([2](#_ENREF_2)), rabbit anti-FABP1 (Sigma), mouse anti-cleaved-caspase-3 (Cell Signalling), mouse anti-mucin2 (Epitomics), mouse anti-cytokeratin 20 (Ks20.8; Dako), rabbit anti-OLFM4 (Abcam),rabbit anti-oct4 (Cell Signalling), rabbit anti-Ki67 (Novocastra), mouse anti-epithelial antigen (EpCAM, cloneBER-EP4; Dako), mouse anti-GFP (Roche), mouse anit-p16 (clone C20, Santa Cruz), mouse anti-p21WAF (clone OP68, Calbiochem) and mouse anti-β-actin (AC-15; Novis Biologicals). The AldefluorTM reagent was purchased from STEMCELL Technologies and used according to the manufacturer’s instructions. The general Caspase Inhibitor

Z-VAD-FMK (R&D systems) was used at a concentration of 25µM and added directly after making colonospheres single cell.

**Flow cytometry and cell sorting**

Aldefluor®-positive cells were analyzed according to the manufacturer’s protocol by using the ALDH substrate BAAA (1 μmol/l per 1×106 cells)(STEMCELL Technologies). Negative control samples were co-incubated with diethylaminobenzaldehyde (DEAB; 50 mM). The cell sorting experiments were conducted with FACS Aria IIU (BD), using FACS Diva version 6.1 software, and the expression was analyzed using a FACScalibur (BD Biosciences, San Diego, USA). Dead cells were excluded using viability marker 7-aminoactinomycin D (7-AAD) (R&D, Detroit, MI) and cell doublets and clumps were excluded using doublet discrimination gating. For analysis of xenograft material, anti-human epithelial antigen (Ep-CAM, clone BER-EP4; Dako) was used as a marker to select for epithelial cells.

**Immunohistochemistry**

For anti-human GPx2 ([4](#_ENREF_2)) and anti-human cytokeratin 20 (Ks20.8; M7019 Dako), antigen retrieval was performed by boiling samples for 20 min in HIER Citrate Buffer pH 6.0 (Monosan®). The anti-human GPx2 was diluted 1/5000 in PBS 0.05% BSA and incubated overnight at 4ºC, and anti-human cytokeratin 20 was diluted 1/50 in PBS 0.05% BSA and incubated overnight at 4 ºC. Secondary antibodies (polymer HRP conjugated anti-rabbit (for GPx2) and anti-mouse (for CK20), Powervision) were added for 30 min at room temperature. Samples were developed using DAB, counterstained with haematoxylin and mounted.

**Bioinformatic analyses**

All analyses were performed using the R2 microarray analysis and visualization platform (<http://r2.amc.nl>). For generation of the GPx2-coexpression signature we identified all genes that were significantly co-expressed with GPx2 in two datasets ([5](#_ENREF_4), [6](#_ENREF_5)). The cut-off for maximum p values for single gene associations was set to e-6 and False Discovery Rate was used to correct for multiple testing. The 100 genes most significantly co-expressed with GPx2 in both datasets showed a 53-gene overlap (Supplementary Table S3). For k-means clustering the 53-gene GPx2 co-expression signature was uploaded into R2 and was used as a gene category to cluster the tumors into 3 groups in 10x100 draws. The clustering was stored as a track. For generation of the differentiation signature we identified all genes that were significantly co-expressed with CDX2 *and* KRT20 *and* VIL1. The cut-off for maximum p values for single gene associations was set to e-6 and False Discovery Rate was used to correct for multiple testing. The 200 genes most significantly co-expressed with CDX2, KRT20 and VIL1 yielded an overlap of 63 genes (Supplementary Table S4). This list was uploaded into R2 as a category. To compare expression levels of genes in tumor subgroups we used the option ‘view gene in groups’ and selecting either the GPx2 subgroups or the CCS subgroups as a track.

**References**

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