Extracellular vesicle release from intestinal organoids is modulated by Apc mutation and other colorectal cancer progression factors

**Supplementary information**

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| miR          | Assay_ID     |
|--------------|--------------|
| mir-10a-5p   | 479241_mir   |
| miR-16-5p    | 477860_mir   |
| miR-21-5p    | 477975_mir   |
| miR-26a-5p   | 477995_mir   |
| miR-34a-5p   | 478048_mir   |
| mir-128-3p   | 477892_mir   |
| mir-146a-5p  | 478399_mir   |
| miR-155-5p   | 483064_mir   |
| mir-181a-5p  | 477857_mir   |
| mir-181b-5p  | 478583_mir   |
| mir-210-3p   | 477970_mir   |
| mir-222-3p   | 477982_mir   |
| mir-532-5p   | 478151_mir   |
| mir-484      | 478308_mir   |

Table S1. Advanced TaqMan miRNA assays and the sequence of primers used to quantify mRNA levels by RT-qPCR.
Table S2. Clinical data of CRC patients. MMR deficiency was determined by immunostaining of the samples for MLH1, MSH2, PMS2 and MSH6 in clinical diagnosis.

| Sample | Sex | Age | Tumor                      | Differentiation grading | Stage        | MMR deficiency |
|--------|-----|-----|----------------------------|-------------------------|--------------|----------------|
| CRC1   | F   | 63  | colon adenocarcinoma       | Grade 2                 | T3N0M0       | no             |
| CRC2   | M   | 74  | colon adenocarcinoma       | Grade 2                 | T3N0M0       | no             |
| CRC3   | M   | 75  | colon adenocarcinoma       | Grade 2                 | T3N2aM0      | no             |
| CRC4   | M   | 74  | colon adenocarcinoma       | Grade 2                 | T3N1M0       | no             |
| CRC5   | F   | 70  | colon adenocarcinoma       | Grade 2                 | T3N0M0       | no             |

Table S3. Characteristics of the CRC patient-derived organoids.*Only codon 12, 13, 18 and 61 were tested for KRAS. Note that none of the cultures survived in the absence of R-Spondin1.

| Sample | p53 mutation | Nutlin sensitivity | KRAS mutation* | EGF dependency |
|--------|--------------|--------------------|----------------|----------------|
| CRC1   | V274A        | no                 | no             | yes            |
| CRC2   | V274A        | no                 | no             | yes            |
| CRC3   | V274A        | no                 | no             | yes            |

Symbol | Description
---|---
CFA1 | Complement factor I OS=Homo sapiens GN=CFI PE=1 SV=2
DHE3 | Glutamate dehydrogenase 1, mitochondrial OS=Homo sapiens GN=GLUD1 PE=1 SV=2
EPCAM | Epithelial cell adhesion molecule OS=Homo sapiens GN=EPCAM PE=1 SV=2
FPRP | Prostaglandin F2 receptor negative regulator OS=Homo sapiens GN=PTGFRN PE=1 SV=2
HEMO | Hemopexin OS=Homo sapiens GN=HPX PE=1 SV=2
HSP7C | Heat shock cognate 71 kDa protein OS=Homo sapiens GN=HSPA8 PE=1 SV=1
IGHA1 | Immunoglobulin heavy constant alpha 1 OS=Homo sapiens GN=IGHA1 PE=1 SV=2
IGHG1 | Immunoglobulin heavy constant gamma 1 OS=Homo sapiens GN=IGHG1 PE=1 SV=1
IGHG4 | Immunoglobulin heavy constant gamma 4 OS=Homo sapiens GN=IGHG4 PE=1 SV=1
IGKC | Immunoglobulin kappa constant OS=Homo sapiens GN=IGKC PE=1 SV=2
IGLC3 | Immunoglobulin lambda constant 3 OS=Homo sapiens GN=IGLC3 PE=1 SV=1
ITIH4 | Inter-alpha-trypsin inhibitor heavy chain H4 OS=Homo sapiens GN=ITIH4 PE=1 SV=4
LAMA1 | Laminin subunit alpha-1 OS=Homo sapiens GN=LAMA1 PE=1 SV=2
LAMB1 | Laminin subunit beta-1 OS=Homo sapiens GN=LAMB1 PE=1 SV=2
| Gene Symbol | Gene Symbol | Gene Symbol |
|-------------|-------------|-------------|
| LAMC1       | Laminin subunit gamma-1 OS=Homo sapiens GN=LAMC1 PE=1 SV=3 |
| LG3BP       | Galectin-3-binding protein OS=Homo sapiens GN=LGALS3BP PE=1 SV=1 |
| NID1        | Nidogen-1 OS=Homo sapiens GN=NID1 PE=1 SV=3 |
| OLFM4       | Olfactomedin-4 OS=Homo sapiens GN=OLFM4 PE=1 SV=1 |
| PDC6I       | Programmed cell death 6-interacting protein OS=Homo sapiens GN=PDCD6IP PE=1 SV=1 |
| PGBM        | Basement membrane-specific heparan sulfate proteoglycan core protein OS=Homo sapiens GN=HSPG2 PE=1 SV=4 |
| SDCB1       | Syntenin-1 OS=Homo sapiens GN=SDCBP PE=1 SV=1 |
| TRFE        | Serotransferrin OS=Homo sapiens GN=TF PE=1 SV=3 |

**Table S4.** List of proteins present in all three CRC organoid samples (org 1-3), but absent in the empty Matrigel controls. See also Figure S3A and Table S5 (separate Excel file).

| ID          | Symbol | p-value | Fold change (EV/noEV) |
|-------------|--------|---------|-----------------------|
| A_33_P3263538 | NA     | 0.000309 | 1.543993487 |
| A_33_P3382105 | NA     | 2.00E-06 | 1.474269217 |
| A_33_P3345743 | NA     | 2.50E-05 | 1.391524844 |
| A_23_P164022  | MYO1C  | 1.20E-05 | 1.378723669 |
| A_33_P3219697 | NA     | 5.30E-05 | 1.375541818 |
| A_33_P3802146 | NA     | 3.40E-05 | 1.372367311 |
| A_33_P3265606 | NA     | 2.40E-05 | 1.353473524 |
| A_33_P3209176 | NA     | 2.20E-05 | 1.3441244 |
| A_33_P3269803 | NA     | 0.000672 | 1.337927555 |
| A_23_P52336 | UNC5B  | 0.000596 | 1.334839854 |
| A_33_P3645805 | NA     | 0.000399 | 1.325619442 |
| A_23_P109907 | ILDR1  | 0.00011  | 1.322560146 |
| A_23_P214168 | COL12A1| 0.000715 | 1.322560146 |
| A_24_P734953 | TRNP1  | 8.80E-05 | 1.322560146 |

**Table S6.** The EX biogenesis gene set. Genes with known function in EX biogenesis / secretion were collected from the literature.
| gene_symbol | gene_name | p-value | log2FoldChange |
|-------------|-----------|---------|----------------|
| A_23_P60079 | ANGPT2    | 0.000106| 1.319507911    |
| A_23_P382199 | VAPA     | 0.000105| 1.31646272     |
| A_23_P146077 | ZNF395   | 7.80E-05| 1.310393404    |
| A_33_P3246883 | NA   | 0.000128| 1.304352069    |
| A_33_P3451157 | NA   | 0.000129| 1.295342252    |
| A_23_P152858 | COPRS    | 0.000523| 1.29352831     |
| A_33_P103718 | DST     | 0.000513| 1.290463977    |
| A_33_P3239267 | NA   | 0.00043 | 1.289370309    |
| A_33_P3421219 | NA   | 0.000215| 1.289370309    |
| A_33_P3752697 | NA   | 0.000404| 1.289370309    |
| A_23_P52727 | NAV2     | 0.000496| 1.286394669    |
| A_33_P69908 | GLRX     | 0.000196| 1.283425898    |
| A_33_P3239267 | NA   | 0.000163| 1.280463977    |
| A_33_P3451157 | NA   | 0.000179| 1.277508892    |
| A_23_P99927  | NOP56    | 0.000156| 1.271619167    |
| A_24_P31704  | KRT80    | 0.000288| 1.271619167    |
| A_33_P3338961 | NA   | 0.000492| 1.271619167    |
| A_32_P41375  | LINC01000| 0.000204| 1.268684494    |
| A_33_P3280066 | NA   | 0.000493| 1.268684494    |
| A_23_P59602  | MIOS     | 0.000291| 1.265756594    |
| A_23_P73780  | IRAK1    | 0.000363| 1.265756594    |
| A_24_P370887 | VAMP3    | 0.000494| 1.265756594    |
| A_33_P3321781 | NA   | 0.000175| 1.265756594    |
| A_33_P3364696 | NA   | 0.000273| 1.262835451    |
| A_23_P135616 | STX18    | 0.000411| 1.25992105     |
| A_23_P28466  | DAW1     | 0.000484| 1.25992105     |
| A_23_P69586  | FAT1     | 0.000385| 1.25992105     |
| A_23_P75380  | AIP      | 0.000385| 1.25992105     |
| A_24_P304051 | GSTO1    | 0.000859| 1.25992105     |
| A_33_P3241596 | NA   | 0.00067 | 1.25992105     |
| A_23_P201342 | DVL1     | 0.000574| 1.257013375    |
| A_23_P162945 | SRP54    | 0.000356| 1.25411241     |
| A_33_P3234487 | NA   | 0.000875| 1.25411241     |
| A_33_P3262191 | NA   | 0.00043 | 1.25411241     |
| A_33_P3317253 | NA   | 0.000388| 1.25411241     |
| A_24_P252130 | PPARD    | 0.000725| 1.251218139    |
| A_33_P3243878 | NA   | 0.000412| 1.251218139    |
| A_33_P3243907 | NA   | 0.000388| 1.251218139    |
| A_33_P3330333 | NA   | 0.00033 | 1.251218139    |
| A_24_P116242 | KLHDC2   | 0.000541| 1.248330549    |
| A_24_P160413 | NA      | 0.000579| 1.248330549    |
| A_33_P3280044 | NA   | 0.000528| 1.248330549    |
| A_33_P3422289 | NA   | 0.000373| 1.245449623    |
| A_23_P166663 | APPL1    | 0.000771| 1.242575344    |
| A_33_P3233841 | NA   | 0.000522| 1.242575344    |
| A_33_P3220437 | NA   | 0.000421| 1.2397077      |
| A_33_P3292179 | NA   | 0.000541| 1.2397077      |
| A_33_P3407324 | NA   | 0.00056 | 1.2397077      |
| A_23_P117971 | NA      | 0.000708| 1.236846674    |
| A_23_P145863 | S100A11  | 0.000596| 1.236846674    |
| A_23_P87742  | IFFO1   | 0.000871| 1.236846674    |
| A_33_P3397795 | NA   | 0.000612| 1.236846674    |
| Gene Symbol | Gene Name | Fold Change |
|-------------|-----------|-------------|
| A_23_P14083 | AMIGO2    | 1.233992249 |
| A_33_P3265956 | NA    | 1.233992249 |
| A_33_P3289561 | NA    | 1.233992249 |
| A_33_P3354499 | NA    | 1.231144413 |
| A_24_P51588 | CCNL2    | 1.22830315  |
| A_33_P3362321 | NA    | 1.22830315  |
| A_33_P3389561 | NA    | 1.225468442 |
| A_33_P3354499 | NA    | 1.222640278 |
| A_24_P51588 | CCNL2    | 1.222640278 |
| A_33_P3362321 | NA    | 1.222640278 |
| A_33_P3389561 | NA    | 1.222640278 |
| A_33_P3354499 | NA    | 1.222640278 |

**Table S7.** Differentially regulated genes between fibroblasts treated with EV-enriched and EV-depleted, CRC-derived medium. Cells were treated for 2 days (p<0.001, no multiple testing correction).
**Figure S1.** EVs derived from colonies can be detected in cultures in 3D matrix. A) Particle number of the medium, supernatant derived from Matrigel or BME matrix, with or without equal numbers of SW1222 cells. Colonies were cultured for 12 days and after changing the medium, samples were collected at 48 h (qNano, 100 nm pore size, n=3, paired t-test). B) The distribution of particle size in supernatant derived from SW1222 colonies after 12 days in Matrigel (grey) or BME (orange). Samples for measurements were collected after 48 h. C) Particle concentrations measured with NanoPore membranes of different pore sizes (qNano, n=4, ANOVA). Supernatants from SW1222 colonies grown in Matrigel were collected after 48 h. The background particle number in Matrigel control was subtracted from the values for each pore size. (D) Colony forming efficiency of SW1222 cells in Matrigel or BME (left panel) or in Matrigel with or without 2.5% EV-free FBS (n=3, paired t-test). E-F) The percentage of positive beads in 2D HT29 (E) or SW1222 (F) cultures with increasing cell numbers (flow cytometry, n=2-3, ANOVA). Since in case of SW1222 cultures, the percentage of CD81+ beads was below 5% even at a high cell number (1 x 10^6 cells), only results with anti-CD63-coated beads were used. G) Particle concentration at different cell numbers in HT29 cell cultures (qNano, 400 nm pore size, n=3, ANOVA). H) The percentage of positive beads coated with anti-CD63 or Streptavidin (Str) and detected by anti-CD63. Beads were incubated in 3D culture supernatant with (SW1222) or without (medium) colonies (flow cytometry, n=3, t-test). In all experiments, 400 μl supernatant from the indicated cell number was used for EV detection.
**Figure S2.** CD81+ EVs can be detected in the medium of 3D CRC colony cultures. A) The morphology of 3D colonies derived from the indicated cell lines (scale bar: 50 μm). B-C) Percentage of CD81+ beads incubated in the supernatant of Matrigel cultures (B) or suspension cultures (C). Data are normalized to 500,000 cells (flow cytometry, n=3). D) Representative flow cytometric measurement of cell surface PTK7 expression in CRC organoid-derived cells (control: isotype control sample).
Figure S3. Proteomic characterization and miRNA profiling of CRC organoid-derived EVs. A) Venn diagram of the detected proteins in three CRC organoid lines. See also Table S4 and S5. B-D) 35-Ct values for the indicated miRNAs after isolating EVs with different methods. UC: serial centrifugation and ultracentrifugation (B), Kit: ExoRNEasy Serum/Plasma Starter Kit (Qiagen (C)), Bead: anti-CD63-coated beads (D). In each case, equal cell numbers and culture volumes were used in the experiments (TaqMan assay, n=3).
**Figure S4.** Survival and expression level analysis of the COL1A1 and COL1A2 genes encoding collagen I. A) Kaplan-Meier survival plot for the indicated genes (low expression: z-score normalized data < -0.5*SD, high expression group: expression level > 0.5*SD, medium: -0.5*SD < z-score normalized value < 0.5*SD). p-value derived from log rank test is shown (probe ID for COL1A1: 202311_s_at, for COL1A2: 202404_s_at). B) Normalized expression level of the indicated genes in the TCGA gene set (Oncomine analysis, www.oncomine.org, reporter A_23_P207521 for COL1A1 and A_23_P255247 for COL1A2 are shown. Note that results were significant for all the reporters).
Figure S5. The EX biogenesis gene set shows no positive enrichment in intestinal tumor cells. Gene Set Enrichment Analysis (GSEA) of an expression dataset from human CRC organoid library, containing adenoma samples as well (A) and from mouse intestinal adenoma cells (B). In both cases the human or mouse adenoma samples were compared to WT controls. Note that KEGG cell cycle and Wnt target gene sets showed a positive enrichment (positive controls). Nominal p-values are indicated.
Figure S6. CRC organoid-derived EVs do not activate fibroblasts. A) Percentage of CD81+ and CD63+ beads, detected by anti-CD81 and anti-CD63 antibodies, respectively (flow cytometry). Beads were incubated in the supernatant or resuspended (in PBS) pellet derived from 3D cultures with or without organoids after UC. Note that positive beads were detected only in the pellet of organoid cultures (n=2). B) Hierarchical clustering of microarray data of human colon fibroblasts. Fibroblasts were treated with EV-enriched and EV-depleted medium from Matrigel without or with the indicated organoids (EVs were collected from 300,000 cells and ultracentrifuged). RNA was isolated after 48 h. C-D) Relative wound area compared to the initial area in confluent fibroblast cultures treated with EV-enriched (EV) or EV-depleted (noEV) medium produced with UC from the indicated organoid cultures. Organoids were cultured in normoxia (C) or hypoxia (D). Control: samples without organoids, no treatment: fibroblasts without CRC medium. E) Relative RNA level of genes associated with fibroblast activation. CRC organoid-derived supernatants (cultured in normoxia or hypoxia for 3 days) were collected, ultracentrifuged and fibroblasts were treated with EV-enriched and EV-depleted medium in normoxia or hypoxia for 2 days. Matr: fibroblasts treated with samples without organoids. Note that CRC-derived EVs have no inducing effect (compare red and green bars). RT-qPCR, n=3. ANOVA and Tukey post hoc test, only the significance of the comparison of CRC noEV and CRC EV is shown.