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# Analysis of extracellular vesicles of frequently used colorectal cancer cell lines

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## Abstract

Colorectal cancer (CRC) ranks as the second most prevalent malignancy globally, highlighting the urgent need for more effective diagnostic and therapeutic strategies, as well as a deeper understanding of its molecular basis. Extensive research has demonstrated that cells actively secrete extracellular vesicles (EVs) to mediate intercellular communication at both proximal and distal sites. In this study, we conducted a comprehensive analysis of the RNA content of small extracellular vesicles (sEVs) secreted into the culture media of five frequently utilised CRC cell lines (RKO, HCT116, HCT15, HT29, and DLD1). RNA sequencing data revealed significant insights into the RNA profiles of these sEVs, identifying nine protein-coding genes and fourteen long non-coding RNA (lncRNA) genes that consistently ranked among the top 30 most abundant across all cell lines. Notably, the genes found in sEVs were highly similar among the cell lines, indicating a conserved molecular signature. Several of these genes have been previously documented in the context of cancer biology, while others represent novel discoveries. These findings provide valuable insights into the molecular cargo of sEVs in CRC, potentially unveiling novel biomarkers and therapeutic targets.

**Keywords** Colorectal cancer, CRC, Extracellular vesicles, Exosomes, Cell line, EV, EV cargo, EV content

## Background

Colorectal cancer (CRC) remains the second leading cause of cancer-related mortality worldwide, with an estimated 1.93 million new cases diagnosed in 2022 alone [1]. This statistic stresses the urgent need for the development of more effective diagnostic and therapeutic strategies, and also for a deeper understanding of CRC's molecular basis. Advances in cell biology have emphasized the essential function of extracellular vesicles (EVs) in intercellular communication, and highlighted their involvement in a wide range of biological processes, including immunological responses [2], inflammation [3], tissue regeneration [4], as well as cancer development [5], and metastasis [6]. EVs carry a cargo of proteins, lipids, and nucleic acids that reflect the physiological state of their parent cells [7], and could be used as potential disease biomarkers or therapeutic agents [8, 9].

In CRC, EVs play an important role in mediating complex interactions within the tumour microenvironment, specifically by facilitating communication between cancer cells and the surrounding stromal and immune cells. This intercellular crosstalk promotes tumour growth, survival, and the establishment of pre-metastatic niches [10–12]. EVs derived from CRC cells can manipulate both local and distant cellular environments, enhancing

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angiogenesis, and metastasis [13, 14]. Additionally, they play a significant role in modulating the response to chemotherapy and affecting the overall therapeutic outcome. Analysing the RNA content of EVs can therefore provide valuable insights into how CRC tumours might respond to specific chemotherapeutic agents, potentially guiding more personalized and effective treatment strategies [11, 15–18]. RNA content of EVs often reflects RNA profile of the host cell, although selective packaging mechanisms also play a role, particularly in cancer pathogenesis, where EVs selectively incorporate and transport oncogenic cargos to target cells [11, 19]. This selective transfer facilitates processes such as the modulation of interactions within the tumour microenvironment, promotion of angiogenesis and development of metastasis, thereby emphasizing the involvement of EVs in tumour progression and metastatic dissemination [12, 20].

This study aimed to describe the RNA content of small EVs (sEVs) released into culture media by frequently used CRC cell lines, by utilising comprehensive sequencing techniques. We compared the molecular profiles of five distinct CRC cell lines and their corresponding sEVs, with sizes typically up to 200 nm [21]. In the analysis of expression data, we focused on protein-coding genes and long non-coding RNAs (lncRNAs), which was our main focus in the previous study [22], and placing the findings in a broader context of CRC, EVs and signalling in cancer.

## Methods

### Cell culture and reagents

Human CRC cell lines were obtained from the American Type Culture Collection (ATCC, USA). RKO, HCT116, HCT15, HT29 and DLD1 cells were cultured at 37 °C and 5% $CO_2$ and supplemented with 10% fetal bovine serum (Gibco, USA) and 1% Penicillin–Streptomycin (Gibco, USA). We followed the ATCC recommendations for the choice of growth media for each of the respective cell line. Cells were in a culture for 48 h before the collection of 200 ml of cell conditioned media.

### Isolation of sEVs from cell conditioned media

For the isolation of sEVs from 200 ml of conditioned media, the following centrifugation steps were carried out at a temperature of 4 °C. First to remove cell organelles and debris, sEV sample was spun at 300 × g for 10 min using an Eppendorf 5810R centrifuge equipped with an A-4–81 rotor. This was followed by a 15-min centrifugation at 2,000 × g to sediment larger particles and microvesicles. Next, a 40-min centrifugation at 10,000 × g was performed in Optima XPN ultracentrifuge, utilising a Rotor 45Ti (both Beckman Coulter, USA). Finally, the sample was centrifuged at 100,000 × g for 100 min, also with the Rotor 45Ti. The visible pellet was resuspended in 1.2 ml of cold PBS that had been previously filtered through 0.45 µm pore cellulose acetate membrane (Corning, USA). A 200 µl aliquot of sEVs was kept for the assessment of EV size and concentration. For enzymatic treatment of the remaining sEV sample, 20 µl of proteinase K (20 mg/ml) and 10 µl of RNase Cocktail Enzyme Mix (20,000 U/ml) were added individually and incubated each 10 min at for 37 °C. The activity of RNase was inhibited by the addition of 8 µl of SUPERase In RNase Inhibitor (20 U/µl) (all Invitrogen, USA).

### Negative stain transmission electron microscopy

Four microliters of sEV fraction were deposited on copper grids that were plasma-cleaned and coated with a carbon film. Next, the grids were stained using 2% uranyl acetate solution. A brief incubation period of 30 s was followed by one-minute staining. The prepared grids were inserted into a Talos F200C transmission electron microscope (ThermoScientific, USA), which was set to operate at an accelerating voltage of 200 kV. Imaging of sEVs was performed using a Ceta-16 M CMOS camera. These images were captured at a nominal magnification of 36,000 times, with an underfocus set to 2–4 µm.

### Multi-angled dynamic light scattering

The assessment of particle size and concentration involved placing 50 µl of the sEV sample into a low-volume quartz batch cuvette, model ZEN2112 (Malvern Panalytical Ltd, UK). Measurements were performed by multi-angle dynamic light scattering method with the Zetasizer Ultra (Malvern Panalytical Ltd, UK), maintaining a steady temperature of 25 °C. Data from the light scattering were recorded at three distinct angles: 173°, 90°, and 13°. Then they were analysed using the ZS Xplorer software, version 2.50 (Malvern Panalytical Ltd, UK). The reported results for hydrodynamic diameter, polydispersity index, and concentration are expressed as the mean of three independent measurements ($n = 3$), along with the standard deviation.

### Isolation of RNA from sEVs

Total RNA was isolated from sEVs by Monarch Total RNA Miniprep Kit (New England Biolabs, USA). First, a volume of lysis buffer equivalent to the sEV volume was pipetted to the sample and mixed by brief vortexing. Next, a volume of ethanol (≥ 95%) corresponding to the volume of the lysed sample was added and mixed thoroughly. The subsequent steps, including DNase treatment, were performed according to the manufacturer's instructions. RNA was eluted with 50 µl of nuclease-free water. RNA concentration was determined using the QuantiFluor RNA System (Promega, USA), with

fluorescence measurements performed on a Quantus Fluorometer following the manufacturer's protocol. Isolated RNA was stored at −80 °C before the library preparation and RT-qPCR analysis.

#### Library preparation and RNA sequencing of sEVs from cell conditioned media

Library preparation for sEV content derived from CRC cell lines started with the RNA input 11.5 ng, with rRNA depletion performed on this initial RNA input using the RiboCop for Human/Mouse/Rat V2 kit (Lexogen, Austria). Following this step, sequencing libraries were constructed using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England BioLabs, USA). Libraries were prepared according to the manufacturer's guidelines with following changes. Fragmentation was carried out for 5 min. Additionally, the UMI universal adapter Kapa (15 µM) was used at a dilution of 50×in 0.1×TE buffer. The libraries received individual barcodes through Kapa UDI Primer Mixes (Roche), and their concentration and quality were evaluated. Fluorometric assessment was performed using the Qubit 4.0 Fluorometer coupled with the Qubit HS DNA Assay Kit (Thermo Fisher Scientific, USA). Electrophoretic analysis was performed utilising the Agilent 2200 TapeStation System alongside the High Sensitivity D1000 ScreenTape (Agilent Technologies, USA). After quality confirmation, each library was diluted to a uniform concentration of 4.5 nM and pooled in equimolar proportions in preparation for clustering. RNA sequencing was carried out using the NextSeq 500/550 High Output Kit v2 on the NextSeq 500/550 system (Illumina, USA), with the run configured for single read sequencing over 75 cycles.

#### RNA-seq data analysis

Bcl files were converted to Fastq format using bcl2fastq v. 2.20.0.422 Illumina software for basecalling. Quality check of raw single-end fastq reads was carried out by FastQC. The adapters and quality trimming of raw fastq reads was performed using Cutadapt v4.3 with Illumina adapter trimming and parameters -m 35 -q 0,20. Trimmed RNA-seq reads were mapped against the human genome (hs38) and Ensembl annotation release 110 using STAR v2.7.11 as splice-aware short read aligner and default parameters except –outFilterMismatchNoverLmax 0.66 and –twopassMode Basic. Quality control after alignment concerning the number and percentage of uniquely- and multi-mapped reads, rRNA contamination, mapped regions, read coverage distribution, strand specificity, gene biotypes, and PCR duplication was performed using several tools namely RSeQC v4.0.0, Picard toolkit v2.25.6 (5), and Qualimap v.2.2.2.

Gene counts have been produced by featureCounts from Subread package v2.0 with parameter-t gene, and further normalised using the Bioconductor package DESeq2 v1.34.0. Genes with a normalised count above a value of 300 for each sample were considered for enrichment analysis. Gene ontology enrichment and KEGG pathway enrichment were obtained using the clusterprofiler package v4.6.2.

#### RT-qPCR validation of sEV content

RNA samples isolated from sEVs were concentrated to 6 µl using a Concentrator Plus 5305 Vacuum Centrifuge (Eppendorf AG, Germany). Complementary DNA was synthesized from 5 µl of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) following the manufacturer's instructions. A preamplification step was performed by mixing 2.5 µl of cDNA with 5 µl of TaqMan PreAmp Master Mix (Applied Biosystems, USA) and 2.5 µl of pooled 200 nM primers synthesized by Integrated DNA Technologies (IDT) (list of primers in Supplementary Table S10), followed by 14 cycles of preamplification. Quantitative PCR reaction mix included 5 µl of PowerUP SYBR Green Master Mix (Applied Biosystems, USA), 2.5 µl of preamplified cDNA diluted 20,000×in 1×TE buffer, 0.5 µl of nuclease-free water, and 2 µl of 1 µM IDT primers. Fluorescence was measured using the QuantStudio 12 K Flex Real-Time PCR system (Applied Biosystems, USA).

#### Statistical analysis of RT-qPCR data

The cycle threshold values were calculated by QuantStudio 12 K Flex software, all RT-qPCR reactions were run in triplicates. The average transcript levels (relative quantity) of all transcripts were normalised using the combination of reference genes GAPDH and ACTB and subsequently analysed by the $2^{-\Delta Ct}$ method. All calculations were performed using GraphPad Prism version 9.00 (GraphPad Software, USA).

## Results

#### Characterization of sEVs isolated from conditioned cell media

To investigate sEVs isolated from 200 ml of conditioned media of five different CRC cell lines (HCT116, DLD1, HT29, RKO, HCT15; for more detailed characteristics see Supplementary Table S9), we employed transmission electron microscopy (TEM) and multi-angled dynamic light scattering (MADLS) to assess particle size distribution, purity and concentration. TEM imaging (shown in Fig. 1a) displayed particles matching the typical size of sEVs and exhibiting the commonly referenced cup-shaped morphology [23]. The detection of sEVs was further supported by MADLS findings. Figure 1b shows

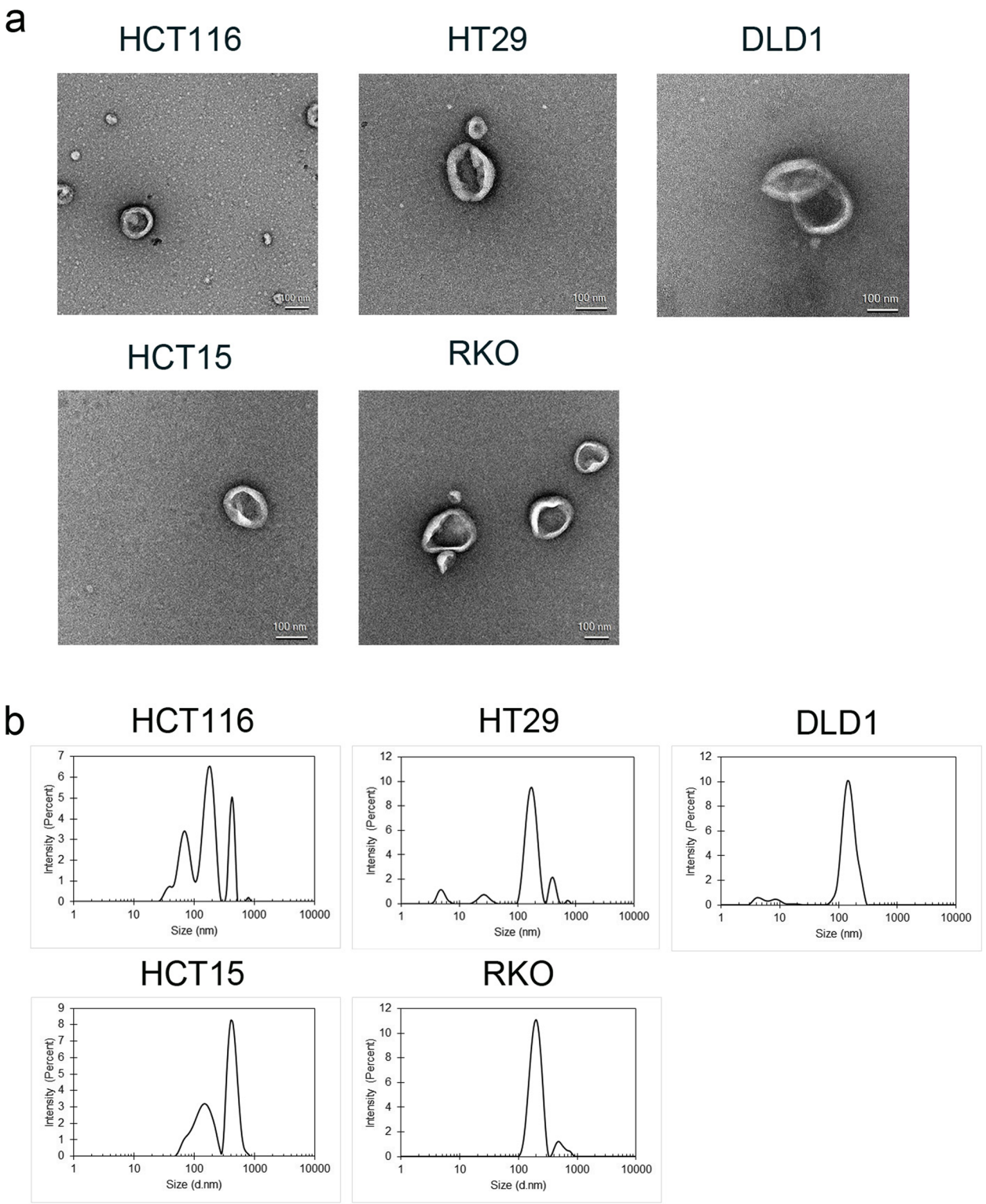


**Fig. 1** Characterization of sEV morphology and particle size. **a** Representative TEM images (scale bar, 100 nm) and (**b**) MADLS graphs of sEVs isolated from conditioned media of five different CRC cell lines

**Table 1** The overview of DLS results. Average particle size (Z-Average), polydispersity index (PDI), and concentration values obtained by MADLS analysis of EVs derived from cell conditioned media

| Cell line | Z-Average (nm) | Polydispersity Index (PDI) | Concentration (particles/ml) |
|---|---|---|---|
| **HCT116** | 161 ± 30 | 0.408 ± 0.032 | $8.2 \times 10^{10}$ |
| **HT29** | 124 ± 3 | 0.582 ± 0.032 | $5.1 \times 10^{15}$ |
| **DLD1** | 119 ± 9 | 0.559 ± 0.028 | $7.6 \times 10^{7}$ |
| **HCT15** | 218 ± 16 | 0.446 ± 0.016 | $1.3 \times 10^{9}$ |
| **RKO** | 194 ± 13 | 0.232 ± 0.085 | $1.1 \times 10^{9}$ |

### RNA sequencing of sEVs released by CRC cell lines

RNA was isolated from five sEV samples obtained from 200 ml of conditioned media for each cell line. Despite the lower starting RNA input from sEVs, we successfully prepared sequencing libraries from all samples. The overview of gene biotypes detected by RNA sequencing is shown in Fig. 2. Importantly, the biotype distribution shows a remarkable similarity across all cell lines. The complete results are included in the Supplementary Information (Supplementary Table S7).

First, we compared the RNA content of sEVs across five different CRC cell lines and identified that all sEV samples shared 2893 genes, including protein-coding genes,

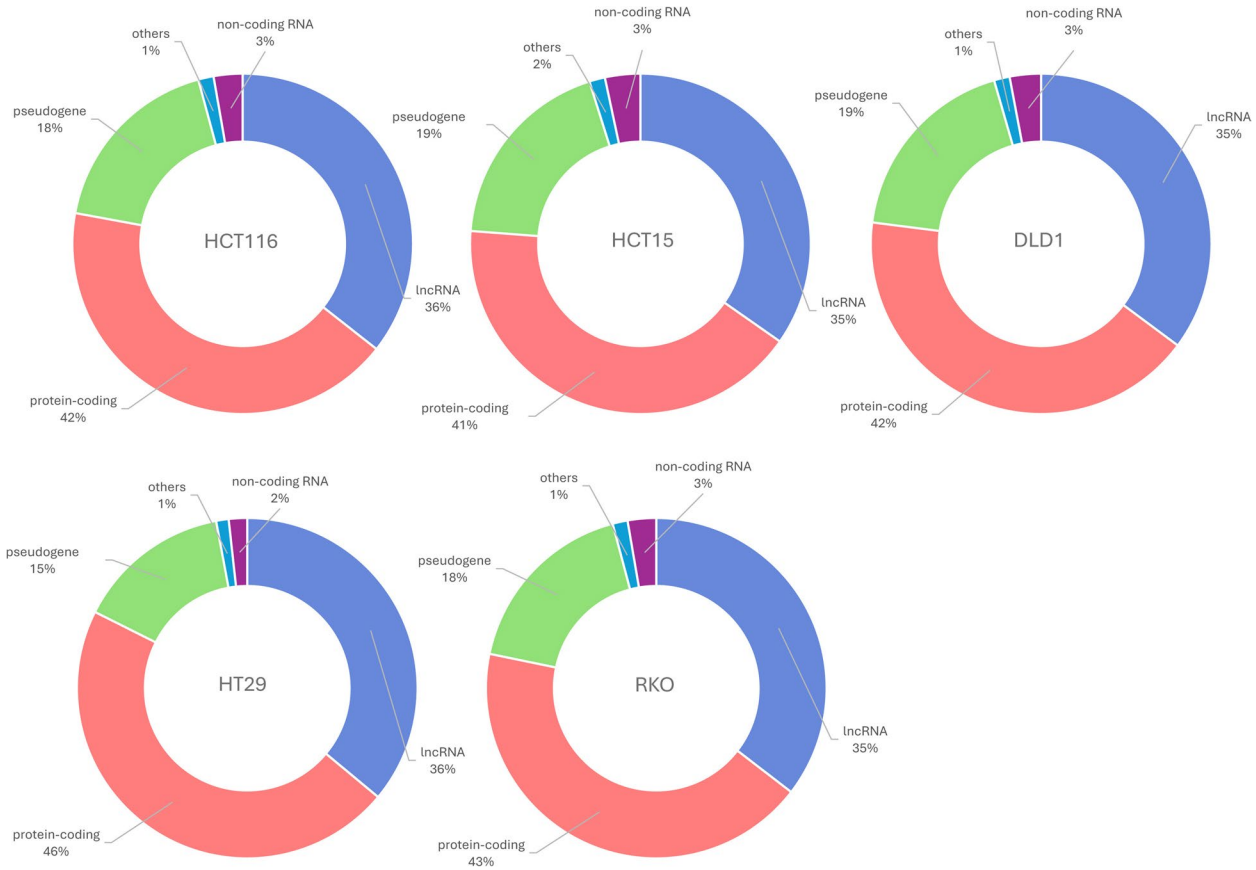


**Fig. 2** Gene biotypes detected in sEVs derived from CRC cells

graphs with a visible peak within the 50–200 nm range, aligning with the expected dimensions of these vesicles. Additionally, there are visible peaks in the size ranges of 4–30 nm and 300–500 nm, suggesting the presence of proteins or larger particles, respectively. Average particle size, particle concentration and the polydispersity index (PDI) showed variations across CRC cell lines, with values specified in Table 1.

lncRNAs, pseudogenes and other types of non-coding RNA with the normalised counts ≥ 300 (Fig. 3a). Notably, the shared sEV content accounted for 64% to 78% of all genes, with HT29 having 64% and HCT116 and HCT15 having up to 78% of their genes in common with the other cell lines.

When we used the same cut-off value for protein-coding genes, we generated a list of 2226 common genes among the sEV samples (Fig. 3b). The similarity in gene

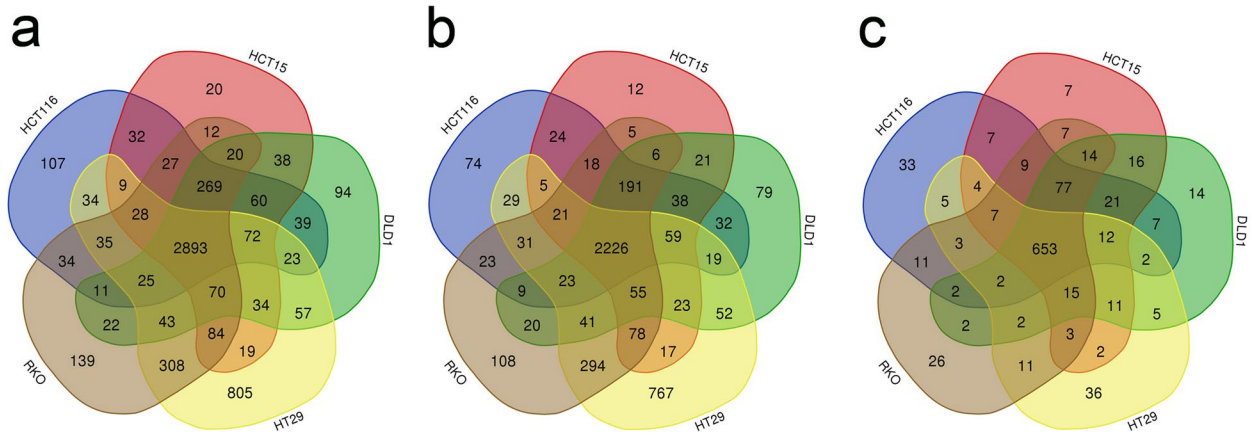


**Fig. 3** RNA content shared across sEVs from five CRC cell lines. Venn diagrams representing the number of common (**a**) protein-coding and non-coding RNAs (**b**) only protein-coding genes, and (**c**) only lncRNAs. The comparison of gene numbers was based on a normalised count cut-off value defined as ≥ 300. Venn diagrams were calculated and drawn using an online tool available at https://bioinformatics.psb.ugent.be/webtools/Venn/

profiles across the cell lines for protein-coding genes ranged from 60% in HT29 to 80% in HCT15. Focusing only on lncRNAs (Fig. 3c), we identified 653 genes that were consistently most abundant in the analysed samples, with the shared content ranging between 76% in HCT116, HCT15, and DLD1 to 84% in RKO.

Next, we analysed the RNA content of sEVs derived from five CRC cell lines to identify the most abundant genes. We focused on two types of genes: protein-coding genes and lncRNAs. For each cell line, we selected the top 30 most abundant genes of each type (Supplementary Tables S1 and S2). To identify the genes consistently elevated in all sEVs released by the cells, we compared these individual top 30 lists. This comparison revealed a significant overlap, with nine protein-coding genes and fourteen lncRNA genes shared across sEV samples derived from CRC cell lines. The high overlap underscores the consistency of the RNA content in sEVs across different CRC cell lines. The list of these most abundant genes is provided in Table 2.

Because the potential of miRNAs as non-invasive biomarkers for early detection and risk assessment of CRC has been extensively studied [8, 9, 17], a table listing all miRNAs detected by RNA-seq of growth medium-released sEVs from the five CRC cell lines studied is provided in the Supplementary Information (Supplementary Table S8). However, we note that the NEBNext Ultra II Directional RNA Library Prep Kit used for library preparation is optimized for longer RNA fragments, which may result in some miRNAs being under- or overrepresented in our dataset.

#### Bioinformatic analysis of sEV RNA content

To investigate the possible biological functions of the RNAs that were contained in the sEVs, three independent gene ontologies (GO terms) including the categories of biological process (BP), molecular function (MF), and cellular component (CC) were used to describe the genes highly enriched in vesicles released by CRC cell lines in growth media. Genes with a normalised count above a value of 300 for each sample were considered for GO analysis ($p$-adjust < 0.05). Dot plots with the highest counts of GO terms (≥ 20) are shown in Figs. 4, 5 and 6. Out of those we selected GO terms that are highly relevant to CRC biology or EV biology. These are listed in Table 3. Additionally, Supplementary Tables S3-S5 contain the full results of GO terms for BP, CC, and MF, including the list of genes enriched within each GO term.

As a next step, we performed KEGG pathway analysis to elucidate the potential biological pathways implicated by the RNA content of sEVs. Only genes with normalised counts exceeding 300 and a p-adjusted value of less than 0.05 were considered for the analysis. Several pathways highly relevant to CRC biology were identified, including the Wnt signalling pathway, RAS signalling pathway, and MAPK signalling pathway. Additional pathways of interest included ErbB signalling, EGFR tyrosine kinase inhibitor resistance, focal adhesion, regulation of the actin cytoskeleton, adherens junction, and tight junction (Fig. 7). These findings are detailed in Supplementary Table S6, which provides comprehensive results of the KEGG pathway analysis, and the list of genes enriched within each pathway.

The analysis is complemented by an interaction map based on protein–protein interactions of protein-coding

**Table 2** List of top protein-coding and lncRNA genes detected as common content in sEVs across all 5 CRC cell lines. Some of the genes have already been described in the literature in relation to cancer biology, or specifically to CRC pathogenesis. Detailed results of RT-qPCR validation of the most abundant transcripts are given in the Supplementary Figures S1-S2

| Ensembl ID | Gene symbol | NCBI Gene ID | Alternative gene symbol | Gene name | References | RT-qPCR validation |
|---|---|---|---|---|---|---|
| **The list of protein-coding genes** | | | | | | |
| ENSG00000174469 | CNTNAP2 | 26047 | CDFE; NRXN4; AUTS15; CASPR2; PTHSL1 | **contactin associated protein 2** | [24–29] | N/A |
| ENSG00000183230 | CTNNA3 | 29119 | ARVD13; VR22 | **catenin alpha 3** | [30–33] | NV |
| ENSG00000150672 | DLG2 | 1740 | PSD93; PSD-93; PPP1R58; chapsyn-110 | **discs large MAGUK scaffold protein 2** | [34] | Validated |
| ENSG00000173406 | DAB1 | 1600 | SCA37 | **DAB adaptor protein 1** | [35, 36] | Validated |
| ENSG00000188107 | EYS | 346007 | RP25; SPAM; EGFL10; EGFL11; C6orf178; C6orf179; C6orf180; bA74E24.1; dJ22I17.2; bA166P24.2; bA307F22.3; dJ1018A4.2; dJ303F19.1 | **eyes shut homolog** | [37–39] | N/A |
| ENSG00000168702 | LRP1B | 53353 | LRP-1B; LRPDIT; LRP-DIT | **LDL receptor related protein 1B** | [40–45] | N/A |
| ENSG00000153707 | PTPRD | 5789 | HPTP; PTPD; HPTPD; HPTPDELTA; RPTPDELTA; R-PTP-delta | **protein tyrosine phosphatase receptor type D** | [46–48] | Validated |
| ENSG00000078328 | RBFOX1 | 54715 | 2BP1; FOX1; A2BP1; FOX-1; HRNBP1 | **RNA binding fox-1 homolog 1** | [49–51] | N/A |
| ENSG00000140836 | ZFHX3 | 463 | ATBT; SCA4; ATBF1; ATFB8; ZFH-3; ZNF927; C16orf47 | **zinc finger homeobox 3** | [52–54] | Validated |
| **The list of lncRNAs** | | | | | | |
| ENSG00000272168 | CASC15 | 401237 | CANT; LINC00340; lnc-SOX4-1 | **cancer susceptibility 15** | [55–59] | NV |
| ENSG00000229140 | CCDC26 | 137196 | RAM; GLM7 | **CCDC26 long non-coding RNA** | [60, 61] | N/A |
| ENSG00000176124 | DLEU1 | 10301 | BCMS; DLB1; LEU1; LEU2; XTP6; BCMS1; DLEU2; LINC00021; NCRNA00021 | **deleted in lymphocytic leukemia 1** | [62–65] | Validated |
| ENSG00000228262 | LINC01320 | 104355288 | - | **long intergenic non-protein coding RNA 1320** | [66] | N/A |
| ENSG00000234323 | LINC01505 | 100996590 | - | **long intergenic non-protein coding RNA 1505** | - | N/A |
| ENSG00000204929 | LINC02934 | 105369168 | - | **long intergenic non-protein coding RNA 2934** | - | N/A |
| ENSG00000254101 | LINC02055 | 107986980 | - | **long intergenic non-protein coding RNA 2055** | - | N/A |
| ENSG00000271860 | LOC101927314 | 101927314 | - | **uncharacterized LOC101927314** | - | N/A |
| ENSG00000228566 | LOC124902439 | 124902439 | - | **uncharacterized LOC124902439** | - | Validated |
| ENSG00000224184 | MIR3681HG | 100506457 | - | **MIR3681 host gene** | - | N/A |
| ENSG00000231918 | NRXN1-DT | 73010 | - | **NRXN1 divergent transcript** | - | N/A |
| ENSG00000253438 | PCAT1 | 100750225 | PCA1; PiHL; PCAT-1 | **prostate cancer associated transcript 1** | [67–73] | N/A |
| ENSG00000285939 | - | - | - | - | - | Validated |
| ENSG00000286481 | - | - | - | - | - | N/A |

*Validated* validated by RT-qPCR, *NV* not validated by RT-qPCR, *N/A* not applicable

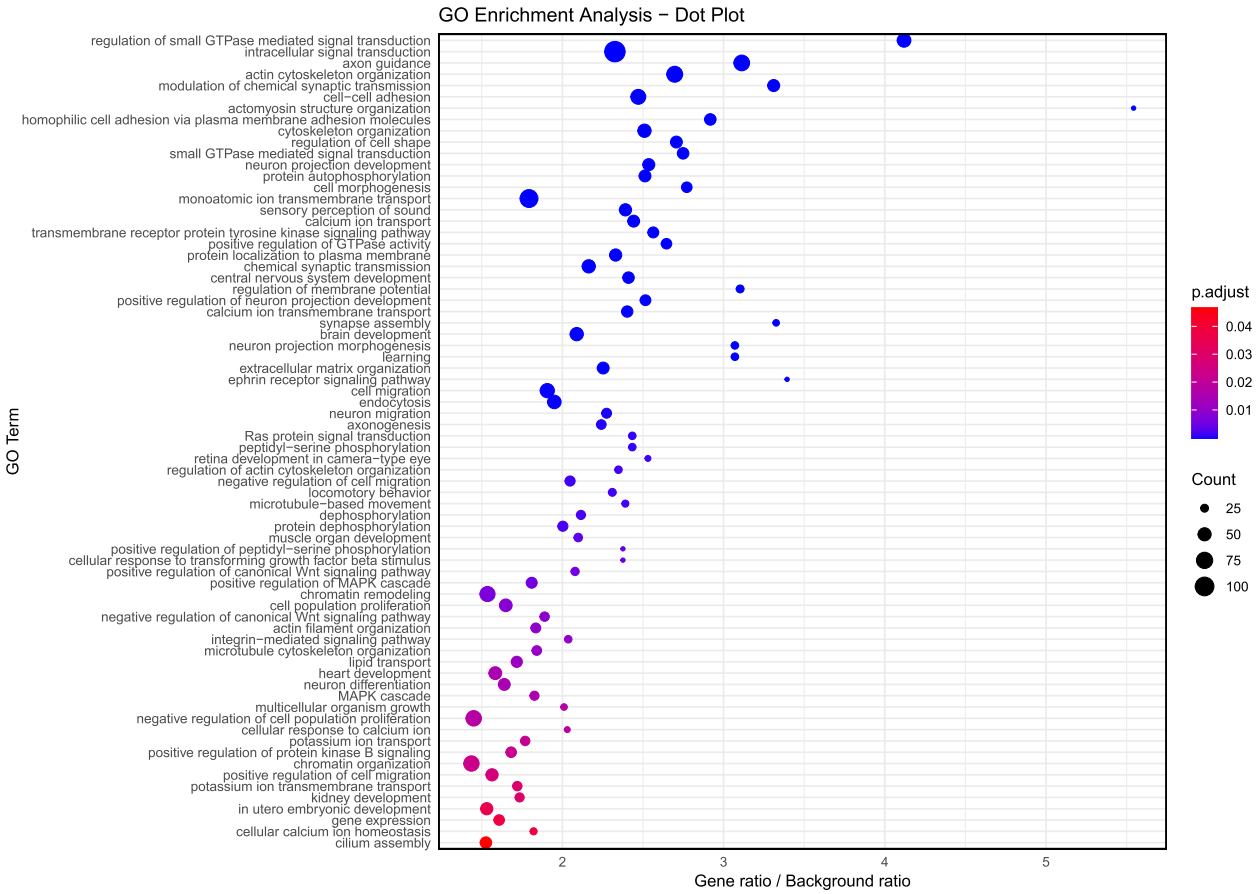


**Fig. 4** The gene ontology (GO) enrichment analysis of abundant genes detected in CRC-derived sEVs, focusing on biological processes (*p*-adjust < 0.05)

genes detected by RNA-seq analysis of the sEV content (Supplementary Figure S3) and a list of genes unique to preselected groups of basic features (level of aggressiveness and MSI-status) for the CRC cell lines used in our study (Supplementary Table S11).

## Discussion

Our analysis of sEVs derived from five different CRC cell lines revealed significant insights into the RNA content. Specifically, we identified nine protein-coding genes and fourteen lncRNAs that were consistently among the top 30 most abundant genes across all cell lines (Table 2). Some of these common genes have already been described in the literature in the context of cancer biology, while others appear to be novel.

Among the most abundant protein-coding genes, we identified nine genes, of which the majority have been described in the literature in relation to CRC or in the context of EV-associated cargo transport. The first protein-coding gene detected in our study is the giant surface receptor LRP1B (LDL receptor-related protein 1B), one of the most frequently altered genes in human cancers overall [40], including CRC [41–43]. LRP1B has been shown to play a suppressive role in colon cancer progression by negatively regulating beta-catenin/TCF (T-cell factor) signalling [44]. It is of note that the Wnt/beta-catenin pathway is recognized to be the central mechanism that drives CRC carcinogenesis and is considered a promising therapeutic target in CRC because most CRC patients have mutations in at least one gene involved in the Wnt signalling pathway, such as the beta-catenin and APC (adenomatous polyposis coli) genes [45].

Alongside LRP1B, CTNNA3 (catenin alpha 3), which plays a role in cell-substrate adhesion and may function as a cytoskeletal linker capable of modulating cell migration [30], was identified in sEVs isolated from conditioned cell media. This gene was found to be located in common fragile sites (CFSs) – large regions of significant genomic instability [31] and has been reported to function as a tumour suppressor in hepatocellular carcinoma (HCC) [32]. Skuja et al. [33] has recently shown that CTNNA3 is also frequently deleted in metastatic CRC.

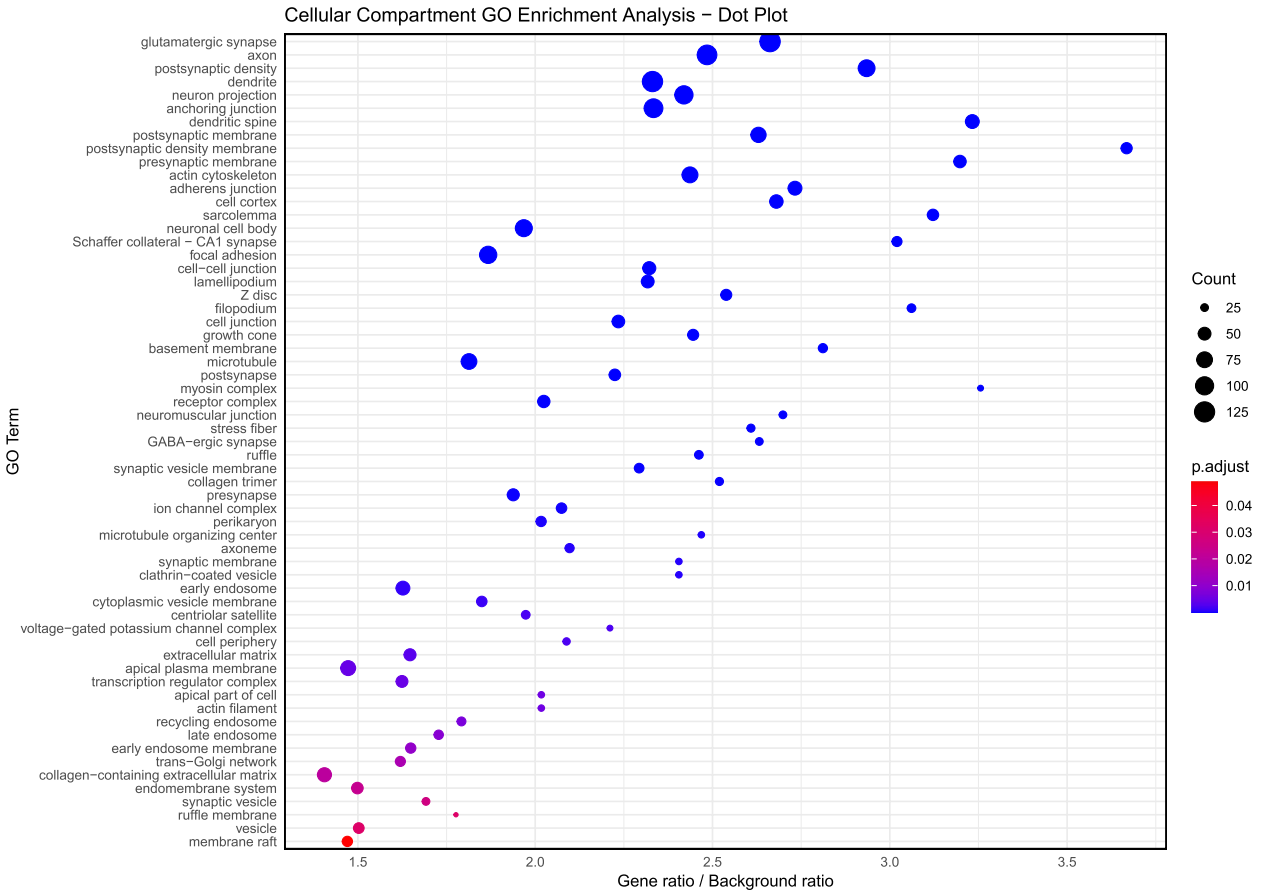


**Fig. 5** The gene ontology enrichment analysis of abundant genes detected in CRC-derived sEVs, focusing on cellular compartment (*p*-adjust < 0.05)

DAB1 (DAB adaptor protein 1), another protein-coding gene detected in our study, is one of the genes induced by NOTCH signalling in CRC and genetic depletion of this protein leads to suppression of cancer invasion and metastasis in CRC mouse model [35]. For CRC, DAB1 has been suggested to contribute to epithelial-mesenchymal transition via the reelin-DAB1 signalling pathway [36].

EYS (eyes shut homolog), the next protein-coding gene we identified, is also known as epidermal growth factor-like protein 10 or 11 (EGFL10, EGFL11). The EYS protein contains multiple epidermal growth factor (EGF)-like domains. In this context, we would like to mention the important role of the epidermal growth factor receptor (EGFR), which is a key factor in cell proliferation and development. Specifically for CRC, Hansen et al. [37] outlined the predictive value of EGFL7 with respect to first-line chemotherapy and bevacizumab treatment in patients with metastatic CRC. In the context of studying formin-like protein 2 (FMNL2), which is often associated with metastasis, He et al. [38] described that the EGFL6/CKAP4/ERK axis is involved in CRC and could therefore be a candidate therapeutic target for CRC. Another study closely related to inflammatory bowel disease (IBD) examined the effect of colon-derived EVs on fibroblast proliferation [39]. Comparative proteomics was utilised to analyse the protein content of CRC EVs isolated from healthy mice and mice with dextran sulphate sodium-induced colitis (IBD-EVs). The results showed that 109 proteins were upregulated in IBD-EVs, namely EGFR.

A recent study by Keane and colleagues [34] suggested that DLG2 (discs large MAGUK scaffold protein 2), one of the common protein-coding genes found in sEVs released from our cell lines, has an immune function in CRC (inflammasome). The authors reported that DLG2 expression is suppressed in IBD and ulcerative colitis as well as in CRC tissue, with the lowest DLG2 expression observed in larger size adenomas, compared to healthy tissues. They further showed that silencing DLG2 led to increased proliferation of colon cancer cells in vitro.

RBFOX1 (RNA binding fox-1 homolog 1) is a member of Fox-1 family of RNA-binding proteins that are evolutionarily conserved and regulate tissue-specific alternative splicing. The link between alterations in RBFOX1

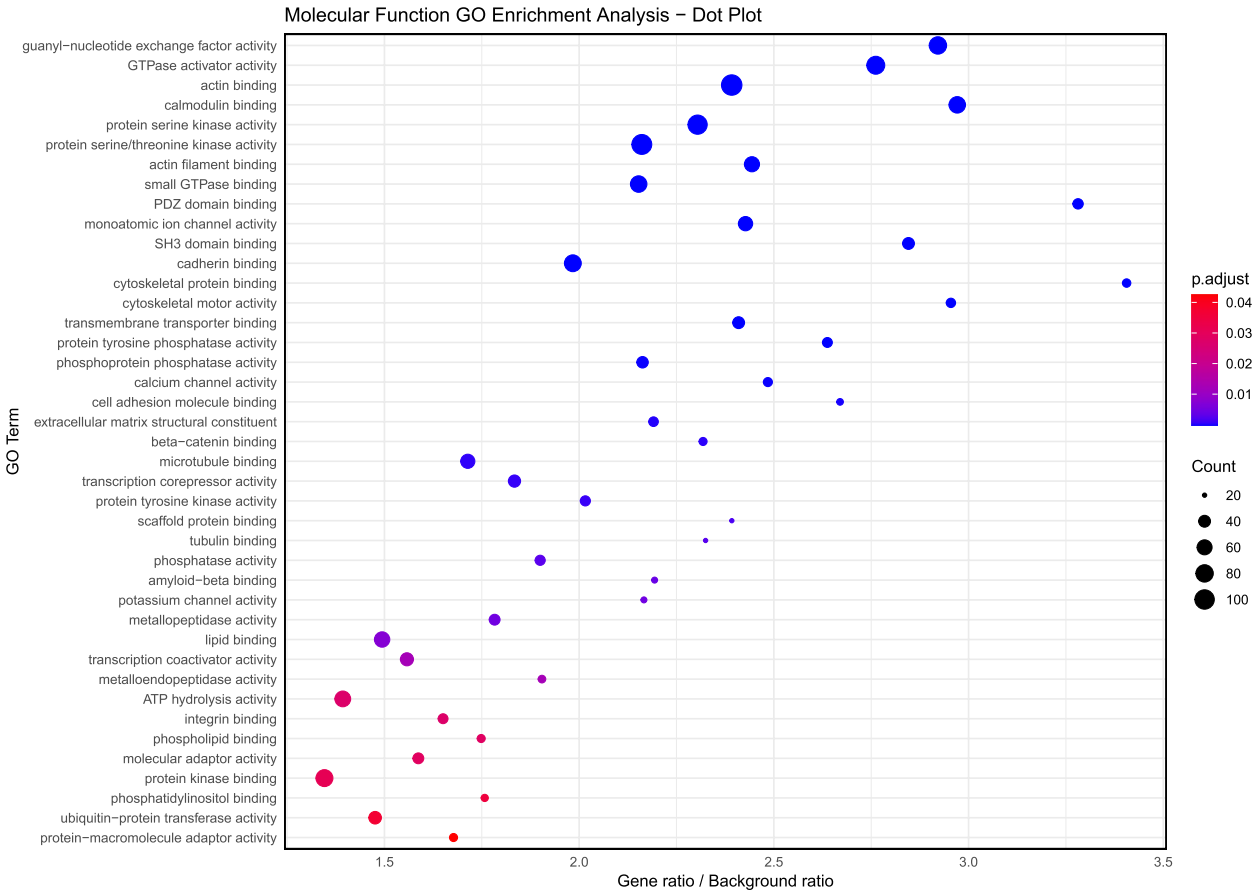


**Fig. 6** The gene ontology enrichment analysis of abundant genes detected in CRC-derived sEVs, focusing on molecular function ($p$-adjust < 0.05)

gene and CRC has been described in several studies [49–51].

RNA coding for PTPRD (protein tyrosine phosphatase receptor type D) was detected at high levels in the sEV content. This gene has been cited in the literature for its prognostic potential in metastatic CRC, either in predicting patient resistance to bevacizumab [46] or in predicting CRC liver metastasis potential based on the distribution of somatic mutations [47]. It is also of considerable interest that the lncRNA PTPRD-AS1 has recently been mentioned as one of five potential lncRNA biomarkers associated with genomic instability in CRC [48].

We have also identified CNTNAP2 (contactin associated protein 2), a member of the neurexin family that functions as a cell adhesion molecule and a receptor in the vertebrate nervous system. Alongside PTPRD, CNTNAP2 has also been reported as a marker of tumour aggressiveness in oligodendrogliomas [24], and in one case study of occult bowel cancer, strong positivity of antibodies against CNTNAP2 was also reported [25]. More generally, downregulation of contactins (CNTN) has been found in CRC stromal tissue [26], CNTN3 has been linked to prognosis and tumour-infiltrating immune cells in CRC [27] and changes in CNTN1 methylation have also been reported in CRC patient samples [28, 29].

Finally, ZFHX3 (zinc finger homeobox 3) is known to negatively regulate MUC5AC gene (mucin 5AC, oligomeric mucus/gel-forming) in gastric cancer by binding to an AT motif-like element in the MUC5AC promoter [52]. Expression of mucin is tissue specific and is closely linked to tumour growth, migration, invasion, and adhesion. Specifically, differential expression and altered glycosylation pattern of MUC5AC are closely associated with CRC development [53] and chemoresistance [54].

Among the identified lncRNAs, that have already been discussed in the literature, PCAT-1 (prostate cancer associated transcript 1) is a lncRNA that has been identified for its significant role in cancer biology. It is overexpressed in several types of cancers, including CRC [67], gastric cancer [68], prostate cancer [69], and lung cancer [70]. In these cancers, PCAT-1 has been associated with promoting tumour progression, metastasis, and chemoresistance and its high levels have been correlated with

**Table 3** Selected gene ontology (GO) terms enriched in RNA content of sEVs derived from CRC cell lines that have high relevance to either CRC pathogenesis or EV biogenesis

| GO ID | Term | Note on function of the term in the context of CRC | Reference |
|---|---|---|---|
| GO:0007265 | RAS Protein Signal Transduction | Often mutated pathway in CRC, leading to increased proliferation and survival of cancer cells | [74] |
| GO:0043410 | Positive Regulation of MAPK Cascade | Often mutated pathway in CRC, leading to increased proliferation and survival of cancer cells | [75] |
| GO:0030335 | Positive Regulation of Cell Migration | Essential for metastasis, which is the leading cause of mortality in CRC patients | [76] |
| GO:0090090 | Positive Regulation of Canonical Wnt Signalling Pathway | Dysregulation of the Wnt pathway in CRC is a hallmark of the disease | [77] |
| GO:0030198/ GO:0031012 | Extracellular Matrix Organisation /Extracellular Matrix | Structural changes in the tumour microenvironment promote cancer progression and metastasis | [78] |
| GO:0005769 | Early Endosome | Role in the formation and release of EVs, which are essential for intercellular communication in cancer cells | [79] |
| GO:0005802 | Trans-Golgi Network | Essential for EV biogenesis, influencing the cargo they carry and their role in communication of tumour cells | [80] |
| GO:0015629 / GO:0003779 | Actin Cytoskeleton / Actin Binding | Involved in the maintenance of cell shape, motility and division. Its reorganisation is essential for tumour cell invasion and migration. EV content may influence actin dynamics in recipient cells and promote metastatic behaviour | [81] |
| GO:0005925 | Focal Adhesion | Focal adhesion sites play a key role in cell migration and invasion, processes that accompany and condition tumorigenesis | [81] |
| GO:0004713 | Protein Tyrosine Kinase Activity | Tyrosine kinases are key to signalling pathways that regulate growth, cell differentiation and survival. In cancers, including CRC, they are often dysregulated. EVs can carry tyrosine kinases or activate these pathways in recipient cells to promote oncogenic signalling | [82] |
| GO:0005096 / GO:0031267 | GTPase Activator Activity / Small GTPase Binding | GTPases are involved in a variety of cellular processes, including cell growth and migration. In CRC, small GTPases are often dysregulated, which promotes tumour progression. GTPase activators regulate these proteins and are essential to the functioning of these signal transduction pathways. EVs may carry small GTPases or modulators that affect signalling in recipient cells | [83] |

poor prognosis and reduced overall survival in patients . Focusing on the role of PCAT-1 in CRC, Ge et al. [71] found that PCAT-1 was overexpressed in 64% of CRC samples, particularly in cases with distant metastasis, marking it as an independent prognostic factor. Qiao et al. [72] showed that down-regulation of PCAT-1 in CRC cells inhibited proliferation and induced cell cycle arrest and apoptosis, while in vivo experiments indicated slower tumour growth. Conversely, Barbagallo et al. [73] reported no significant difference in PCAT-1 expression between CRC tissues and normal tissues or between serum EVs from patients and healthy individuals.

DLEU1 (deleted in lymphocytic leukemia 1) was another abundant lncRNA in sEVs from CRC cells. While its exact function is still under investigation, recent studies have shown it to play a role in the development and progression of various cancers [62–64]. In the context of CRC, the study by Liu and colleagues [65] found DLEU1 upregulated in CRC tissues, particularly in advanced stages, with higher expression linked to lower survival rates, suggesting its potential as a biomarker for CRC prognosis. DLEU1 played a critical role in regulating malignant behaviour of CRC cells.

Similarly, in the study by Hu et al. [66], LINC01320 (long intergenic non-protein coding RNA 1320) was upregulated in gastric cancer tissues and cell lines, correlating with lower patient survival rates. Its overexpression promoted cancer cell proliferation, migration, and invasion, while knockdown inhibited these processes. METTL14-mediated m6A modifications enhanced

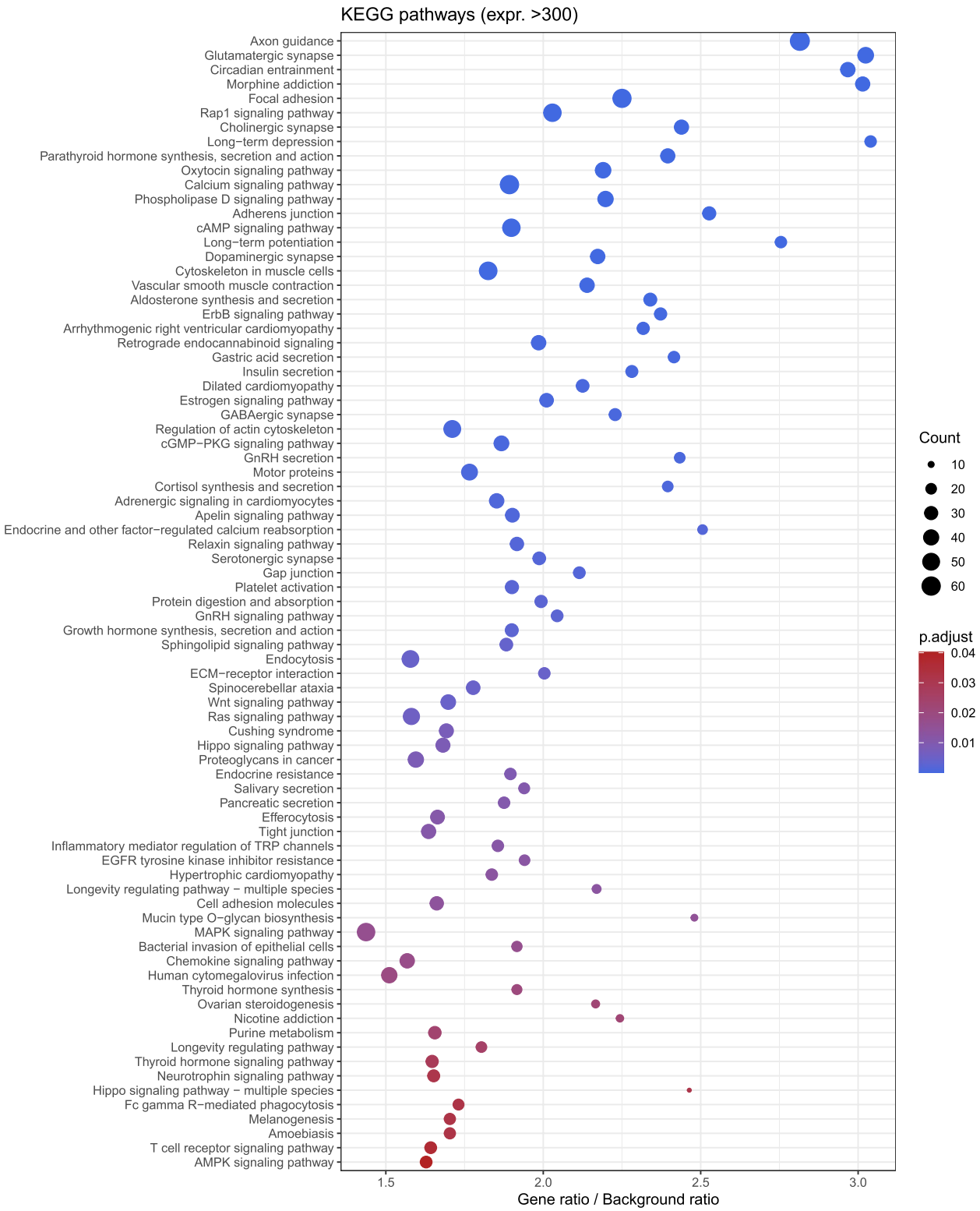


**Fig. 7** The KEGG pathway enrichment analysis of abundant genes detected in CRC-derived sEVs (*p*-adjust < 0.05)

LINC01320 stability, allowing it to sponge miR-495-5p and increase RAB19 expression. This METTL14/LINC01320/miR-495-5p/RAB19 axis promoted aggressive gastric cancer traits, suggesting potential therapeutic targets.

CASC15 (cancer susceptibility 15) also plays a crucial role in various cancers. The study by Jing et al. [55] revealed that this lncRNA was significantly upregulated in colon cancer tissues and cell lines, correlating with advanced TNM stages and metastasis. CASC15 promoted colon cancer cell proliferation, migration, and invasion by acting as a competing endogenous RNA (ceRNA) for miR-4310, upregulating LGR5 expression and activating the Wnt/beta-catenin signalling pathway. Knockdown of CASC15 significantly inhibited these oncogenic processes in vitro and reduced tumour growth in vivo. Fernando et al. [56] found CASC15 upregulated in RUNX1-rearranged acute leukemia, influencing cellular survival, proliferation, and differentiation by regulating SOX4 expression via the transcription factor YY1. Knockdown of CASC15 decreased SOX4 expression and significantly altered the global transcriptome. He et al. [57] discovered CASC15 upregulated in HCC, correlating with larger tumour size, advanced stage, metastasis, and worse prognosis. CASC15 promoted cell proliferation by aiding the G1/S transition and inhibited apoptosis, enhancing cell migration and invasion through the EMT pathway. Lin et al. [58] identified CASC15 as an oncogenic lncRNA in ovarian cancer, promoting EMT and metastasis via the TGF-β/SMAD3 pathway. CASC15 knockdown significantly reduced metastatic spread in mouse models. Lessard et al. [59] found CASC15 upregulated in advanced melanomas, associated with shorter disease-free survival. CASC15 regulated melanoma cell phenotype switching and correlated with key genes like MITF and SOX10, affecting proliferation and invasiveness.

The study by Yan et al. [60] showed that downregulation of CCDC26 (CCDC26 long non-coding RNA) in gastrointestinal stromal tumour (GIST) cells treated with imatinib led to increased drug resistance through upregulation of IGF-1R, establishing a crucial CCDC26/IGF-1R axis for imatinib sensitivity. Similarly, Cao et al. [61] found that CCDC26 enhanced imatinib sensitivity by regulating c-KIT expression, with its knockdown leading to increased resistance via upregulation of c-KIT. Both studies highlighted CCDC26's role in modulating drug response and its potential as a therapeutic target to overcome imatinib resistance in GIST.

Here, we chose to focus on several GO terms enriched in sEV RNA content derived from CRC cell lines that are of high relevance to CRC pathogenesis (Table 3). The RAS protein signal transduction pathway is a key driver of CRC progression, with frequent mutations in RAS genes leading to constitutive activation of downstream signalling pathways such as the MAPK cascade [84]. Our GO analysis identified several essential genes enriched within this pathway that are relevant to CRC. For instance, SOS1 acts as a guanine nucleotide exchange factor (GEF) for RAS proteins, promoting their activation and subsequent signalling through the MAPK pathway [85]. Similarly, RAPGEF1 and RAPGEF2 activate Rap1, influencing cell adhesion and migration, which are essential for metastasis [86]. Our findings also highlight the roles of RALGPS1 and RALGPS2, which activate Ral GTPases involved in cytoskeletal dynamics and vesicle trafficking. These processes are essential for cancer cell migration and metastasis [87].

We have also identified several genes enriched within the Positive Regulation of MAPK Cascade GO term. The MAPK cascade is essential in CRC progression, regulating cell proliferation, differentiation, and survival. Our GO analysis revealed several important genes enriched in this pathway relevant to CRC, including EGFR, MAP2K1, FGFR2, IGF1R, KSR1, ROCK1, and ROCK2. EGFR, a key receptor, activates the MAPK pathway, and its overexpression and activation leads to increased proliferation in CRC. Additionally, MAP2K1 (MEK1) functions downstream to activate ERK1/2, driving synthesis of D1 and promoting cell division [75]. Scaffolding protein KSR1 enhances MAPK signalling efficiency by promoting cell proliferation and oncogenic potential through the RAS/RAF pathway. KSR1 also facilitates signal propagation by assembling the RAF/MEK/ERK complex at the plasma membrane [88]. FGFR2 and IGF1R, both receptor tyrosine kinases, also activate the MAPK pathway, supporting CRC cell proliferation [89, 90]. Additionally, ROCK1 and ROCK2 regulate cytoskeletal dynamics and cell motility, facilitating metastasis in CRC [91].

Moreover, a closer examination of the Positive Regulation of Canonical Wnt Signalling Pathway GO term revealed several key genes highly relevant to CRC. Notably, YAP1 and FGFR2 are essential in enhancing Wnt signalling, driving CRC cell proliferation and survival [92, 93]. Overexpression of EGFR further amplifies oncogenic pathways, including Wnt, contributing to tumour growth. Additionally, SMAD3 interacts with the Wnt pathway, influencing cell differentiation and proliferation in CRC [94]. DKK2 and GPC3 are modulators of Wnt signalling, with roles in tumour growth and metastasis [95, 96], while RSPO2 acts as a potent activator of Wnt signalling, frequently overexpressed in CRC [97].

Next, we focused on genes involved in GTPase Activator Activity and Small GTPase Binding pathways. Among these genes is DOCK3 (dedicator of cytokinesis 3), which is classified as a mini-driver. Mini-drivers are genes

with weak tumour-promoting effects that can enhance oncogenesis when they occur alongside other driver genes [98]. Transcript levels of potential biomarker gene IQGAP2 (IQ motif containing GTPase activating protein 2) is known to be reduced across different cancers including CRC [99]. And finally, DIAPH3 (diaphanous related formin 3) is also considered a potential biomarker gene, with its suggested role in tumour initiation and the metastatic process [100, 101].

Furthermore, our KEGG pathway analysis of RNA-seq data provided additional insights into the molecular mechanisms involved in CRC pathogenesis and reinforced our GO analysis results. The data revealed several pathways important to cancer development and progression that we have already discussed, such as the Wnt signalling pathway, MAPK pathway, and RAS signalling pathway. As described earlier, these pathways are essential in regulating cell proliferation, differentiation, migration, and survival, all of which are crucial processes in cancer biology. Additionally, pathways such as ErbB signalling, EGFR tyrosine kinase inhibitor resistance, and Rap1 signalling were identified, highlighting their role in cancer cell signalling and survival. Meanwhile, pathways involved in cell adhesion and motility, including focal adhesion, regulation of the actin cytoskeleton, adherens junction, and tight junction, underscore their relevance to the metastatic potential of CRC cells.

## Conclusions

Our study provides new insights into the molecular content of sEVs secreted into culture media by commonly used CRC cell lines. Using high-resolution techniques such as TEM to assess the typical size and morphology of sEVs, and MADLS for particle size distribution and concentration, we confirmed sEV characteristics in detail. RNA sequencing of these sEVs revealed a remarkably high overlap in the RNA content, with the top nine protein-coding genes and fourteen lncRNA genes consistently present across all CRC cell lines. These genes include both known CRC/cancer-related genes and newly discovered ones, indicating their potential as biomarkers or therapeutic targets. In the discussion, we explore the molecules identified in our study, examining their significance and roles in various molecular processes from multiple perspectives. Our findings highlight the importance of sEVs in CRC intercellular communication and suggest new avenues for diagnosis and therapeutic development.

### Abbreviations

| | |
|---|---|
| CRC | Colorectal cancer |
| GO | Gene ontology |
| lncRNAs | Long non-coding RNAs |
| MADLS | Multi-angled dynamic light scattering |
| MSI | Microsatellite instability |
| sEVs | Small extracellular vesicles |
| TEM | Transmission electron microscopy |
| PDI | Polydispersity index |

## Supplementary Information

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Supplementary Material 1.

Supplementary Material 2

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### Authors' contributions

MB (Marie Boudná) performed experimental work, analysed and interpreted data, and wrote the manuscript, NB, RJ, and VB performed bioinformatic analyses, TS, KK, TM, and RB performed experimental work, JK performed DLS analysis; MB (Miroslav Boudný) and PVF developed the concept and design, KS performed experimental work, wrote the manuscript and substantively revised it, OS designed the manuscript and substantively revised it.

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### Data availability

The data supporting the results are contained in the files: Supplementary_Information_Boudna_2025; Supplementary_Tables S1-S8_Boudna_2025.xlsx. Raw sequencing data were generated at CEITEC Genomics Core Facility and are publicly available at the Sequence Read Archive under accession number PRJNA1128448.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare no competing interests.

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