Cavin-1 attenuates the EV export of oncogenic mir-148a by modulating export of RNA-binding proteins. Given that RNA-binding proteins select for targets by binding conserved RNA sequences, known as motifs, miR-148a and other RNA targets will share a motif that allows specifically for their export over other microRNAs.

Methods and Materials: 5-10 pages. Including controls, technical controls and biological controls.

## Reagents:

Trypsin-EDTA (Gibco), Roswell Park Memorial Institute (RPMI) 1640 media, Fetal Bovine Serum (FBS) (Bovogen), Phosphate Buffered Saline (PBS) (Amresco Inc), Geneticin G418 Antibiotic (Invitrogen). Rabbit anti-hnRNP K (Abcam).

## Cell culture:

Previously generated PC3 cell lines, containing GFP or GFP tagged cavin-1, were assessed through western immunoblotting for GFP to determine stable GFP expressivity. These cell lines were cultured in 5% FBS/RPMI1640 media in a 5% CO2 incubator set to 37°C. G418 antibiotic drug was added to these cultured cells to select for GFP expressing cells, making a total concentration of 0.1mg/mL. Detachment of the cells during passaging was completed using 0.25% Trypsin-EDTA solution.

## Differential miRNA expression:

Previously collected RNA-seq data for small RNAs, generated by Illumina sequencing, had been aligned and assessed for raw counts for miRNAs in cell and EVs. An R package, DESeq2, had normalized these counts to fit a negative binomial distribution and excluded microRNA data that possessed low to no counts (≤10 counts) for miRNA species across the triplicates and conditions (GFP and cavin-1), allowing for only relevant microRNAs to be assessed. Applying the function makes comparisons of expression between GFP and cavin-1 cell conditions and returns this in the form of log2 fold change (FC), Wald test p-value and a false discovery rate corrected p-value. This analysis was completed separately for cell and EV miRNA content. By using the log2FC values for each miR, comparisons were made between cell and exosome expression by taking the difference in the form of FCcell-FCexo. Add bit about frequency plot and cut offs. Graphs were completed using the ggplot package from R, including a 95% confidence interval.

## Extracellular Vesicle Extraction and RNA extraction:

Cells were grown to 70% confluency prior to the addition of fresh serum free RPMI1640 media on 15cm Petri dishes. The conditioned media was collected after 24hrs of incubation and concentrated in a 10kDa ultracentrifugation filter tube (Sigma) until 1mL of concentrated media was achieved. This was processed through an exoRNeasy midi kit (Qiagen) to extract the total EV RNA as per manufacturer’s instruction. A sample of these cells were also collected for comparison. The total cellular RNA was collected using the MiRvana kit as per manufactures’ instruction (Invitrogen). Nanodrop was used to assess to the purity and concentration of the RNA, where samples with an A260/280 approximating 1.8 were be used for further experimentation.

## Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) and preparation:

Poly-adenylation was completed using the E.coli polyadenylation enzyme and associated buffers (NEB) using a standard protocol (Balcells *et al.* 2011). This was immediately followed by cDNA conversion using the Superscript II Reverse Transcriptase and 0.1μg/μL oligo DT (Invitrogen) as per standard protocol (reference). RT-qPCR was performed on the samples with primers specific to miR-363-3p, 148a-3p, 200a-3p, 30a-3p and 574-5p (IDT). Mir-125a-3p was used as the reference gene due to producing the same level of expression in EV derived from both GFP and cavin-1 PC3 cells based on the RNA-seq data. Delta delta CT statistics were completed by comparing between GFP and cavin-1 cell lines for the target and reference genes. Bar graphs generated by GraphPad Prism 6 and statistics calculated using a non-parametric two-sided T-test (Mann-Whitney T-test).

Motif Discovery and Assessment:

TAMO (Tools for Analysis of MOtifs) package was used to determine shared RNA-motifs within the differentially exported miRNA data set. The MEME algorithm was used to find a motif 4 to 10 nucleotides in length mapped amongst the inputted miRNA sequences with at least 70% conservation. The resulting motif was compared to the sequences of all expressed miRNAs in the PC3 cells to determine specificity to the differentially exported miRNAs using the sitemap algorithm. Sequence logos were generated using WebLogo (http://weblogo.berkeley.edu/).

(For tips on how to write the bioinformatics parts : www.nature.com/articles/srep26090?WT.feed\_name=subjects\_computational-biology-and-bioinformatics)

## Proteomic Analysis:

Previously published liquid chromatography tandem mass spectrometry results for GFP and cavin-1 cell lines analyzed the proteomic content of the EVs excreted from these cells (Inder paper reference). The fold change difference inflicted by cavin-1 was generated (mean GFP/Cavin-1) for each protein detected and p-value determined by two-sided paired Student t-test. Each protein in this data set were analyzed using the biomaRt R package for Gene Ontology (GO) annotation to determine RNA-binding ability (GO:0003723).

## Co-localization by Immunofluorescence Confocal Microscopy:

Cell were grown to 70% confluency on coverslips prior to fixation with 4% PFA for 30minutes and washing with PBS. 0.1% Triton-X in 3% BSA in PBS was added to the coverslips to block and permeabilize the cells. After 30 minutes of incubation, the coverslips were washed and primary antibodies in blocking solution (3% BSA/PBS) were then incubated with the coverslips for 1 hour at room temperature. Coverslips were then washed 3 times with PBS prior to incubation with secondary antibodies in blocking buffer for 1 hour in the dark at room temperature. After washing 3 times in PBS, 1:1000 dilution of DAPI in blocking solution was incubated with coverslips for 10minutes in the dark, followed by additional PBS and MilliQ water washing. Excess water was removed by Kimwipe prior to mounting on slides with 8μL Prolong Diamond (Invitrogen). Slides were dried for 24 hours at 37°C prior to imaging with the Olympus Confocal microscope.

## MicroRNA In situ Hybridization:

Cells were grown to 70% confluency on coverslips prior to fixation with cold 100% methanol. Coverslips were then washed thrice with PBS and incubated in the dark overnight at room temperature in 50pmole Cy5 conjugated antagomiR in oligo hybridization buffer; 50mM NaCl, 1mM Tris-Cl (pH 8.0), 0.1mM EDTA (pH 8). Cy5-scrambled oligo was used as a negative control. Excess antagomiR was removed by washing thrice in PBS before 4% PFA fixation for 30minutes. Subsequent steps were performed as per immunofluorescence protocol for hnRNPK localisation with Alexa Fluor 568 secondary antibody.

Immunoprecipitation:

List antibodies etc set up, controls. No biotinylated mir control.

## Western blotting:

|  |  |  |
| --- | --- | --- |
| Antibody/oligo | Method | Dilution |
| hnRNP K (mouse) |  |  |
| hnRNP K (rabbit) |  |  |
| Normal Rabbit IgG |  |  |
|  |  |  |
|  |  |  |
|  |  |  |

Results: Experimental data with explanations to make the data comprehendible with stats. 2000w

**microRNAs are selectively exported from prostate cancer cells.**

miR-148a was previously found to be selectively exported from the PC3 cell line where this export was truncated by ectopic expression of cavin-1. However, this analysis had not considered other miRs that may also be moderated by this system. To determine all miRNAs that are being modified in the PC3-EVs by cavin-1 expression, ‘Our lab conducted a comprehensive RNA-seq analysis to quantify the miRNAs in EV and cognate cells’. 95 mirs were detected in EVs from PC3 cell lines (refer to figure here big one with 95mirs). Comparison between GFP and cavin-1 cell lines revealed a total of 12 significantly (p ≤0.05) modified miRs in the EVs, including miR-148a. Refer to previous rationale/example of mir\_148a not changed in cell? Want to see if same trend persists. ~~However, decreases in EV-miR content could be a product of cellular expression decreases~~. Comparison between cellular and EV modification induced by cavin-1 reveals a subset of microRNAs that are dramatically reduced in the EVs with little modification of total cellular expression. Further comparisons between all miRs identified in EVs (n=95) to the cellular levels reveals a total of 19 miRs that may be modified selectively. Generally, miRNAs present in the EVs change proportionately to the cellular expression changes induced by cavin-1, however several species present with a dramatic decrease in EVs compared to cells where 5 of these are significantly modified between cell lines. These are the miRs likely to be acted upon by a selective export mechanism, attenuated by cavin-1 expression.

5 significantly modified miRs were selected for validation across the three groups; miR-30a-5p, miR-148a-3p, miR-200a-3p, miR-574-5p and miR-363-3p. RT-qPCR of these targets confirms whether the cavin-1 induced modification of these miRs were reproducible. Here, the trend first displayed by the RNA-seq data is maintained, shown by a decreased presence of miR-30a-5p, -148a-3p and -200a-3p in EVs between the cell lines compared to the cellular content. Inversely, miR-574 was increased due to the presence of cavin-1 in the exosome, and miR-363 is confirmed to be not differentially exported by cavin-1. This establishes that some miRs are indeed selectively exported from PC3 cells, where cavin-1 modulated this export.

**Distinct sequence motifs are overrepresented in differentially exported microRNAs.**

Protein to RNA interactions are dictated by specific nucleic acid sequences, or motifs, that are conserved across the targeted RNAs. Here, we attempted to assess whether the selectively exported miRNAs share a sequence motif to explain their selective export. #Motif discovery with only 5 miRs was inadequate for significant motif discovery, so I’ve considered additional differential miRs according to frequency plot (explain the whole FCcell-FCexo thing) and z-score, may need to shorten sentence. Motif discovery was used to define stretches of RNA that are shared amongst the differentially exported miRNAs that would be targeted by the miR export protein that do not overlap with any of the non-exported miRs. This analysis returned two distinct motifs that are enriched in the miR group that possess reduced export upon cavin-1 expression (figureX). These motifs are present within 14 of the 19 miRs within this group with minimal (n=1) hits in the non-differentially exported miR group. This suggests potential binding sites the export protein may be able to bind to evoke specificity and selectivity of the targets.

**Candidate proteins are present in exosome with RNA binding ability.**

To identify candidate proteins that could mediate this selective miR export, EV protein content was assessed for differential export upon cavin-1 expression and RNA-binding ability. Previously published proteomic data determined the identity of EV proteins from PC3 GFP and PC3 cavin-1 cell lines. Comparing between the cell lines returned a total of 120 significantly differentially exported proteins, where 109 of these proteins were decreased upon cavin-1 expression. Additionally, these proteins were further analyzed by GO analysis to determine whether these proteins had previously reported RNA-binding capacities. Together, this yields a total of 5 differentially exported RNA-binding proteins. Interestingly, further investigation into the RNA binding specificity of hnRNPK revealed, through mutagenesis assays, its affinity to bind to sequence: AGTGTG. Upon comparison to the known motif, using the sitemap algorithm, hnRNPK matches Xpercent to the predicted binding motif. Hereby, hnRNPK was considered a viable candidate protein to mediate the selective export of miRs.

**hnRNPK sub-cellular localization modified in cavin-1 PC3 line.**

Immunofluorescence was performed using hnRNP K specific antibodies to determine cellular localization changed between GFP and cavin-1 cell lines which may explain differential export. An initial observation of hnRNPK subcellular localization revealed a distinct change between cell lines, from punctate like structures in PC3 GFP to a perinuclear focus in cavin-1+ cell lines. Further co-localization studies were performed to determine what these structures were. CD9 is a commonly used marker for multivesicular bodies and exosome formation. hnRNPK appears to co-localize with the CD9 protein in GFP PC3 cells, which indicates presence in the multivesicular bodies that is not occurring in the PC3 cavin-1 cell line. However, the hnRNPK in PC cavin-1 was found present in endoplasmic reticulum, shown by strong overlap with ER resident protein, ERp44. Therefore, change in subcellular localization modified by cavin-1 could explain the differential export of hnRNPK and miRNAs.

**hnRNPK co-localizes with selectively exported microRNAs.**

**hnRNPK binds microRNAs. ??**