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). DharmaFECT I (Dharmacon). Rabbit anti-FUS and 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. 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).

## 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 also analyzed using the biomaRt R package for Gene Ontology (GO) annotation to determine RNA-binding ability. Proteins that were decreased in EV fractions and possess RNA-binding ability (based on GO term) were deemed candidates for miRNA escort activity.

Motif Discovery and Assessment:

An open source computational framework, called TAMO (Tools for Analysis of MOtifs) was used to determine shared RNA-motifs within the differentially exported miRNA data set. The X algorithm was used to find a motif 4 to 10 nucleotides in length mapped amongst the inputted miRNA sequences. This motif was compared to the sequences of all expressed miRNAs in the PC3 cells to determine specificity to the differentially exported miRNAs using the Y algorithm.

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

Unix command line and Python programming languages were used to run TAMO, which combines the use of MEME, AlignACE and MDscan algorithms.

## Pull down assay:

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

## Western blotting:

## Transfection of Biotinylated miRNA:

Basically a lipofectamine process and any optimization test.

## Colocalization 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). 24 hours at 37°C dried these slides prior to imaging with the Olympus Confocal microscope.

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| --- | --- | --- |
| Antibody/oligo | Method | Dilution |
| hnRNP K (anti-mouse) |  |  |
|  |  |  |
|  |  |  |
|  |  |  |
|  |  |  |
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Results: Experimental data with explanations to make the data comprehendible with stats. 2000w

**Select miRs are selectively exported from prostate cancer cells.**

RNA-seq allows for raw count quantification by aligning sub-sequences of RNA to a reference gene. This allows for accurate quantification of microRNAs expressed in both cell lines, EV and cellular transcriptome. Comparison between GFP and cavin-1 cell lines revealed a total of 12 significantly (p ≤0.05) modified miRs in the exosome and 28 differentially expressed miRs in the cell. Comparing all available miRs for analysis (n=95) between cellular and EV expression (log2FC) revealed three groupings based on export; increased miRs in the exosome in cavin-1 expressive cells, miRs with no distinct differential export, and miRs decreased in the exosome in cavin-1 cells. Generally, miRNAs present in the EVs change proportionately to the cellular modification induced by cavin-1 expression, 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 modified by cavin-1 expression.

5 highly abundant and 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 these miRs could be a focus for further experimentation. 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 exosomes 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 miRs that can be utilized in subsequent experimentation.

**Distinct motifs are present in differentially exported miRs.**

Motif discovery defines stretches of RNA sequence that are shared amongst the miR differential export groupings (figX). This analysis returned two distinct motifs that are enriched in the miR group that possess decreased export upon cavin-1 expression; AgTGCa and TrmAgAwCy. These motifs are present within 12 of the 20 miRs within this group with minimal (n=1) hits in the non-differentially exported miR group. This suggests potential binding sites are present within that miR group that are responsible for the export. While the analysis other two miR export groups did also reveal shared motifs, the number of miRs that matched those motifs were minimal.

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

Previously published liquid chromatography MS/MS analysis of the exosome and lipid raft proteomic content was assessed for candidate proteins. Here, the candidate proteins were selected based on previously published RNA-binding knowledge and moderated presence in the lipid raft and exosomes between the cell lines. How to present this?

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

Immunofluorescence was performed using FUS and hnRNP K specific antibodies to determine cellular localization changed between GFP and Cavin-1 cell lines.

Presence and absence in imaging between cell lines.