Control of selective microRNA release via exosomes and microvesicles

AIMS AND BACKGROUND

This project **aims** to understand the mechanism of caveolae and cholesterol in regulating the selective loading of microRNA (miRNA) to extracellular vesicles (exosomes and microvesicles) to effect cell-to-cell communication.

Recent studies confirmed the biological importance of extracellular vesicles and their miRNA cargos in intercellular communication, but little is known about the mechanisms governing cargo loading for different extracellular vesicle types. Our previous studies on caveolae forming proteins, caveolin-1 and cavin-1, revealed a surprisingly selective secretion of protein and miRNA cargos via extracellular vesicles, indicative of specific cargo loading mechanisms. Caveolae are a type of membrane lipid rafts, cholesterol-rich nano/microdomains, which has been implicated in mediating extracellular vesicle release. Through lipid raft and extracellular vesicle proteomics analyses, we identified a new surface marker for cell surface-derived microvesicles, as well as a group of RNA-binding proteins (RBPs) which may function to selectively load miRNAs to specific types of extracellular vesicles. In addition to altered lipid raft proteome, we also observed subcellular re-distribution of cholesterol in our model cell system. These findings provide a defined cellular system in which to test the **hypothesis** that differential expression of caveolae genes alters lipid distribution and locazliation of RBPs, thereby changing the loading of specific miRNAs into extracellular vesicles and the patterns of intercellular communication.

Recent progress has established the patho/physiological importance of long range inter-cellular communication through the transfer of active proteins, RNA and lipids via membrane-bound vesicles [6]. The extracellular vesicle system has some advantages over the classical endocrine system and cytokines. Firstly, encasing of proteins and RNA inside a membrane increases their stability in blood and interstitial fluid. Secondly, the surface of the vesicles may contain specific information 'addressing' the vesicle to target locations. For example, we have found specific homing of prostate cancer-derived vesicles to bone and lung [4]. Thus, there is a burgeoning field of cell biology exploring the diagnostic and therapeutic potential of extracellular vesicles [5]. While the biological roles of extracellular vesicles are now established [7], the exact mechanisms of cargo loading are only beginning to be elucidated.

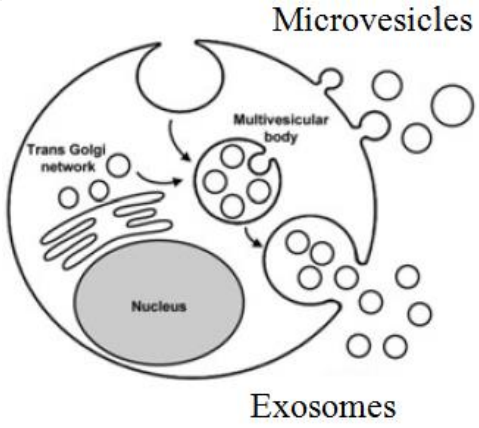

Figure 1. Extracellular vesicle release via exosomes and microvesicles [5].

Two major types of extracellular vesicles are defined by their route of release, termed exosomes and microvesicles, respectively [8] (**Figure 1**). The more well-known term **'exosome'** denotes vesicles of endocytic origin, formed by inward budding the membrane to form multivesicular bodies, and then released by fusion of the limiting membrane with the plasma membrane. (Note, the term 'exosome' is also used in cell biology to denote the ribonuclease complex, which is not discussed in this proposal.) Vesicles that protrude and shed from the cell surface are denoted **'microvesicles'** (also called microparticles and ectosomes). Researchers have begun to decipher the molecular determinants for exosome and microvesicle sorting and release, with **one major caveat** that separation of exosomes and microvesicles post-release is not simple experimentally, and most existing studies examined a mixed population of exosomes/microvesicles despite the use of specific names [8]. For example, many papers cite and utilise size differentiation, with microvesicles being larger. However, recent work including ours [4, 9], indicate that the diameters of microvesicles and exosomes overlap. Furthermore, many of the markers previously thought to be specific to exosomes have now been implicated in microvesicle release also, for example, TSG101 [10]. Several cargo loading mechanisms have been reported, including lipid-binding domains and membrane anchors [11], a glycan signature [12], a 25 nucleotide 'zipcode'-like sequence for mRNA enrichment [13], and a GGAG motif for miRNA recruitment by sumoylated hnRNPA2B1 [14]. Importantly, the molecular contents of extracellular vesicles are regulated, for example in response to environmental pH [15], hypoxia [16, 17] and heat [18]. However, the mechanism(s) of regulation is not yet defined.

Extracellular vesicles are enclosed by a lipid bilayer membrane enriched in cholesterol, sphingolipids and ceramide [8]. This lipid composition is similar to **lipid rafts,** dynamic nanometre diameter lateral membrane nanodomains which may coalesce to larger microdomains in response to stimuli, leading to signal transduction and downstream functional changes. A role for lipid rafts in regulating extracellular vesicle release is supported by data from cholesterol addition and depletion experiments and by neutral sphinomyelinase treatment [19, 20].

Caveolin-1 binds cholesterol and is one of the cholesterol transporters involved in the delivery of cholesterol to the plasma membrane [21], where it forms sub-microscopic pits termed caveolae. Formation of caveolae requires two family of proteins, caveolins and cavins, with highly selective tissue-specific expression patterns [22]. Cavins are cytoplasmic accessory proteins which regulate caveolae [23]. Our initial work has focussed on cavin-1 which stabilizes caveolae at the plasma membrane [24]. Dysfunction of caveolin-1 or cavin-1 are associated with varied pathologies such as cancer, muscular dystrophy and atherosclerosis [23], indicating the involvement of caveolae in fundamental cell function. As caveolae are found on the plasma membrane, the current dogma of caveolae function focusses on signal and mechano transduction from the cell surface. However, accumulating data suggest that caveolae dysfunction may have a broader impact on cellular organelles. For example, loss of caveolin-1 leads to elevated mitochondrial cholesterol [25], while our work show that formation of caveolae by expression of cavin-1 in caveolin-1-positive PC3 cells altered the functional extracellular vesicle content, both protein and miRNA [4, 26, 2].

In order to elucidate the mechanisms of selective cargo insertion and release via the exosome and microvesicle pathways, a well-characterised experimental paradigm is required. Recent studies have begun to examine the contents of sub-classes of extracellular vesicles. For example, vesicles harvested from apical versus basolateral plasma membrane of polarized epithelial cells show distinct molecular signatures [27, 28]. Here, we describe ***a well-characterised experimental system using cavin-1 expression in PC3 cells, and propose a research program to understand how cavin-1 modulates extracellular vesicle content, in particular miRNA.***

Preliminary Studies

CI Hill's research program on the molecular mechanisms of caveolae forming proteins in health and disease, partly through the support of an ARC Future Fellowship, has established and characterised the PC3 cell system as a model system to understand the role of caveolae in cancer biology (**Figure 2**). This cell line was selected because of its unique expression pattern of caveolin-1^{+}/cavin-1^{-}, which mirrors what we observed in advanced prostate cancer tissues [2]. In all healthy tissues and cell lines examined, the expression of caveolin-1 and cavin-1 are highly correlated [22]. We showed that in the absence of cavin-1, caveolae do not form in PC3 cells, yet caveolin-1 localizes to the plasma membrane [24]. Ectopic expression of cavin-1 in PC3 cells attenuated the aggressive phenotype [1-3]. Cavin-1 reversal of phenotype depended on the presence of caveolin-1 because it had no functional effect in LNCaP prostate cancer cells without caveolin-1 expression [2].

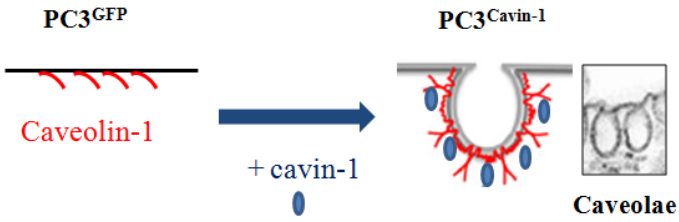

Figure 2. PC3 experimental system. Compared to PC3^{GFP} control, PC3$^{Cavin-1}$ cells show reduced in vivo metastasis, migration, anchorage-independent growth, angiogenesis and stromal fibroblasts [1-3].

The observed reduction in angiogenesis and stromal myofibroblasts associated with ectopic cavin-1 expression suggests a component of paracrine action [2]. While caveolin-1 was released in extracellular vesicles in agreement with a previous study [29], cavin-1 itself was not released, suggesting an indirect mechanism. The observed redistribution of cellular cholesterol suggest that cavin-1 induced lipid raft remodelling. To decipher the molecular mechanisms, we undertook subcellular proteomics and found that cavin-1 altered subsets of proteins in extracellular vesicles and lipid raft, without changing total expression levels [26]. This result indicates that cavin-1 altered cellular trafficking. Because PC3$^{Cavin-1}$ cells was less aggressive (Figure 2), we were most interested in the down-regulated proteins. Only 16 out of 300+ proteins were down-regulated in both extracellular vesicle and lipid raft fractions, with 50% of these being cytoskeleton-related proteins

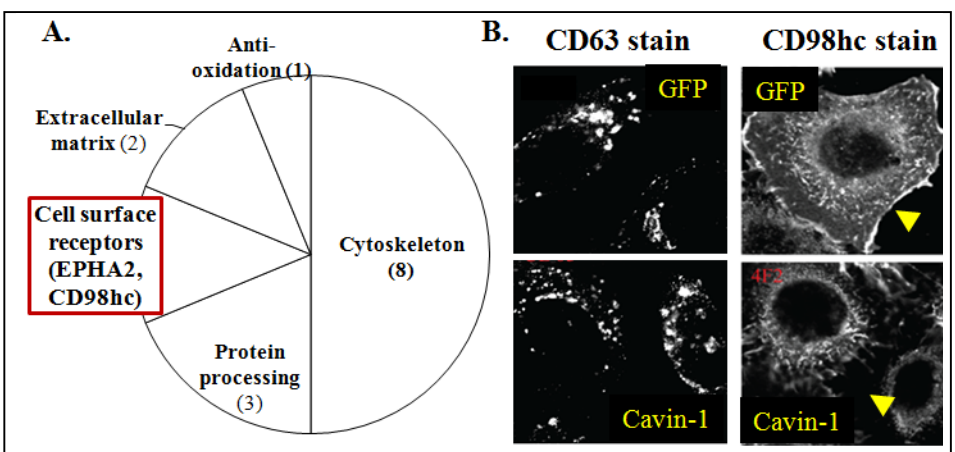

Figure 3. Cavin-1 specifically down-regulates surface-derived microvesicles marked by CD98hc. (A) Cavin-1 down-regulated 16 proteins in both extracellular vesicle and lipid raft fractions, including 2 membrane proteins. **(B)** Cavin-1 reduced the plasma membrane level of CD98hc (arrows) but not total expression level [4]. Distribution of multivesicular body/exosome marker CD63 was not altered (left panel).

(**Figure 3A**), in agreement with our publication reporting alteration of cytoskeleton-lipid raft linkages upon cavin-1 expression [26]. The two cell surface receptors (EphA2 and CD98hc) identified amongst the 16 proteins down-regulated in both the extracellular vesicle and lipid raft fractions were investigated further as potential markers of cavin-1-sensitive vesicles. The proteomics results were confirmed by immunoblotting of both proteins in subcellular fractions. Furthermore, CD98hc was detected on the surface of extracellular vesicles by immuno-electron microscopy (EM) [4] (labelled 4F2 in the figures. EphA2 antibody was not suitable for immuno-EM). Quantitation of the diameter and number for all vesicles and the CD98hc-labelled population showed no significant difference between $PC3^{Cavin-1}$ and $PC3^{GFP}$ cells [4]. Both proteins localized to the cell surface in $PC3^{GFP}$ cells, which was reduced in $PC3^{Cavin-1}$ cells (arrow, **Figure 3B**). In contrast, cavin-1 expression not have a bulk effect on the distribution of CD63, a classic marker of the multivesicular body/exosome pathway (**Figure 3B**). Taken together, these data suggest that ***cavin-1 modulate cargo-loading in the microvesicle pathway by altering protein/lipid trafficking, without significant impact on the exosome pathway.*** Identification of CD98hc as a novel microvesicle marker provides an unique opportunity to examine microvesicles and exosomes (using CD63) from the same cell system.

In addition to proteins, extracellular vesicles are enriched in small non-coding RNAs which functions in post-transcriptional gene regulation in target cells [30, 6]. We undertook a pilot small RNA sequencing experiment of both the cellular and extracellular vesicle small RNAs to determine if cavin-1 also altered specific RNA secretion. Initial analysis focussed on the microRNA (21-25 nucleotide) class of small RNAs which is the most well characterised. Statistical analysis on number of reads per miRNA revealed that 97 miRNAs were significantly changed in $PC3^{Cavin-1}$ cells compared to $PC3^{GFP}$ cells (**Figure 4A**). Furthermore, comparison of the altered miRNAs in extracellular versus cellular fractions revealed that only 23 miRNAs overlap between the 97 and 39 altered miRNAs in the extracellular vesicle and total cellular miRNAome in $PC\text{-}3^{cavin-1}$ cells (**Figure 4B**). This means that while down-regulated miRNA expression can lead to secretion changes, there is a significant level of post-transcriptional regulation. We chose miRNA-148a as a candidate for validation by qRT-PCR, which confirmed the ***selective reduction in miR-148a vesicle secretion but not total cellular level*** [4].

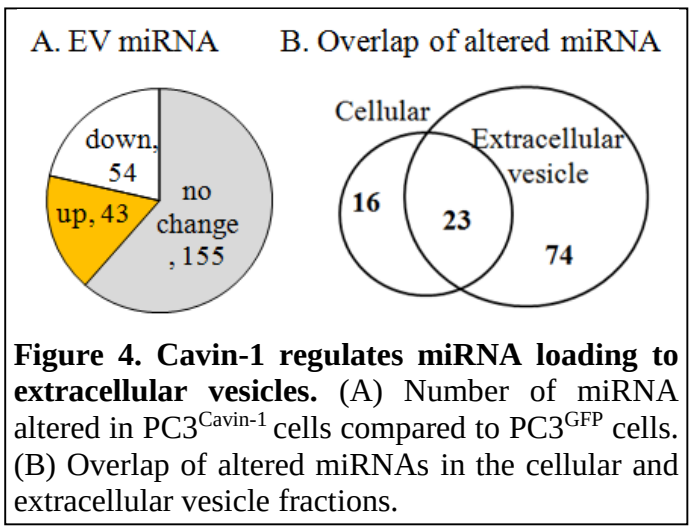

Figure 4. Cavin-1 regulates miRNA loading to extracellular vesicles. (A) Number of miRNA altered in $PC3^{Cavin-1}$ cells compared to $PC3^{GFP}$ cells. (B) Overlap of altered miRNAs in the cellular and extracellular vesicle fractions.

A recent study identified hnRNPA2B1 as a miRNA-escort protein which controls the loading of specific RNAs to exosomes [14]. By comparing our PC3 cell extracellular vesicle proteomics data to the recently published RNA binding protein compendium [31], we identified 14 RNA binding proteins including hnRNPA2B1. Interestingly, 13 out of the 14 RBPs are known to directly interact (**Figure 5a**). Indeed, analysis of the subcellular protein-protein interaction (PPI) networks revealed a strikingly high inter-connectivity within the subcellular fractions (extracellular vesicle or lipid raft), which is hardly ever observed for total proteome networks. This result further validates PPI network as a biologically relevant tool to reveal molecular mechanisms. To identify putative **RBP-miRNA interactions** in the extracellular vesicles, we searched the mature sequences of the 97 altered miRNAs for known RNA-binding motifs for the 14 RBPs. This analysis showed that **FUS (fused in sarcoma)** has the highest number of known miRNA interactions out of the list (**Figure 5b**). FUS is also the most reduced RBP in extracellular vesicles in $PC3^{Cavin-1}$ cells (Ratio cavin1:GFP of 0.42±0.09, p-value 0.03). FUS is generally located in the nucleus, however, according to the extracellular vesicle database EVpedia [32], FUS proteins has been in reported in 16 extracellular vesicle proteomics experiments. FUS has been reported to contribute to the biogenesis of a specific subset of miRNAs by binding to primary miRNA at the sites of their transcription in the nucleus [33]. *However,*

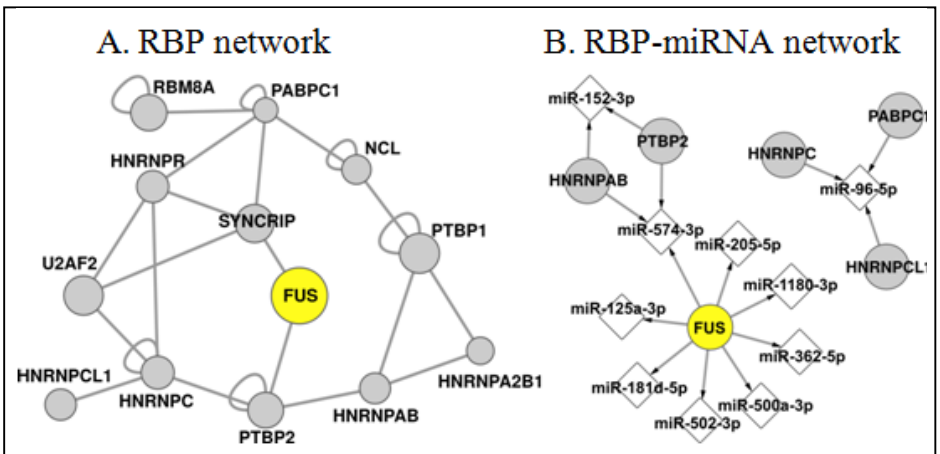

Figure 5. RNA-binding proteins (RBP) in extracellular vesicles. (A) RBP interactions in PC3 extracellular vesicle data. (B) Predicted miRNA-RBP interactions using miRNA seq data

there has been no evidence that FUS binds to mature miRNAs and regulates their transport and localization in the subcellular compartments. Nevertheless, we found that 66 out of 97 miRNAs (68%) differentially expressed in PC3$^{cavin-1}$ cells have also been shown to be differentially expressed in the FUS knockdown study [33], including miR-500a and miR-574 which contained the FUS-interaction GGUG motif (**Figure 5b**). These results strongly suggest a ***potential functional role for FUS in escorting a subset of miRNA to extracellular vesicles.***

To examine the potential lipid raft-mediated mechanism of cavin-1 action, we looked for known FUS-interacting proteins in the lipid raft proteome. This analysis showed that FUS is predicted to be a highly connected interactor of identified lipid raft proteins. Furthermore, mining our lipid raft proteomics database [34] revealed that FUS has been reported in three published lipid raft proteomics experiments [35-37]. Interestingly, all three studies used cancer cell models (LNCaP prostate cancer cells, Jurkat and NB4 leukemia cells), while the extracellular vesicle studies identified FUS in body fluids, normal and cancer cell models. This result may suggest ***an altered lipid raft environment in cancer cells leads to recruitment of FUS and altered extracellular vesicle miRNA composition***.

RESEARCH PROJECT

This proposal meets the objectives of the Discovery Projects scheme as it will ***expand Australia's knowledge base and research capability*** through cutting edge ***basic research and research training***. The assembled team includes two ARC Future Fellows (CI Hill, PI Cloonan), an early/mid-career systems biologist (CI Cristino), and two ***international collaborators*** (PI Foster and PI Wenk) with established reputation in organelle proteomics and lipidomics, respectively.

To test the **hypothesis** that differential expression of caveolae genes alters lipid distribution and locazliation of RBPs, thereby changing the loading of specific miRNAs into extracellular vesicles, **three independent aims** have been designed. *These are to be carried out concurrently over the 3-year grant.* Aims 1 and 3 will be PhD projects co-supervised by Brisbane-based CIs and PIs, with scope for creativity within the framework outlined. The requested cell biology research associate will assist in laboratory training of the PhD candidates, and drive the experimental work for Aim 2.

Aim 1. Examine the effect of cavin-1 expression on exosomes versus microvesicles.

To test the hypothesis that cavin-1 differentially regulates exosomes and microvesicles, we will use CD63 and CD98hc to capture vesicles for molecular and physical characterisation. Immunocapture conditions have been established for CD63 in long-term collaborator Prof Rob Parton's laboratory (**Figure 6**). Similar conditions will be used to capture CD98hc vesicles. A suitable commercial CD98hc antibody has already been identified and published for immunoblotting and immuno-EM [4]. After establishing immunocapture, CD63-positive exosomes and CD98hc-positive microvesicles will be isolated from the secretome of equal number of PC3$^{Cavin-1}$ and PC3^{GFP}cells as determined by cell counting. The following characterisation will be performed.

A. Examine the vesicle **morphology** and measure vesicle diameters by EM with collaborator Prof Rob Parton [4].

B. Measure the **number** of vesicles of each type using a novel tunable alternating current electrohydrodynamic (ac-EHD) methodology method with collaborator Prof Matt Trau [38].

C. Compare the vesicle ***proteome***. We will utilise label free proteomics quantitation strategy [39, 24, 40] rather than the previous stable isotope labelling with amino acids in culture (SILAC) method [41, 26] in order to evaluate the lipidome and miRNAome in similar samples. Proteomics sample process and LC-MS/MS will be performed as previously described [26]. Label-free quantitation analysis will be performed using Scaffold software.

D. Profile the vesicle ***lipidome*** with PI Wenk. Lipids will be extracted using a modified Bligh and Dyer protocol, and then analysed by HPLC-ESI-MS/MS with spiked in labelled synthetic standards as previously described [43, 44]. Signal intensities for each lipid species will be extracted, normalized to the representative internal standards and expressed as a molar fraction of the total amount of measured lipids.

E. Profile the vesicle ***miRNAome*** using next generation RNA sequencing. RNA in the fractions will be extracted using Trizol, and the small RNA will be

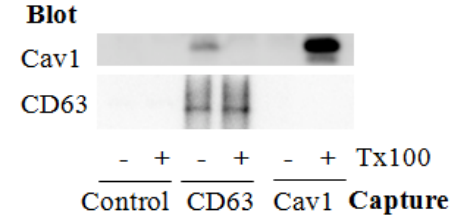

Figure 6. Vesicle immunocapture. PC3 extracellular vesicles were immunocaptured using CD63, Caveolin-1 (Cav1) or irrelevant antibody (Control) under native (-Tx100) or detergent-solubilized (+Tx100) conditions. Presence of Cav1 and CD63 were detected by immunblotting. Note Cav1 could only be co-isolated under native conditions (whole vesicle).

captured for sequencing using the NEBNext Small RNA Library Prep Kit. Libraries will be size selected using a Perkin Elmer Labchip XT. As miRNA sequencing of total RNA pools saturates at approximately 2 million mapped reads when collapsing isomiRs [45], we will aim for a depth of 1 million mapped reads per sample to ensure complete coverage. Libraries will be pooled and multiplexed for sequencing on an Illumina NextSeq mid-output run. miRNAs will be mapped to the latest version of miRBase and the human genome using methodology established in PI Cloonan's group. Differential expression will be detected using EdgeR.

Computational analysis. Comparison between the exosome and microvesicle profiles, multi-omic network analysis will be guided by CI Cristino, as described in the preliminary study above. These analyses may reveal the pathways involved in exosome versus microvesicle pathways, as well as the mechanism of cavin-1 effect. Furthermore, we will examine the differential miRNAs for potential motifs specific for sorting to exosomes versus microvesicles, similar to the derivation of the GGAG motif for hnRNPA2B1 recruitment [14].

The above work is expected to require the first 2 years of a PhD. In the final year, the PhD candidate has the creative freedom to undertake experimental validation of selected targets using molecular cell biology techniques as described for Aim 2 below. For example, immunofluorescence microscopy will be used to localize cargos unique to microvesicles to the cell surface. Lipid composition will be modulated using lipid modifying enzymes. Microvesicle-specific miRNA targeting motif will be tested by expressing tagged construct of the miRNA sequence. The investigative team has the experience in cell biology techniques to guide the candidate.

Outcomes. Completion of this aim will provide novel molecular information on the exosomes versus microvesicles from the same cell type, which may also inform on the regulatory pathways and mechanisms of cargo selection. These are a current knowledge gaps in the field.

Aim 2. Investigate RNA-binding proteins as cavin-1 regulated miRNA-escort proteins to microvesicles.

Our preliminary computational analysis has revealed a number of RNA-binding proteins in extracellular vesicles (Figure 4A), including the previously reported miRNA chaperone hnRNPA2B1 [14]. For experimental evaluation, we will begin with the most highly down-regulated RBP, FUS. Based on present data, we expect that FUS is responsible for miRNA recruitment to microvesicles (hence FUS should be localised to the plasma membrane), while some of the other RBPs such as hnRNPA2B1 (no change by cavin-1, ratio 0.87±0.27, p-value 0.92) may chaperone miRNA to the exosomes. The following experiments utilise hnRNPA2B1 as a known miRNA-exosome chaperone protein (positive control) when evaluating the ***novel role of FUS as miRNA chaperone to microvesicles, regulated by cavin-1***. In the unlikely case that FUS fails to validate in step A (unlikely as every altered protein from the proteomics screen so far has validated by immunoblotting), we will examine other hnRNP proteins also identified in the extracellular vesicle proteomics experiments.

A. Confirm FUS and hnRNPA2B1 in extracellular vesicles (exosomes versus microvesicles) and confirm regulation by cavin-1.

Quantitative immunoblotting will be performed on extracellular vesicles. Comparison will be made between $PC3^{Cavin\text{-}1}$ and $PC3^{GFP}$cells. After confirmation for total extracellular vesicles, the experiment will be extend to CD63- and CD98hc-captured vesicles, to test the hypothesis that FUS is specific for microvesicles. In addition to the above biochemical methods, microscopy experiments will be performed to determine if cellular FUS and hnRNPA2B1 can be detected at the multivesicular body for exosome formation, or at the plasma membrane for microvesicles. Since FUS is primarily a nuclear protein, any potential cytoplasmic signal is likely to be comparatively too weak to be observed by simple immuno-fluorescence microscopy experiments. Therefore, we will utilise the Duolink proximity ligation assay to determine the co-localization of FUS/hnRNPA2B1 with the markers CD98hc and CD63. The laboratories of CI Hill and collaborator Parton have already established this technique (unpublished). A known FUS-interacting protein such as DROSHA will be used as positive control [33].

B. Characterise the FUS-miRNA interactome.

To confirm the binding of miRNA to FUS, immunoprecipitation (IP) of FUS will be performed, followed by characterisation of the bound miRNAs. The experimental procedures will be adapted from the recently published paper on exosomal hnRNPA2B1, using UV to crosslink the miRNA to its binding protein [14]. Both the extracellular vesicle and cellular post-nuclear supernatant fractions (avoiding the abundant non-relevant nuclear FUS) will be used as starting material, to determine if FUS binds the same set of miRNAs. Successful co-immunoprecipitation will be confirmed by qPCR of the expected target miRNAs (shown in Figure 4B). For full characterisation of the FUS-miRNA

interactome, sequencing will be performed after eluting the bound RNA with Trizol. Library preparation and quality control will be performed as for Aim 1E.

C. Determine if FUS and hnRNPA2B1 localizes to lipid rafts, and the modulation by cavin-1.
To determine if FUS recruitment to lipid rafts is regulated by cavin-1 expression, lipid raft fractions of $PC3^{Cavin\text{-}1}$ and $PC3^{GFP}$cells will be prepared [24, 41, 26, 42] and immunoblotted for FUS. Interestingly, hnRNPA2B1 has also been reported in lipid raft proteomics studies as determined using RaftProt [34]. We will also probe for hnRNPA2B1 to determine if it is regulated by cavin-1. Since FUS lacks a lipid binding motif, we hypothesized that it is recruited to lipid rafts by a lipid-binding protein. Mining of our PC3 lipid raft proteome through Pfam identified several proteins with lipid binding motifs, including Annexin A5 being a known FUS-interacting protein.

D. Functional validation of FUS.
Overexpression and knockdown of FUS in $PC3^{Cavin\text{-}1}$ or $PC3^{GFP}$cells will be performed to test the hypothesis that FUS chaperones miRNA to microvesicles. After confirming protein levels by immunoblotting of the cell lysate, candidate miRNAs in the total cell and extracellular vesicle fractions will be measured by qPCR as previously described [4]. If confirmed, the global effect of FUS modulation on miRNA secretion will be investigated, then compared to the FUS bound miRNA list from 2B.

Outcomes. These experiments will confirm or refute ***the hypothesis that FUS is a lipid raft-regulated miRNA chaperone for microvesicle-mediated miRNA release***.

Aim 3. Determine the cavin-1 induced pathways by correlating intracellular molecular profiles with exosome and microvesicle molecular profiles.
Aims 1 and 2 are designed to provide novel characterisation of exosomes versus microvesicles, and determine the microvesicle miRNA binding proteins. In order to understand how cavin-1 expression differentially regulates the exosome and microvesicle contents, we need to examine the cellular effects of cavin-1. Our previous work suggests that cavin-1 acts indirectly on vesicle release as it is not released itself [4, 26]. Cavin-1 function is dependent on caveolin-1 [2] and $PC3^{Cavin\text{-}1}$ cells show redistribution of cholesterol, and specific alterations in a subset of lipid raft proteins including associated cytoskeletal proteins [26]. Taken together, these data suggest that cavin-1 expression alters multiple cellular trafficking pathways with one of the mechanisms being modulation of lipid rafts and cytoskeletal organisation. Apart from the observed cholesterol redistribution, microscopic analysis of compartment markers such as Rab5 (early endosome), CD63 (multivesicular body, example image in Figure 4) revealed no observable difference between $PC3^{Cavin\text{-}1}$ and $PC3^{GFP}$cells. Hence we have designed the following systematic subcellular analysis to evaluate the effect of cavin-1 expression on cytoplasmic organelles.

A. Subcellular fractionation and molecular profiling.
Isolating highly pure cytoplamic organelles of the secretory pathway is technically challenging and will result in low yields. To ensure feasibility, we have chosen the correlation profiling method which assigns group (organelle) membership of the analytes (proteome, lipidome) based on correlation of the distribution profile with marker proteins. PI Foster is one of the international leaders in this methodology including computational methods [46, 47]. During this project, we will optimise a single sucrose gradient procedure to separate post-nuclear supernatant into different profiles correlating with the cytoplamic organelles. Feasibility of this technique in our hands is demonstrated in the pilot experiment shown in **Figure 6**, which plots the relative intensity of each organelle marker (y-axis) plotted against fraction number (x-axis). Complete separation is not necessary, as long as the organelle markers show different profiles, since we will be able to use an algorithm to assign the lipid and protein species to organelles according to the profiles. For example, the endosome marker is highly abundant in

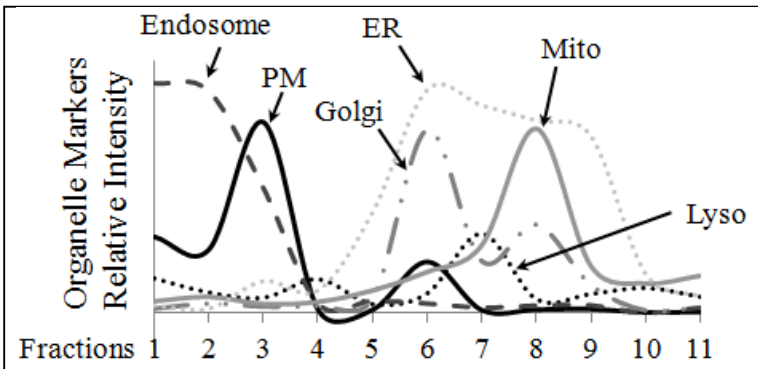

Figure 6. Subcellular Fractionation by Correlation Profiling. Post-nuclear supernatant fraction of HEK293 cells was separated by a 4 hour spin on a 0.2M-2M sucrose gradient. Fractions were collected from the top of the gradient and labeled 1-11. **A)** Selected organelle marker proteins were analysed by dot blot and relative abundance plotted against fraction number. The markers used were: Endosome, EEA1; PM (plasma membrane), Moesin; Golgi, GOLGA2; Mitochondria, CoxIV; Endoplasmic reticulun (ER), Calnexin; Lysosome, LAMP1.

fractions 1 and 2, dropping to non-detectable in fraction 4. On the other hand, the plasma membrane (PM) marker showed a sharp peak at fraction 3. By using the algorithm developed by the lab of PI Fostser [47] to classify the molecular profiles corresponding to each of the organelle markers, we can classify the detected molecules as sharing the same profile as one of the organelle markers. Thus, at the end of the correlation profiling computation we will generate lists of proteins and lipids for each organelle.

In the first 6 months of this project, the subcellular fractionation methodology will be fully optimised by comparing it with other well-characterised organelle markers to ensure the best markers are chosen for correlation profiling. For normalization, each gradient will be loaded with an equal number of cells as determined by cell counting prior to homogenisation. Following optimisation and standardization, biological triplicate gradients will be prepared from $PC3^{Cavin\text{-}1}$ and $PC3^{GFP}$cells. Fractions will be confirmed by dot blot of organelle markers, then subjected to proteomics and lipidomics analysis as outlined in Aim 1. In a pilot LC-MS/MS analysis, we identified 350 and 480 proteins from fractions 4 and 5, respectively. Spectral intensity and spectral count of the organelle markers correlated with the dot blot results in Figure 6.

B. Computational analysis and hypothesis generation.
To answer the question of which compartments are altered by cavin-1 expression, organelle protein and lipid composition will be compared between $PC3^{Cavin\text{-}1}$ and $PC3^{GFP}$cells. As a positive control, we expect cholesterol to move from the plasma membrane in $PC3^{GFP}$cells to an internal compartment in $PC3^{Cavin\text{-}1}$ cells.

The molecular networks will be built from the molecules identified in each fraction and cell model. All human protein-protein interaction data found in the BioGRID database [48, 49] associated with the candidate proteins will be retrieved and the PPI networks will be constructed by representing proteins as nodes and physical interactions as undirected edges as previously described [50, 51]. Cytoscape program [52] and the ClueGO plugin [53] will be used for network visualization and functional enrichment analysis, respectively. During this project, computational tools for lipid-protein interaction mapping will be developed with PI Wenk, and made available to the research community. We anticipate these novel tools to be highly utilised. The statistical analysis of functional and topological properties of the molecular networks (i.e. PPI, lipid-protein) will be performed by comparing to similar networks from randomly selected lists of molecules [50, 51].

C. Experimental evaluation of hypothesis.
Sections A and B will require approximately 2 years to complete, leaving the PhD candidate the final year of creative, knowledge-driven research, in which s/he will experimentally test a hypothesis generated from the above systematic analysis. For example, redistribution of cholesterol from the plasma membrane to the endosome compartment, together with increase in ESCRT or specific Rab proteins in endosomes will lead to the hypothesis that cavin-1 expression re-wired the endosomal trafficking with identified molecular mediators (ESCRT or Rab) which can be knocked down in functional evaluation of the hypothesis.

Outcomes. This aim will establish novel methodologies for the combined lipid-protein correlation profiling of cytoplasmic organelles. It may reveal novel mechanisms of cavin-1 action.

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