The role of PKCα localisation in Prostate Cancer

Honours Thesis

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30th October 2014

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Co-supervisor: Rob Parton

Statement of Authorship

I, Amanda Oliver confirm that the work presented in this research proposal/research report has been performed and interpreted solely by myself except where explicitly identified to the contrary*. I confirm that this work is submitted in partial fulfilment for the degree of BSc Hons in Biomedical science and has not been submitted elsewhere in any other form for the fulfillment of any other degree or qualification.

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I, Michelle Hill confirm that I have seen a copy of the work presented in this research report as the supervisor of Amanda Oliver.

Date: 30/10/14

Signature: 

Lab notebooks and experimental files are stored with the University of Queensland Diamantina Institute at the Translational Research Institute.

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List of abbreviations

AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
BSA	Bovine serum albumin
CAV1, CAV2, CAV3	Caveolin -1, -2, -3
CKAP4	Cytoskeleton associated protein 4
DAG	Diacylglycerol
DAPI	4’,6-diamindino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRM	Detergent-resistant membrane
ECL	Enhanced chemiluminescent
ECM	Extracellular matrix
EDTA	Edetic acid
EGF/EGFR	Epidermal growth factor/Epidermal growth factor receptor
emPAI	Exponentially modified protein abundance index
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinases
ERM	Ezrin, radixin and moesin
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
GFP	Green fluorescent protein
HRP	Horseradish peroxidase
IF	Immunofluorescence
KEGG	Kyoto Encyclopedia of Genes and Genomes
MMP-9	Matrix metalloproteinase 9
NPM	Nucleophosmin
Opti-MEM	Opti-Minimal Essential Medium
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PKC, PKCα, PKCδ	Protein Kinase C – alpha, delta
PKCDBP	Protein Kinase C Binding Protein (cavin-3)
PLA	Proximity ligation assay
PLD	Phospholipase D
PPI	Protein-protein interactions
PS	Phosphatidylserine
PS	Phosphatidylserine
PTRF	Polymerase transcript release factor (cavin-1)
PVDF	Polyvinylidene fluoride
RPMI	Rosewell Park Memorial Institute
SDPR	Serum deprivation response protein (cavin-2)
SDS	Sodiujm dodecyl sulfate
SDS-PAGE	SDS Polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SFK	Src family kinase
SRBC	sdr-related gene product that binds to c-kinase (cavin-3)
STRING	Search Tool for the retrieval of Interacting Genes/Proteins
TBST	Tris buffered saline + Tween 20
WB	Western blot

Abstract

While localised prostate tumours are almost always successfully treated, treatment options for late stage metastatic prostate tumours are limited. Therapeutic targets specific for advanced prostate cancer are therefore required to decrease the number of prostate cancer related deaths. Cavin protein family members, cavin-1, cavin-2 and cavin-3, have been implicated as tumour supressors in prostate cancer. Along with caveolin-1 (CAV1), cavin-1 expression is required to form caveolae, a cholesterol rich membrane microdomain (lipid raft). Furthermore, cavins -2 and -3 are thought to stabilise caveolae in a cavin-1 dependent manner. Intriguingly, caveolin-1 is a known prostate tumour promoter and is associated with advanced, metastatic prostate cancer. Introduction of cavin-1 expression into CAV1-positive prostate cancer cells suppresses tumourigenicity, partly by a reduction in migration. Additionally, cavin-1 expression recruits protein kinase C alpha (PKCα), a known regulator of cell migration, to cholesterol rich membrane fractions known as lipid rafts. Cavin-2 and cavin-3 have also been identified as binding partners of PKCα and PKCδ respectively.

Therefore, the current project aimed to connect the potential link between cavin-mediated PKCα recruitment to lipid rafts and prostate cancer cell migration. To do this, cavin family members were expressed individually and in combination in the aggressive, CAV1 positive prostate cancer model, PC3 cells. Using functional assays it was shown that migration is reduced upon expression of cavins -1, -2 and -3 compared to control cells. Furthermore, adhesion of cavin-1 cells to fibronectin and collagen was increased, consistent with an increase in integrin α6β4 phosphorylation in the lipid rafts based on analysis of unpublished proteomics results. Bioinformatics analysis of potential protein protein interaction networks showed a potential role for other proteins involved in linking the cytoskeleton to the

membrane and extracellular matrix. Due to its connection with cavins, the localisation of PKCα was investigated in each cell line. Using a proximity ligation assay, co-localisation between PKCα and CAV1 only in cavin-1 expressing PC3 cells was demonstrated. It was previously established that cavin-1 expression induced PKCα recruitment to lipid rafts but this is the first study to demonstrate that this recruitment is caveolae specific. The same analysis of PKCδ, which binds to cavin-3, showed the first reported co-localisation between PKCδ and CAV1. This co-localisation occurred in all cell lines and was therefore independent of cavin expression.

Together, these results suggest that PKCα co-localisation with CAV1 may play a part in adhesion and migration in cavin-1 expressing cells. Furthermore, a role for cavin-2 and cavin-3 in reducing migration of PC3 cells was established in further support of a role in prostate tumour suppression.

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Introduction

Limited therapies exist for advanced prostate cancer

Prostate cancer is the second most diagnosed cancer in males worldwide (Jemal et al., 2011). While mortalities from prostate cancer only account for 6% of total cancer deaths the survival of patients with advanced disease is poor (Gravis et al., 2014; Jemal et al., 2011; Wu et al., 2014). Active surveillance, prostectomy, radiation therapy and androgen deprivation therapy are all successful treatments for localised tumours (Liu et al., 2013; Lund et al., 2014) however, a subset of tumours advance to a castration resistant, metastatic stage for which treatment options are limited (Karantanos et al., 2014; Taplin, 2007). The best treatments for advanced tumours prolong survival for only a few months on average (Patel et al., 2014). To improve the survival prospects in these patients, new therapeutic targets specific for advanced tumours are needed. Recently, the proteins involved in caveolae formation, caveolins and cavins, have been implicated in the progression of prostate cancer and may provide direction for such therapies (Aung et al., 2011; Bryant et al., 2011; Moon et al., 2013; Nassar et al., 2013a; Nassar et al., 2013b).

Caveolae: a lipid raft microdomain implicated in prostate cancer

Caveolae are a type of cholesterol rich membrane microdomain (lipid raft) characterised by small invaginations in the plasma membrane (Parton and del Pozo, 2013; Parton and Simons, 2007) (Figure 1). These microdomains cover the plasma membrane of various cell types including endothelial cells, adipocytes and muscle cells (Parton and del Pozo, 2013; Thorn et al., 2003). The functions of caveolae include endocytosis, mechanotransduction

and various signal transduction events (Calizo and Scarlata, 2012; Echarri and Del Pozo, 2006; Le Lay et al., 2006; Sun et al., 2010). Two distinct protein families, caveolins and cavins, have been identified in the formation and structure of caveolae.

Caveolins

Caveolins are a family of integral membrane proteins consisting of three members, caveolin-1 (CAV1), caveolin-2 (CAV2) and caveolin-3 (CAV3) (Rothberg et al., 1992; Scherer et al., 1996; Way and Parton, 1996). Caveolae formation requires CAV1 and CAV3 expression and homo-oligomerisation in non-muscle and muscle cell types respectively. Caveolae formation is lost upon genetic ablation of CAV1 and CAV3 (Drab et al., 2001; Galbiati et al., 2001; Razani et al., 2002). Furthermore, ectopic expression of CAV1 causes de novo formation of caveolae in lymphocytes (Fra et al., 1995). CAV1 and CAV3, but not CAV2, are therefore essential for caveolae formation.

Cavins

Recently, the cavin protein family was established as coat proteins that stabilise caveolae (Bastiani et al., 2009; Hill et al., 2008). The four existing members of this family, cavin-1, cavin-2, cavin-3 and cavin-4 are differentially expressed depending on the cell type (Bastiani et al., 2009). When co-expressed with cavin-1, cavins form distinct heteromeric complexes in the cytosol and associate with caveolae in the presence of CAV1 (Bastiani et al., 2009). Cavin-4 is expressed predominantly in muscle cells (Bastiani et al., 2009). Cavins -1, -2 and -3 are expressed in non-muscle cell types and exist in two distinct complexes, cavin-1 and -2 or cavin-1 and -3 (Gambin et al., 2014; Ludwig et al., 2013). Of the four cavin family members, only cavin-1 is essential for caveolar formation (Bastiani et al., 2009; Hill et al., 2008; Liu et al., 2008). Cavin-2 and -3 are not essential for formation but are thought to influence

caveolar tubulation and curvature, and trafficking and internalisation respectively (Hansen et al., 2009; Hernandez et al., 2013; McMahon et al., 2009). In normal tissues, CAV1 is co-expressed with cavin-1, leading to caveolar formation (Bastiani et al., 2009; Hill et al., 2008). However, without cavin-1 expression, CAV1 lies on a planar surface which has been termed non-caveolar caveolin-1 (Hill et al., 2008; Moon et al., 2013). Intriguingly, this expression pattern contributes to prostate tumour progression (Figure 1c).

Caveolins as tumour promoters and cavins as tumour suppressors

In prostate cancer, caveolins and cavins have been reported as tumour promoters and suppressors, respectively (Table 1). Neither CAV1 nor cavin-1 is expressed in normal prostate epithelia however overexpression of CAV1 in advanced tumours is common (Moon et al., 2013). This expression pattern is mirrored in the aggressive prostate cancer cell model, PC3 cells (Hill et al., 2008). CAV1 overexpression *in vitro* promotes tumour growth, anchorage independence, angiogenesis and insensitivity to androgen depletion therapy (Li et al., 2009; Li et al., 2001; Moon et al., 2013; Nasu et al., 1998; Tahir et al., 2009; Timme et al., 2000). In addition, *in vivo* studies show that decreased CAV1 expression results in reduced tumour burden, metastasis and androgen insensitivity (Watanabe et al., 2009; Williams et al., 2005). As a result, CAV1 has been suggested as a biomarker, indicator of therapy efficacy and therapeutic target (Kuo et al., 2012; Tahir et al., 2013; Yang et al., 1999).

Excitingly, cavin-1 expression can attenuate the tumour promoting effects of CAV1 overexpression in prostate cancer. Ectopic expression in PC3 cells reduces proliferation, migration and anchorage independent growth *in vitro* (Aung et al., 2011; Hill et al., 2012; Moon et al., 2013). In an *in vivo* mouse xenograft model, expression of cavin-1 in PC3 cells

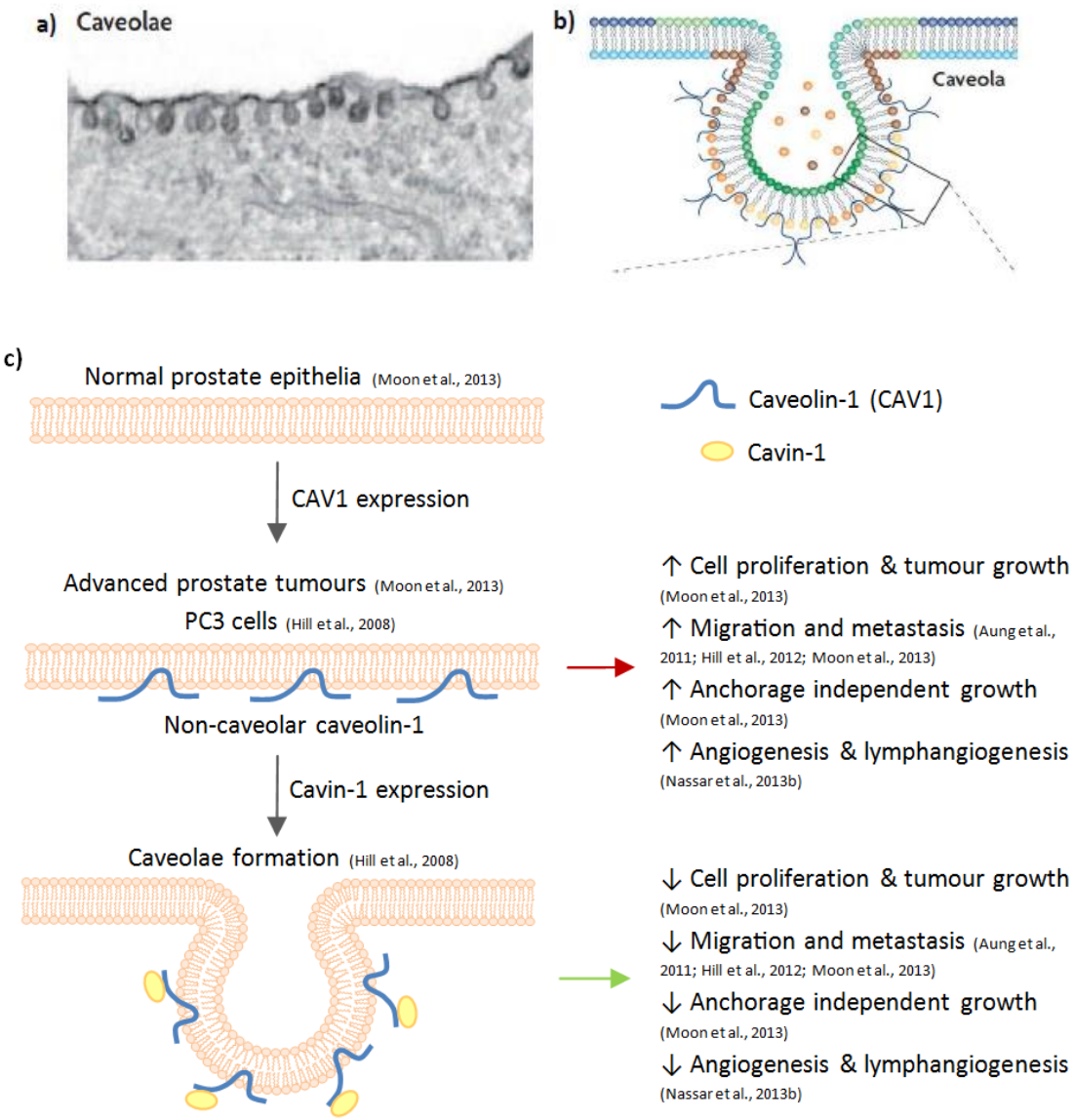

Figure 1: *Caveolae, caveolins and cavins in prostate cancer progression.* Caveolae are small invaginations located in membrane lipid rafts (Parton and Simons, 2007). The main proteins in caveolae, caveolins and cavins, are implicated in prostate tumour progression and suppression respectively. **a)** Electron micrograph of caveolae in adipocytes (Adapted from Parton and Simons, 2007). **b)** Structure of caveolae, 60-80nm diameter invaginations in the membrane (Parton and Simons, 2007). The blue hairpins represent caveolin-1, the first identified structural protein in caveolae (Rothberg et al., 1992) (Adapted from Parton and Simons, 2007). **c)** In normal prostate epithelia there is no expression of caveolins or cavins. However, in advanced prostate cancer progression CAV1 overexpression is common, this organisation is termed non-caveolar caveolin-1. Cavin-1 expression in caveolin-1 positive prostate cancer cells induces the formation of caveolae and reduces tumourigenesis.

decreased tumour size, metastasis, angiogenesis and lyphangiogenesis (Moon et al., 2013; Nassar et al., 2013b). Currently, a small amount of research has suggested possible mechanisms by which cavin-1 expression reduces migration in PC3 cells. Secretion of matrix metalloproteinase 9 (MMP-9) was decreased by expression of cavin-1 and to a lesser extent cavins 2-4 (Aung et al., 2011). Cavin-1 expression results in a decrease in markers of epithelial to mesenchymal transition (EMT), a process by which cells gain migratory and invasive properties (Hill et al., 2012). Proteomics results suggest that cavin-1 expression in PC3 cells decreases localisation of cytoskeletal proteins in lipid rafts (Inder et al., 2012). However, further mechanisms, as well as possible roles for cavins -2 or -3, remains to be investigated.

The roles of cavins -2 and -3 in prostate tumour suppression are not well characterised. However, there is evidence that cavin-2 and -3 are downregulated in prostate cancer (Altintas et al., 2013; Li et al., 2008; Mishra et al., 2010). There are reports that both, along with cavin-1, are down regulated by epigenetic and unknown mechanisms in various cancer types (Bai et al., 2012; Gianazza et al., 2012; Lee et al., 2008; Lee et al., 2011; Melson et al., 2014; Tong et al., 2010; Xu et al., 2001; Zochbauer-Muller et al., 2005). Furthermore, loss of cavin-3 in fibroblasts reduces ERK and increases Akt signaling and induces aerobic glycolysis, proliferation and resistance to apoptosis (Hernandez et al., 2013). However, the consequences of both cavins -2 and -3 in tumour cells are not yet established.

PKCα: a possible player in reducing prostate cancer cell migration

Given the evidence of cavins as tumour suppressors, their interacting partners and downstream pathways are of interest. Intriguingly, cavin-1, cavin-2 and cavin-3 have all been independently linked with Protein kinase C (PKC), a family serine/threonine kinases

involved in various cellular processes (Fogh et al., 2014; Mellor and Parker, 1998). Cavins -1 and -2 have been implicated with PKCα localisation and function (Hill et al., 2012; Mineo et al., 1998). PKCα is a key regulator of cell morphology, cytoskeletal dynamics and migration (Fogh et al., 2014). Activation of PKCα is dependent upon phosphotidylserine (PS), diacylglycerol (DAG) and calcium which cause conformational changes that expose binding sites for substrates and/or regulatory proteins (Kishimoto et al., 1980; Mellor and Parker, 1998). To modulate this, various membrane associated proteins are involved in tethering PKC isozymes to specific locations (Mochly-Rosen et al., 1991a; Mochly-Rosen et al., 1991b). Alterations in these proteins are associated with abnormalities in PKC activity and/or localisation and can be implicated in cancer development (Egidy et al., 2008; Zhu et al., 2005).

Although PKCα is mostly cytosolic it has been identified in lipid rafts, a localisation that may be mediated by cavin proteins (Melnikov and Sagi-Eisenberg, 2009; Mineo et al., 1998; Prevostel et al., 2000). Cavin-2 and -3 were first identified as binding partners of PKCα and related isozyme PKCδ, respectively (Izumi et al., 1997; Mineo et al., 1998). Recent data from our lab suggests that in PC3 cells only cavin-1 is required to recruit PKCα to lipid rafts (Hill et al., 2012). Knock-down of cavin-1 in NIH3T3 fibroblasts, that express cavins 1-3, resulted in a loss of PKCα from the lipid raft fraction (Hill et al., 2012). Similarly, cavin-1 expression in PC3 cells induces PKCα recruitment to the lipid raft fraction (Hill et al., 2012). Cell lysate and whole membrane PKCα levels were unchanged in both experiments (Hill et al., 2012). Taken together, it is possible that cavin-1 can alter PKCα localisation and this may impact its function, such as that in migration.

Table 1: Summary of caveolin-1 and cavin-1, -2 and -3 in caveolae formation and tumour progression.

Protein	Role in caveolae	Role in tumour progression
CAV1	• Required for formation with cavin-1 (Drab et al., 2001; Fra et al., 1995)	• Expression increases with prostate cancer stage and grade and is a predictor of poor clinical outcome (Gumulec et al., 2012; Tahir et al., 2006; Yang et al., 2005) • Has been suggested as a biomarker for diagnosis, prognosis and therapy efficiency in tissue or serum (Kuo et al., 2012; Tahir et al., 2013; Yang et al., 1999) • Promotes angiogenesis, tumour aggressiveness, invasion and metastasis, resistance to androgen depletion, cell proliferation and colony formation in both *in vitro* and *in vivo* models (Li et al., 2001; Tahir et al., 2006; Tahir et al., 2009; Timme et al., 2000) • Suppression decreases metastasis and tumour burden and increases androgen sensitivity (Li et al., 2001; Nasu et al., 1998; Williams et al., 2005) Reviewed in (Nassar et al., 2013a) and (Goetz et al., 2008)
Cavin-1/ PTRF	• Required for formation with CAV1 (Hill et al., 2008) • Responsible for formation and localisation of cavin complex at caveolae (Bastiani et al., 2009; Gambin et al., 2014; Ludwig et al., 2013)	• Not expressed in prostate cancer and aggressive prostate cancer cell model, PC3 cells (Gould et al., 2010; Hill et al., 2008; Moon et al., 2013) • Reduces proliferation, migration and anchorage dependent growth *in vitro* (Aung et al., 2011; Hill et al., 2012; Moon et al., 2013) • Decreases tumour burden, metastasis, angiogenesis and lymphangiogenesis *in vivo* (Moon et al., 2013; Nassar et al., 2013b) • Reduces secretion of proteases, cytokines and growth regulating proteins (Inder et al., 2012) • Downregulated in breast cancer (promoter hypermethylation)(Bai et al., 2012) Reviewed in (Nassar et al., 2013a)
Cavin-2/ SDPR	• Tubulation and elongation (Hansen et al., 2009) • Forms complex with cavin-1 and is recruited to caveolae (Bastiani et al., 2009; Gambin et al., 2014; Ludwig et al., 2013)	Down regulated in: • Breast, kidney and prostate cancers (Fhl1 regulated mechanism) (Li et al., 2008) • Breast cancer (unknown mechanism) (Bai et al., 2012) • Renal cell carcinoma (reduced serum peptide levels) (Gianazza et al., 2012) • Prostate cancer compared to matched normal prostate tissue (RNA-microarray and qRT-PCR) (Altintas et al., 2013)
Cavin-3/ SRBC/ PKCDPB	• Internalisation and trafficking (McMahon et al., 2009) • Forms complex with cavin-1 and is recruited to caveolae (Bastiani et al., 2009; Gambin et al., 2014; Ludwig et al., 2013)	Down regulated by promoter hypermethylation in: • Breast and lung cancers (Xu et al., 2001) • Prostate cancer cell lines (Mishra et al., 2010) • Ovarian cancer (Tong et al., 2010) • Colorectal (Lee et al., 2011) • Serum from patients with pancreatic cancer (Melson et al., 2014)

Many focal adhesion complex proteins, which are involved in cell adhesion to the extracellular matrix (ECM) are commonly activated by PKCα. These include filamin A, talin, vinculin, integrins and Rho GTPases (Dovas et al., 2010; Litchfield and Ball, 1986; Muriel et al., 2011; Ng et al., 1999; Tigges et al., 2003; Ziegler et al., 2002). Additionally, PKCα phosphorylates cytoskeletal proteins such as actin and α-tubulin which alter cell morphology (De et al., 2014; Larsson, 2006). During cell migration polymerisation of the actin cytoskeleton causing protrusions of the cell, coupled with stabilisation of protrusions by adhering to the ECM occurs in a cyclic fashion (Beningo et al., 2001; Zaidel-Bar et al., 2003). Focal adhesion and cytoskeletal proteins are key players in such a dynamic process, therefore PKCα is a key player in regulating cell migration. This is highlighted in many cancer types in which overexpression of PKCα causes or is associated with an increase in migration (Masur et al., 2001; Ng et al., 1999). Therefore, altered localisation of PKCα in prostate cancer could be a mechanism by which cavin-1 decreases migration in PC3 cells.

Hypothesis and aims

Given the evidence above, I hypothesized that the binding of cavins recruits PKCα to caveolae in cavin-1 expressing cells, leading to a reduction in migration (Figure 2). To address this hypothesis, three specific aims were developed to:

1. Determine the relative abilities of cavin proteins to recruit PKCα to caveolae.
2. Correlate recruitment of PKCα to caveolae with a phenotypic change in migratory and/or adhesive ability using functional assays.
3. Investigate the mechanisms by which the caveolar localisation of PKCα may influence other proteins.

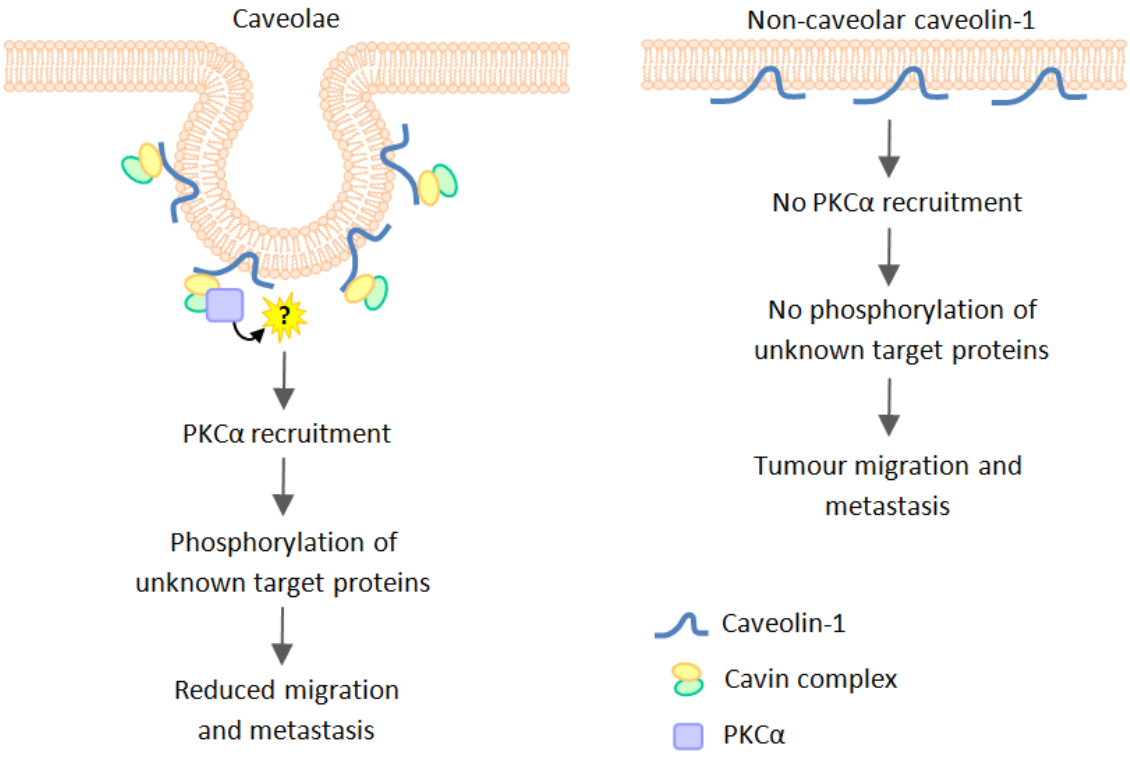

Figure 2: *Proposed mechanism of cavin-1 mediated reduced migration in PC3 cells.* Expression of cavin-1 in PC3 prostate cancer cells induces caveolae formation and reduces migration and metastasis associated with non-caveolar caveolin-1 (Aung et al., 2011; Hill et al., 2008; Hill et al., 2012; Moon et al., 2013). PKCα, a known regulator of cell migration, is recruited to the lipid raft fraction of cavin-1 expressing PC3 cells but not control cells (Hill et al., 2012). This project hypothesized that when recruited to lipid rafts, PKCα phosphorylates unknown target proteins potentially involved in cell morphology and attachment and that this change is partly responsible for the reduction in migration of cavin-1 expressing PC3 cells.

Methods

Reagents

Reagents and antibodies were purchased from the following sources:

Tissue culture reagents: Trypsin-EDTA and Opti-MEM media (Gibco), Roswell Park Memorial Institute (RPMI) 1640 media and Paraformaldehyde (PFA) (Sigma), Geneticin G418 Antibiotic and Lipofectamine 2000 (Invitrogen), Fetal Bovine Serum (FBS) (Bovogen), Phosphate Buffered Saline (PBS) (Amresco Inc).

Antibodies: See Table 2.

Cell culture

PC3 cells stably expressing GFP alone and GFP tagged to cavins were previously generated (Bastiani et al., 2009). Note that cell lines are frequently described as cavin-1, cavin-2 or cavin-3 expressing, this refers to the GFP tagged cavins. Western immunoblotting of GFP was used to determine that expression level of cavins was similar between cell lines (data not shown). All cell lines were cultured in RPMI1640 media containing 5% FBS and were kept in a 5% CO_2 incubator at 37°c. Stable cell lines were cultured with 0.1mg/mL G418 antibiotic to select for GFP expressing cells. Unless otherwise stated, cells were detached from flasks using 0.25% Trypsin-EDTA.

Transient transfection

Expression plasmids for cavin-2-cherry, cavin-3-cherry or cavin-1-flag were previously generated (Bastiani et al., 2009). Cells were grown to 60-80% confluency in a dish or plate appropriate for the amount of cells needed. Lipofectamine 2000 and DNA were incubated separately in Opti-MEM media up to 100µl total volume for 5 minutes before mixing both

solutions and incubating for 20 minutes at room temperature. Lipofectamine-DNA mixture was added to cells and incubated for 4 hours in a 5% CO_2 37°c incubator. Media with Lipofectamine-DNA mixture was removed and replaced with fresh growth media. Transfection efficiency was determined by western blotting and fluorescence microscopy. Lipofectamine:DNA ratio was constant at 2µl Lipofectamine to 1µg DNA. The amount of DNA was dependent upon the plate/dish size (Table 3).

Immunofluorescence Microscopy

For immunofluorescence (IF), cells were grown to 70% confluency on coverslips and fixed in 4% PFA for 20-30 minutes after washing in PBS. After fixation, coverslips were stored until use in PBS at 4°c for up to 4 weeks. Fixed cells were washed a further three times in PBS followed by permeabilising for 10 minutes in 0.02% Triton-X-100/PBS and blocking in 3% BSA/PBS for 30 minutes. Coverslips were then incubated with respective primary antibodies in 3% BSA/PBS for 1 hour at room temperature followed by washing 3 times in PBS and incubating with secondary antibody in 3% BSA/PBS for 1 hour in the dark at room temperature. After washing 3 times in PBS, coverslips were incubated with DAPI for 5-10 minutes in the dark and washed in PBS and MilliQ water. Any excess water was removed with a Kimwipe and coverslips were added to slides with 6ul of prolong diamond (Invitrogen). Slides were dried at 37°c for at least 3 hours and imaged within 3 days on Zeiss Confocal microscope.

PLA assay

Protocol for PLA assay (Invitrogen Duolink) was the same as IF until after incubation with primary antibodies (Figure 3). After incubating 8µl of each secondary antibody from the PLA kit (5x anti-rabbit PLUS and 5x anti-mouse MINUS) in 24µl 3% BSA/PBS for 20

Table 2: Dilutions and source for antibodies used. WB = western blot. IF = immunofluorescence.

Antibody	Dilution for WB	Dilution for IF	Source
Mouse anti-PKCα	1:1000	1:100	BD Transduction Laboratories
Mouse anti-PKCδ	1:1000	1:100	BD Transduction Laboratories
Rabbit anti-caveolin-1	1:3000	1:200	BD Transduction Laboratories
Mouse anti-nucleophosmin		1:100	Zymed
Goat anti-rabbit Alexa Fluor 405, 568 or 647		1:200	Molecular probes/Invitrogen
Rabbit anti-cavin-1/PTRF	1:2000		ProteinTech
Rabbit anti-cavin-2/SDPR	1:100		ProteinTech
Rabbit anti-cavin-3/PKCDBP	1:3000		ProteinTech
Mouse anti-β-actin	1:6000		Sigma
Goat anti-rabbit HRP	1:3000		Invitrogen
Goat anti-mouse HRP	1:3000		Invitrogen

Table 3: Amount of DNA used for transfection for relevant plate/dish size.

Plate/dish size	Amount of DNA (µg)
6 well plate	2µg
10cm dish	5µg
15cm dish	10µg

minutes at room temperature, coverslips were incubated with secondary antibodies at 37°c in a pre-humidified chamber for 1 hour. Following 3x 5 minute washes, coverslips were incubated for 30 minutes at 37°c in a pre-humidified chamber with 8µl 5x ligation stock plus 1µl of ligase in 31µl MilliQ water. Coverslips were washed 3 times in PBS and incubated with amplification solution (8µl amplification stock plus 0.5µl polymerase in 31.5µl MilliQ water) for 100 minutes in the dark at 37°c in a pre-humidified chamber. Coverslips were incubated with DAPI (diluted 1:1000 in PBS) for 5-10 minutes at room temperature in the dark after washing in PBS 3 times (5 minutes each wash). Coverslips were washed in PBS again, rinsed in MilliQ water, dried on a Kimwipe and mounted on slides with 6µl prolong diamond. Slides were dried for at least 3 hours at 37°c and imaged within 3 days on Zeiss Confocal microscope. Five representative images were taken for each replicate and condition. For quantification, the number of PLA signals per cell was counted for 20 cells and normalised by dividing by cell area calculated on image J.

Whole cell lysate collection

Whole cell lysates were collected from cells plated on 10cm dishes at 80% confluency. Dishes were placed on ice and washed twice with cold PBS. Excess PBS was removed from dishes, cells were scraped in 300µl of lysis buffer (1% Triton-X100, 20mM Tris pH7.5, 150mM NaCl, 1x protease inhibitor cocktail, 0.5mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 0.5mM sodium vanadate and 10mM NaF in MilliQ water) and transferred to a 1.5mL tube. Samples were incubated for 30 minutes on ice before spinning at 15,000g for 5 minutes at 4°c. The supernatant was collected, amount of protein estimated using a Bradford assay.

Amount of protein was estimated using Bradford reagent (Biorad). Bradford reagent was

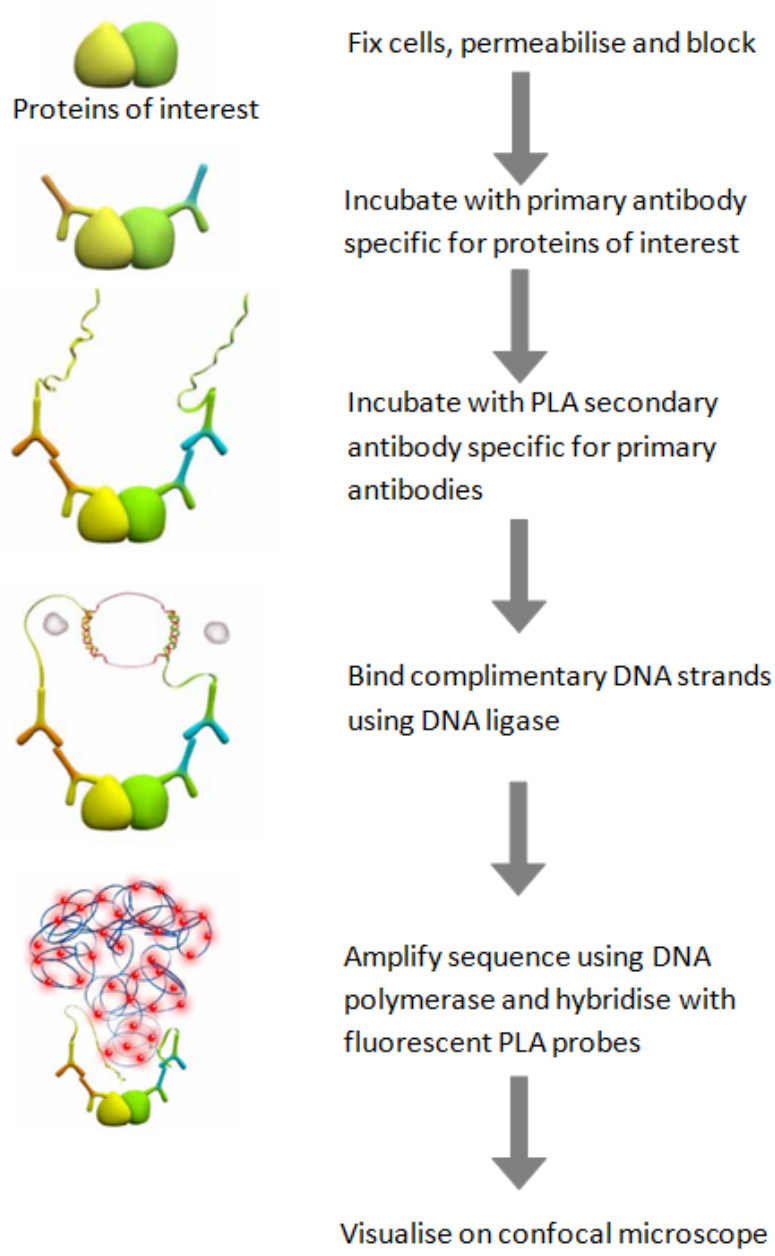

Figure 3: *Schematic of proximity ligation assay (PLA) workflow.* Cells are grown on coverslips and fixed in 4% PFA/PBS, permeabilised in 0.02% Triton-X-100/PBS and blocked in 3% BSA/PBS. Incubation with primary antibodies (raised in mouse and rabbit) and secondary antibodies (PLA probes specific for mouse and rabbit antibodies) targets proteins of interest. When proteins of interest are in close proximity, a ligation reaction binds complimentary DNA strands conjugated to the secondary antibodies. Interactions are amplified by rolling circle DNA replication of the ligated DNA and hybridisation with a fluorescent probe. Co-localisation of proteins of interest is visualised by fluorescent signals using confocal microscopy.

diluted 1:5 in MilliQ water and 200µl was added to 5µl of BSA standards (0, 0.2, 0.4, 0.6, 0.8 and 1µg/µl) and samples in a 96 well plate in duplicate. Absorbance at 595nm was measured using a spectrophotometer (Thermoscientific) and the protein concentration of samples were estimated from a standard curve generated from BSA standards. Samples were then diluted to 1µg/µl in 1x reducing SDS-PAGE sample buffer (2% SDS, 10% glycerol, 0.02% bromophenol blue, 50mM DTT, 40mM Tris pH 6.8) and denatured by boiling for 5 minutes at 95°c.

Western blot analysis

Twenty micrograms of each sample were loaded and ran on 10% SDS-PAGE gels. Proteins were transferred by wet transfer onto a Polyvinylidene fluoride (PVDF) membrane (Millipore) for 90 minutes at 100V. After transfer, membranes were blocked for 30 minutes in 5% skim milk powder in TBST (20mM Tris-HCl, 137mM NaCl, 0.1% Tween-20, pH 7.5). Membranes were incubated with primary antibodies at 4°c overnight. After washing 3x in TBST (5 minutes each wash), membranes were incubated with secondary HRP (horseradish peroxidase) antibody for 1 hour at room temperature. After washing, blots were visualized using enhanced chemiluminescent (ECL) substrates (SuperSignal West Pico/Dura; ThermoScientific) and developed on film (Fuji Super Rx medical x-ray film).

Transwell Migration assay

Assays were performed with 8µm pore, transwell inserts (Corning) in a 24 well plate to measure chemotactic migration. Cell suspensions (1x 10^6 cells/mL) were prepared in serum-free RPMI1640 media containing 0.1% BSA and 100µl was loaded on the top chamber. The bottom chamber contained RPMI1640 media with 10% FBS. Cells were allowed to migrate for 20 hours in a 5% CO_2 37°c incubator. After incubation, filters were washed in PBS and fixed for 10 minutes in 4% PFA. Non-migrating cells were removed from the top of the filter

by swabbing twice with a wet cotton tip and remaining cells were stained with DAPI for 5-10 minutes. Filters were removed and set on slides in 8µl prolong diamond (Invitrogen) with coverslips and viewed using an epifluorescence microscope at 20x magnification. For each filter, 10 images were taken and number of cells was quantified using imageJ.

To determine if there was a difference in growth rate between the cell lines during the time of transmigration assay, cell counting was performed. Cells were counted using a haemocytometer and $2x10^5$ cells were added to a 6-well plate with 2ml of 5% FBS RPMI1640. Following a 20 hour incubation at 37°c (5% CO_2) cells were detached with 250µl of Trypsin-EDTA. Trypsin was neutralised by 750µl growth media and cell number was counted using a haemocytometer.

Adhesion assay

Poly-D-lysine (Sigma), fibronectin (Sigma) and collagen (Sigma) were used to coat a 96 well plate for adhesion assays. For coating, 1.6ug (ie. 5µg/cm^2) of each protein was added in duplicate wells for 5 conditions (4 cell lines and a blank) and incubated for 1 hour at room temperature. Plates were washed in PBS and blocked with 0.1%BSA in PBS for 1 hour at 37°c, then kept at 4°c with 100µl PBS in each well until use.

Cells were detached from flasks with versene (Gibco) at 37°c for 15 minutes. After adding growth media to dilute versene, cells were counted using a haemocytometer, diluted to $4x10^5$ cells/mL and pelleted at 20g. Supernatant was removed and cell pellet was resuspended in serum free RPMI1640 with 0.1% BSA. One hundred µl of cells ($4x10^4$ cells) were added to coated plates in duplicate wells and incubated for 30 minutes in a 5% CO_2 37°c incubator. Unattached cells were removed with the media, and the wells were washed twice in PBS and stained with Crystal violet (Sigma) for 10 minutes. Crystal violet was

removed and wells were washed with PBS until colourless on removal. Cells were lysed with 100µl DMSO and absorbance was read at 570nm using a spectrophotometer.

Bioinformatics analysis

The results from a previous proteomics experiment conducted by Dorothy Loo (former Hill Lab member) were utilised for this analysis. Briefly, the detergent-resistant membrane fractions (DRMs) from cavin-1 and GFP expressing PC3 cells were collected and PKC substrates were immunoprecipitated with a PKC substrate specific antibody. The isolated proteins were identified using mass spectrometry and analysed using Spectrum Mill (Agilent) by Dorothy Loo. Three replicate experiments were performed for each condition. The output from Spectrum Mill was then analysed in Scaffold to determine significantly altered proteins in the cavin-1-GFP expressing cells compared to the GFP expressing cells using the quantitative analysis tool with normalisation. Significantly altered proteins were entered into a protein-protein interaction (PPI) database, STRING (string-db.org), along with PKCα and cavin-1/PTRF to generate a PPI network (Franceschini et al., 2013; Jensen et al., 2009; Snel et al., 2000; Szklarczyk et al., 2011; von Mering et al., 2003; von Mering et al., 2007; von Mering et al., 2005). The interaction score cut off was set to the lowest threshold (≥0.150) to see all potential interactions. Ten predicted functional partners were added using predictions from STRING to see potential networks influenced by PKCα at lipid rafts.

Statistical analysis

Unless stated, all results were analysed with GraphPad Prism 6. Two-way, unpaired Student's t-tests were used to obtain p-values. All errors written in text and displayed in graphs represent the standard error of the mean (SEM). Significance cut off was set at $p = 0.05$.

Results

Focal adhesion forming proteins are phosphorylated by PKC

Cavin-1 expression in caveolin-1 positive prostate cancer cell line, PC3 cells, results in the formation of caveolae in lipid rafts and reduces tumourigenesis (Aung et al., 2011; Hill et al., 2012; Moon et al., 2013; Nassar et al., 2013a). PKCα is recruited to lipid rafts in cavin-1 expressing cells (Hill et al., 2012), and may be a mediator of cavin-1 action as it is known to regulate cell migration. To determine potential targets of PKCα at caveolae in PC3 cells, results of a previous experiment performed in the lab (by Dorothy Loo) were analysed using bioinformatics. For this experiment, the DRMs from GFP and cavin-1 expressing PC3 cells were collected and PKC substrates were immunopreciptated by a PKC substrate specific antibody. The resulting proteins isolated from this immunoprecipitation experiment were then identified using mass spectrometry. Scaffold was used to generate a list of significantly altered proteins based on normalized Protein Abundance Index (emPAI) (Ishihama et al., 2005). A total of 153 proteins were identified from 3 replicate experiments. From this list of proteins, 13 were elevated in the cavin-1 expressing PC3 cells compared to the control (Table 4). However only 3 of these proteins, integrin α6 ($p < 0.0001$), cytoskeleton-associated protein 4 (CKAP4) ($p = 0.027$) and integrin β4 ($p = 0.028$) were significantly elevated as assessed by student's t-test.

To generate a PPI network map from the significantly altered proteins, the online PPI network database STRING (string-db.org) was used. STRING makes predictions on interacting partners based on imported interactions from existing databases, associations in functional genomics and textmining from published literature (Franceschini et al., 2013; Jensen et al., 2009; Snel et al., 2000; Szklarczyk et al., 2011; von Mering et al., 2003; von

Mering et al., 2007; von Mering et al., 2005). In addition, STRING calculates an 'interaction score' or confidence score that is referenced to the probability of finding both proteins in the same Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (Kanehisa et al., 2004; von Mering et al., 2005). The significantly altered proteins, along with PKCα and cavin-1, were uploaded into STRING. The PPI network generated predicted interactions between PKCα and both integrin α6 (0.906 interaction score) and integrin β4 (0.975 interaction score) (Figure 4a). A high probability interaction was also detected between integrin α6 and integrin β4 (0.999 interaction score). Furthermore, a possible interaction between PKCα and cavin-1 was predicted based on textmining of existing literature (0.156 interaction score). Further to this analysis, the top 10 predicted functional partners for this protein network were added using an inbuilt function of STRING (Figure 4b). From this analysis predicted partners of the original network were integrin β1, plectin-1, collagen type XVII α1, phospholipase D1, integrin β7, ezrin, gap junction protein α1, phospholipase D2, retinoic acid receptor and ras related C3 botulinum toxin substrate 1 (interaction scores ≥ 0.988).

Cavin-1 expression increases adhesion to collagen and fibronectin

The proteomics results showed an increase in phosphorylation of adhesion proteins in the lipid rafts of cavin-1 PC3 cells. As the effects of cavins on cell adhesion have not yet been documented, we went on to examine the effects of cavin expression on PC3 cell adhesion to ECM substrates, collagen and fibronectin. To test this, $4x10^4$ cells were plated in duplicate on 96 well plates coated with collagen, fibronectin or poly-D-lysine. After 30 minutes, non-adhering cells were removed and remaining cells were stained with crystal violet. Differences in adhesion relative to GFP expressing cells were then determined by spectrophotometry of wells after homogenizing with DMSO. Since cells bind non-specifically

Table 4: Proteins identified by mass spectrometry that were elevated in the lipid rafts of cavin-1 expressing PC3 cells compared the control after immunoprecipitation with a PKC substrate antibody.

Protein name	Accession number	Uniprot ID	MW (kDa)	p-value (t-test)
Integrin alpha-6	ITA6_HUMAN	P23229	127	< 0.0001
Cytoskeleton-associated protein 4	CKAP4_HUMAN	Q07065	66	0.027
Integrin beta-4	ITB4_HUMAN	P16144	202	0.028
NADH-ubiquinone oxidoreductase 75 kDa subunit	NDUS1_HUMAN	P28331	79	0.057
Moesin	MOES_HUMAN	P26038	68	0.12
Desmoglein-2	DSG2_HUMAN	Q14126	122	0.13
Clathrin heavy chain 1	CLH1_HUMAN	Q00610	192	0.14
Caveolin-1	CAV1_HUMAN	Q03135	20	0.22
Myeloid-associated differentiation marker	MYADM_HUMAN	Q96S97	35	0.28
Myosin-9	MYH9_HUMAN	P35579	227	0.29
Unconventional myosin-Ib	MYO1B_HUMAN	O43795	132	0.30
Unconventional myosin-Ic	MYO1C_HUMAN	O00159	122	0.37

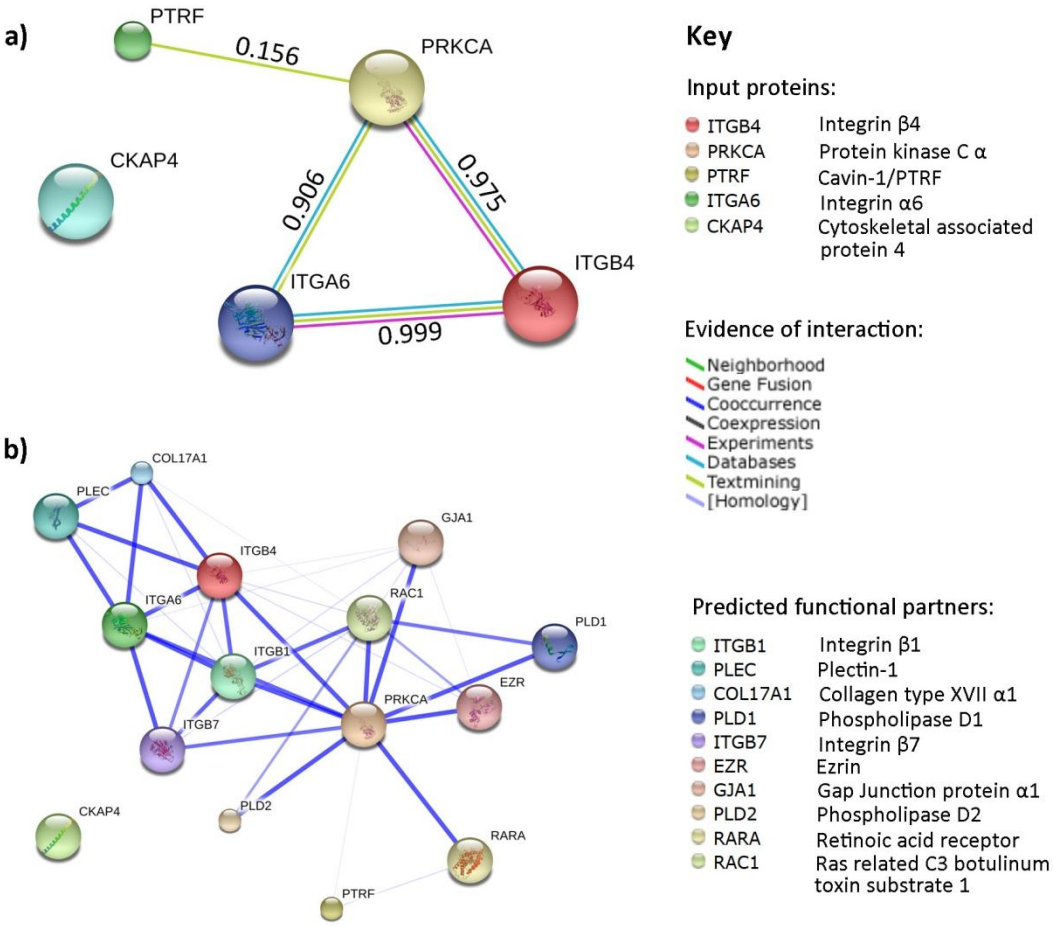

Figure 4: *PKCα is predicted to interact with cell adhesion proteins in the lipid rafts of cavin-1 expressing PC3 cells.* Bioinformatic analysis using STRING (string-db.org) of mass spectrometry data characterizing the PKC substrates in the lipid rafts of cavin-1 and GFP expressing PC3 cells. **a)** Network of significantly elevated proteins in cavin-1 PC3 cells, basis of interaction is represented with coloured lines. The interaction score, indicating the confidence of predicted interaction, is displayed between each interacting protein. **b)** The top 10 predicted functional partners of the interaction network shown in a) above. Thickness of lines between interacting partners indicates the confidence of the interaction, thicker lines mean higher confidence in the predicted interaction.

to poly-D-lysine, it was used as a positive control to confirm that there were no differences in number of cells plated or crystal violet staining between cell lines (Figure 5d). Adhesion of cavin-1 expressing PC3 cells was significantly increased by 1.445 (± 0.099) fold on fibronectin ($p = 0.0109$) and 1.481 (± 0.0822) fold on collagen ($p = 0.0043$) compared to GFP PC3 cells (Figure 5d). Similarly, adhesion of cavin-1 PC3 cells on both fibronectin and collagen was significantly increased compared to cavin-2 expressing PC3 cells ($p \leq 0.0329$). Attachment of cavin-3 PC3 cells to collagen was significantly increased by 1.242 (± 0.064) fold ($p = 0.0192$) compared to GFP control cells. However, no significant difference was observed between cavin-3 and GFP PC3 cells on attachment to fibronectin ($p = 0.1028$). There was no significant difference adhesion to either fibronectin ($p = 0.3571$) or collagen ($p = 0.7400$) between GFP and cavin-2 expressing PC3 cells. This result suggests that cavin-2 expression decreases migration but has no effect on adhesion to ECM proteins in PC3 cells.

Expression of cavins in PC3 cells reduces migration

Next, we wanted to examine the migratory ability of each PC3 cell line. While the effects of cavin-1 in PC3 cell migration are established (Aung et al., 2011; Hill et al., 2012), there are currently no reports on the effects of related cavins, cavin-2 and cavin-3. A transwell migration assay was used to determine differences in 3D migration by chemotaxis to serum containing media over a 20 hour period for each PC3 stable cell line. Before proceeding with the migration assay, a proliferation assay over 20 hours based on cell counting was used. Differences in proliferation between cavin-1 and GFP control PC3 cells have been previously established (Moon et al., 2013). Furthermore, unpublished data from our lab shows that expression of cavins -2 and -3 in PC3 cells causes a dramatic reduction in cell proliferation. Therefore, I wanted to account for any possible effects from proliferation that could be

mistaken for differences in migration. There was no significant difference between proliferation in GFP, cavin-1 and cavin-3 cell lines over 20 hours (Figure 6). However, cavin-2 expression significantly reduced cell number after 20 hours compared to GFP ($p = 0.0075$), cavin-1 ($p = 0.0363$) and cavin-3 ($p= 0.0061$) cell lines. The fold difference in cavin expressing cell lines compared to GFP PC3 cell number was used to normalise the results of the migration assay.

After allowing cells to migrate for 20 hours towards serum containing media through a porous filter, cells were fixed and non-migrating cells remaining on the top of the filter were removed. For quantification, remaining cells were stained with DAPI and number of cells per 20x magnification counted over 10 fields. On average, there were 52.13 (± 4.31) GFP expressing PC3 cells per field (Figure 5). As expected (Aung et al., 2011; Hill et al., 2012), cavin-1 expressing PC3 cells showed a significantly reduced number of migrated cells, 25.90 (± 2.72) per field, compared to GFP expressing PC3 cells after adjusting for proliferation ($p = 0.0029$). Significantly reduced migration was also observed, when normalised for differences in proliferation, in the cavin-2 (19.92 ± 1.12 cells per field, $p = 0.0019$) and cavin-3 (29.47 ± 3.531 cells per field, $p = 0.0152$) expressing cell lines compared to the control. While there was a significant reduction in cavin-2 cell lines compared to cavin-1 and cavin-3 cell lines in the raw data, when adjusted for proliferation, this difference was no longer significant. Therefore, cavin-1, cavin-2 and cavin-3 reduced PC3 cell transmigration to a similar extent.

Further to the stable cell lines, we wanted to test the effects of cavin co-expression on adhesion and migration by creating transient co-expressing cells. Cavin-1 PC3 cells were successfully transfected with cavin-2-cherry and cavin-3-cherry constructs using lipofectamine 2000 (Figure 7). However, cells subjected to this treatment showed

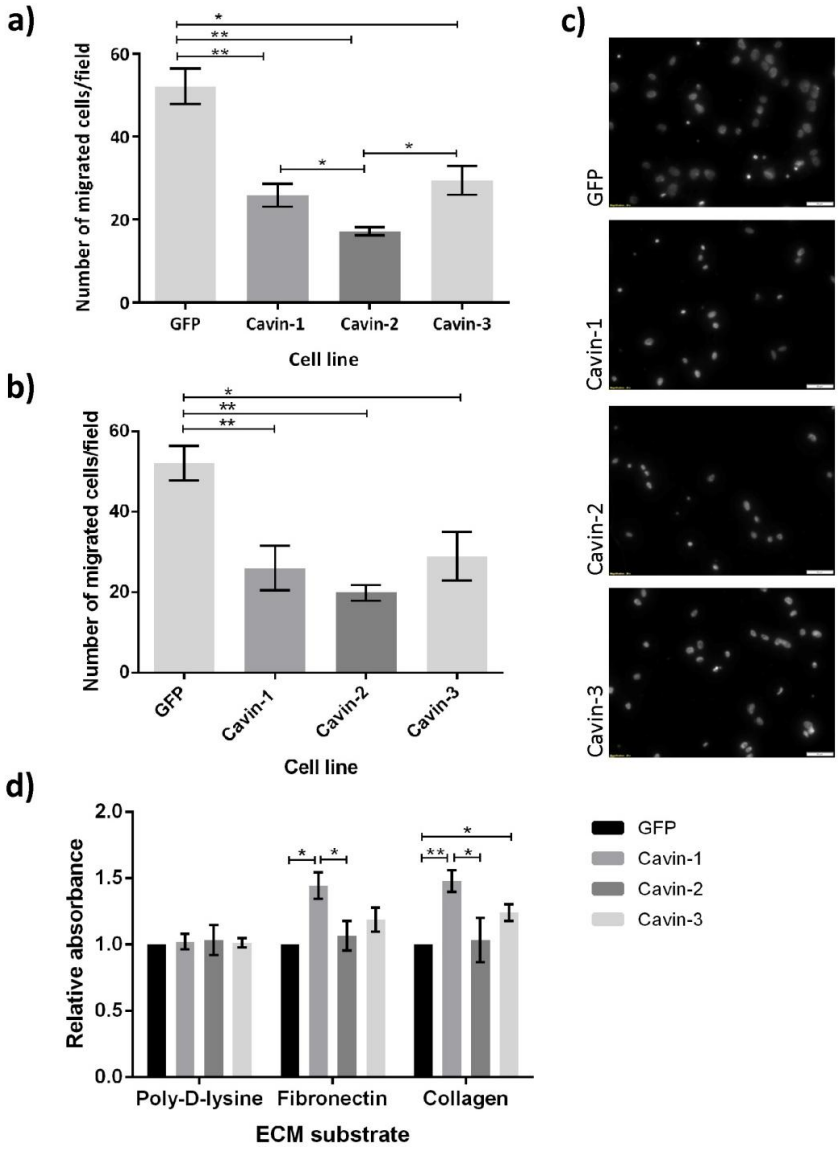

Figure 5: *Effects of cavin expression in PC3 cells on cell migration and adhesion.* **a)** Transwell migration over 20 hours towards serum containing media was measured for GFP, cavin-1, cavin-2 and cavin-3 expressing PC3 cells. Migration was quantified based on the number of cells migrated to the lower chamber seen through fluorescence microscopy of DAPI stained cells (10 fields at 20x magnification). Expression of all cavins reduced migration compared to GFP expression in PC3 cells. Migration of cavin-2 cells was further reduced compared to cavin-1 and cavin-3 PC3 cells. **b)** Results of the migration assay were normalised to account for differences in proliferation over 20 hours (see figure 6). After normalisation, there was no siginificant difference in migration of cavin-1, cavin-2 and cavin-3 PC3 cells. **c)** Representative fluorescent microscopy images of DAPI stained cells from transwell migration assay. Scale bar represents 50µm. **d)** Adhesion of PC3 cell lines to extracellular matrix (ECM) substrates fibronectin and collagen was determined by measuring absorbance (595nm) of crystal violet stained cells after 30 minutes incubation in 96 well plates coated with respective proteins. Poly-D-lysine was used as a positive control. Cavin-1 expressing PC3 cells showed significantly increased adhesion to both collagen and fibronectin compared to GFP and cavin-2 expressing PC3 cells. Cavin-3 cells showed increased adhesion to collagen compared to GFP cells. All data is represented as mean ± SEM from 3 replicate experiments. * p = 0.01-0.05, ** p = 0.001-0.01

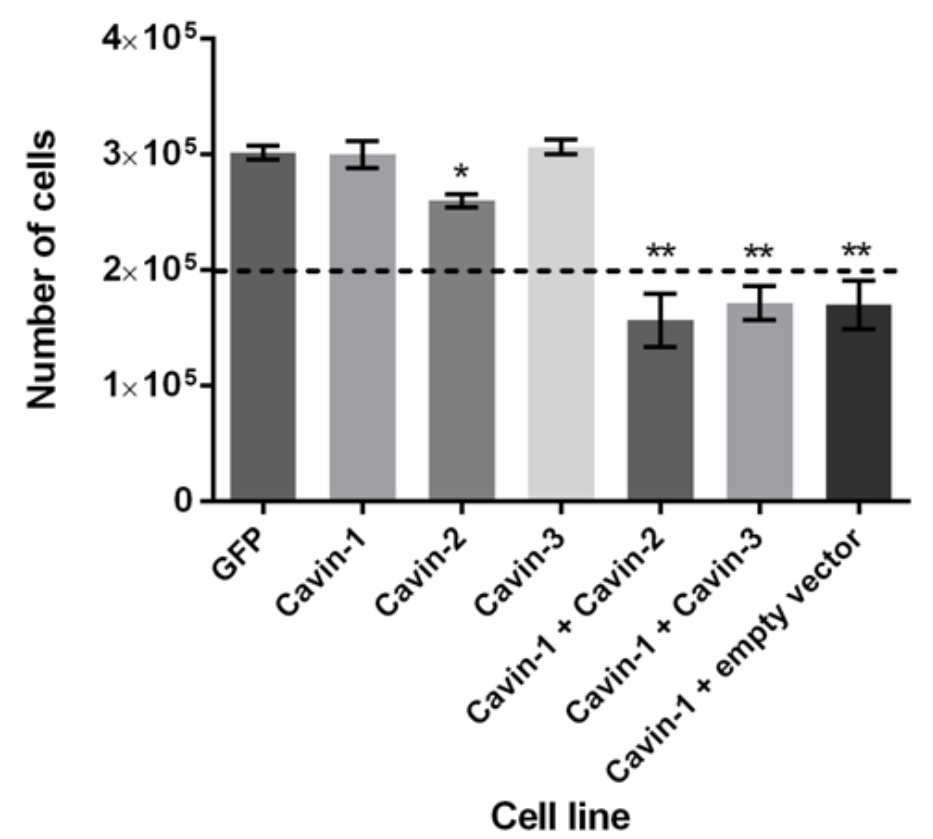

Figure 6: *Cell proliferation assay of PC3 cell lines over 20 hours.* PC3 cell lines ($2x10^5$ cells) were plated in 6 well plates in growth media, after 20 hours the cell number was counted to determine differences in proliferation. Cell number in lipofectamine transfected cell lines decreased from starting number after 20 hours. Cavin-2 expressing PC3 cell line showed reduced proliferation compared to GFP, cavin-1 and cavin-3 expressing lines. *p 0.01-0.05, **p 0.001-0.01 compared to GFP, cavin-1 and cavin-2 expressing PC3 cells. Data is represented as mean ± SEM from 3 replicate experiments. Dotted line represents the number of cells plated at 0 hours.

significantly reduced proliferation compared to all PC3 stable cell lines (Figure 6). After 20 hours, the number of cells originally loaded on a 6 well plate ($2x10^5$ cells) was decreased, indicating cell death. This reduction in cell number was also seen in the empty vector control transfection. Therefore, the cell lines produced from this method could not be used for functional assays due to toxicity of Lipofectamine reagents.

Differential localisation of cavins in PC3 cells

To observe cellular localisation of cavins when expressed in PC3 cells the GFP-tagged cavin expressing PC3 cell lines were used. Expression of GFP alone showed cytosolic localisation spread evenly throughout the cell (Figure 8a). Cavin-1-GFP was localised in puncta on the cell surface, as previously described (Hill et al., 2008). Cavin-2-GFP was mostly detected in the cytosol however membrane localisation, particularly at cell protrusions, was also observed (arrow, Figure 8a). Lastly, cavin-3-GFP localisation was dispersed in the cytosol, similar to GFP expression alone (Figure 8a). It is already known that cavins form a complex and localise at caveolae when cavin-1 and CAV1 are expressed (Bastiani et al., 2009; Gambin et al., 2014; Ludwig et al., 2013). The cherry-tagged cavin co-expressing cell lines, we used to see if localisation of cavin-2 and -3 would be altered when expressed with cavin-1. Upon transfection in cavin-1 PC3 cells, localisation of cavin-2 and -3 was altered and showed distinct puncta localisation which co-localised with cavin-1 expression (Figure 8b). Furthermore, CAV1 also co-localised with both cavins-2 or -3 and cavin-1 in puncta when stained for using IF.

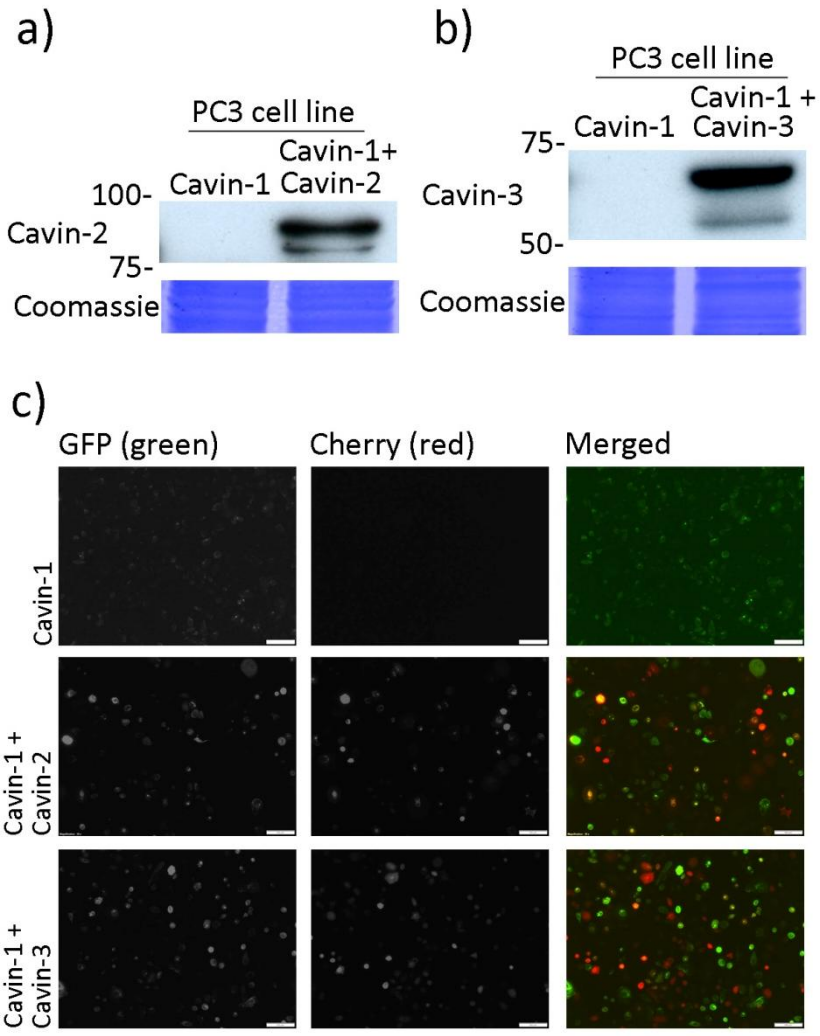

Figure 7: *Cavin-1 PC3 cells were successfully transfected with cavin-2-cherry and cavin-3-cherry.* **a)** Cavin-2 was detected only in the whole cell lysate (WCL) (20µg) of cavin-1-GFP expressing PC3 cells transfected with a cavin-2-cherry construct using Lipofectamine 2000. **b)** Cavin-3 was detected in the WCL (20µg) of cavin-1-GFP PC3 cells transfected using Lipofectamine 2000 with cavin-3-cherry. Size on blots is in kDa. **c)** Fluorescence microscopy image of cavin-1-GFP PC3 cells and cavin-1-GFP PC3 cells transfected with either cavin-2-cherry or cavin-3-cherry. Scale bar represents 100µm.

Expression of PKCα and PKCδ is unchanged by expression of cavins

Cavins are known to interact with PKC isozymes PKCα and PKCδ (Hill et al., 2012; Izumi et al., 1997; Mineo et al., 1998). First, the expression of PKCα and PKCδ in the stable PC3 cell lines was confirmed and any differences in expression levels determined. The cell lysates of each stable cell line were collected and compared by western immunoblotting using equal amounts of protein (20μg). Relative to GFP PC3 cells, expression of all cavins increased PKCα expression (Figure 9). However, this increase was not consistent across replicates and was therefore insignificant. Furthermore, PKCδ expression levels were unchanged by the expression of cavins relative to GFP expressing control PC3 cells (Figure 9).

PKC alpha co-localises with caveolin-1 only in cavin-1 expressing PC3 cells

It was previously reported that cavin-1 expression in PC3 cells recruits PKCα to the lipid raft fraction, from which it is normally absent (Hill et al., 2012). As detergent resistant membrane collection cannot distinguish planar lipids from caveolae, we wanted to see if PKCα co-localises with CAV1 and therefore caveolae by using IF confocal microscopy. CAV1 co-localised with cavin-1 when expressed in PC3 cells (appendix Figure 1). PKCα was expressed widely throughout the cell and formed concentrated pools within the cell. No definite co-localisation between PKCα and CAV1 was observed in any cell line. However, because PKCα is widely expressed throughout the cell and only a small amount of total PKCα is expected in lipid rafts, it was difficult to make conclusions based on these results.

To overcome the difficulties in co-localising PKCα and CAV1, a proximity ligation assay (PLA) was used. This assay uses primary antibodies specific for proteins of interest and secondary antibodies to complementary single stranded DNA (Soderberg et al., 2006). When

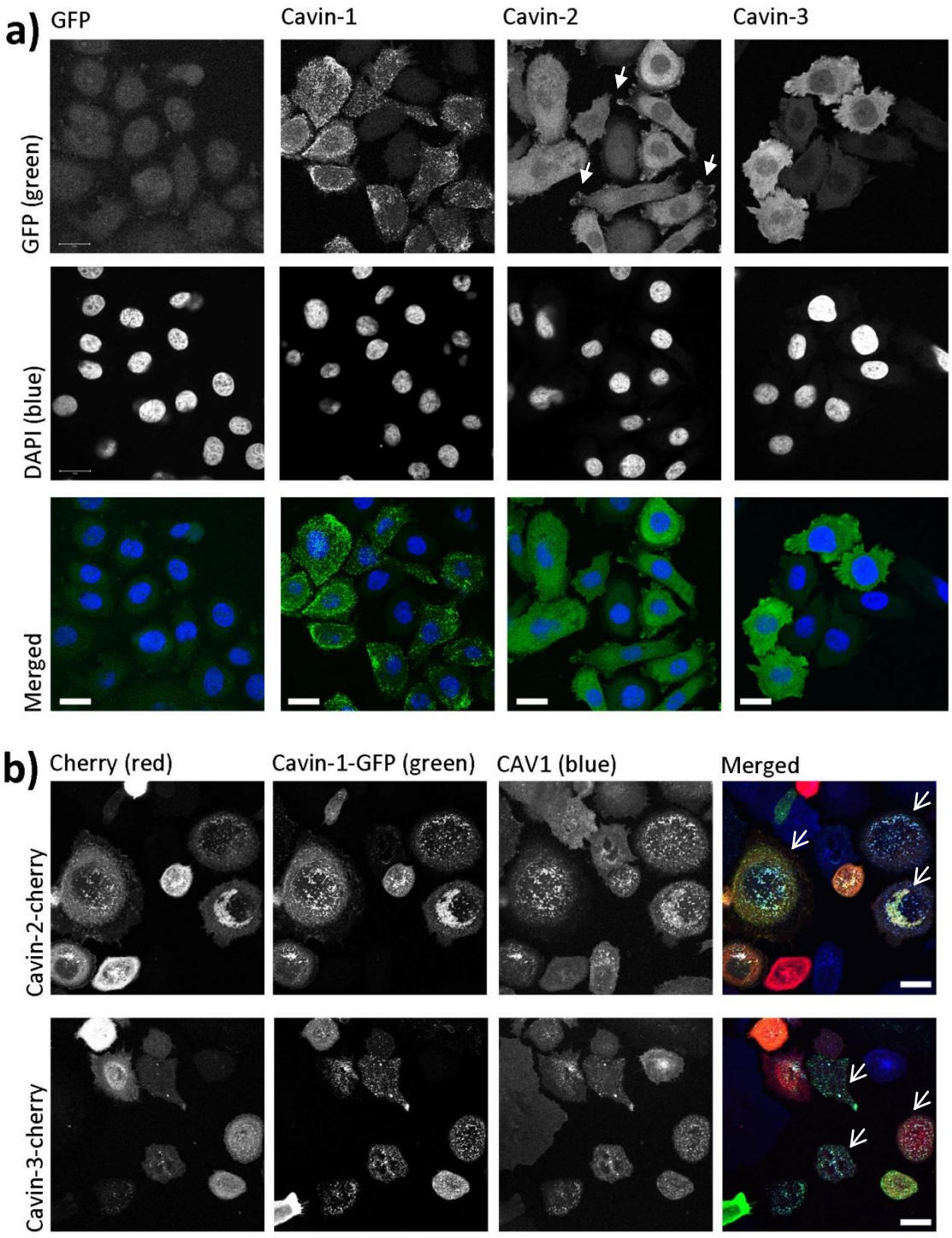

Figure 8: *Expression pattern of cavins in PC3 cells.* **a)** Confocal microscopy images of PC3 cells expressing GFP, cavin-1-GFP, cavin-2-GFP and cavin-3-GFP and stained with DAPI. Closed arrows highlight membrane localisation of cavin-2. **b)** Confocal microscopy images of cavin-1-GFP expressing PC3 cells transfected with either cavin-2-cherry of cavin-3-cherry and stained with caveolin-1. Co-expression with cavin-1 alters cavin-2 and cavin-3 localisation to co-localise with both cavin-1 and caveolin-1. Open arrows indicate examples of co-expressing cells. Scale bar represents 20µm.

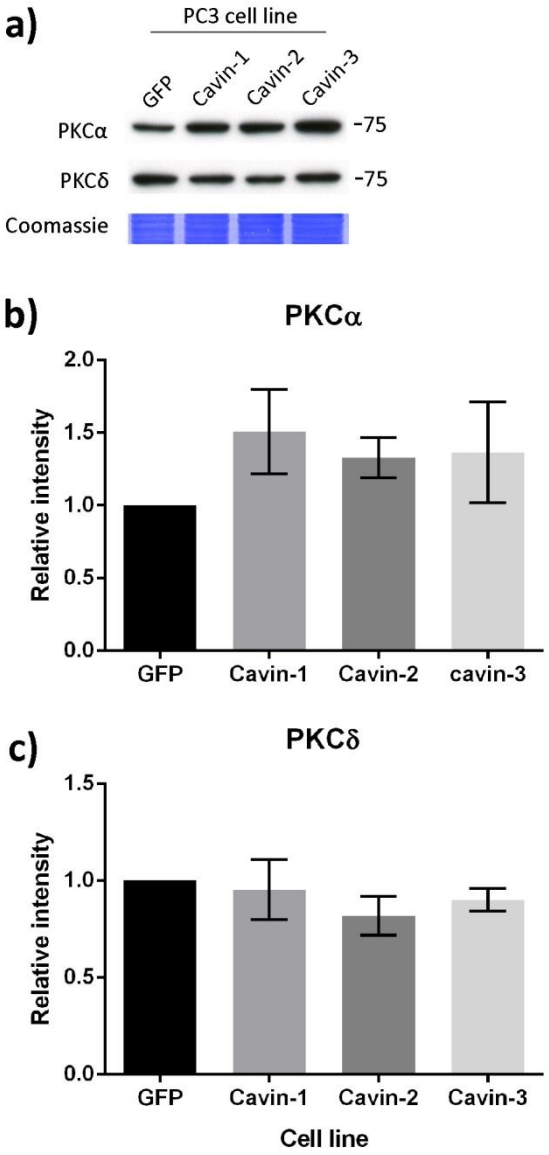

Figure 9: *PKCα and PKCδ expression levels are unchanged by cavin expression in PC3 cells.* **a)** Representative western blots of PKCα and PKCδ in the whole cell lysate (WCL) (20µg) of PC3 cell lines. Size on blots is in kDa. **b)** Quantitation of PKCα expression from WCL of PC3 cells calculated from band intensity on image J. No significant difference in PKCα expression was seen between cell lines. **c)** Quantitation of PKCδ expression from WCL of PC3 cells. There was no significant difference in PKCδ expression between cell lines. Data represents mean ± SEM for 3 replicate experiments.

the secondary antibodies are in close proximity, the complementary DNA strands can be joined by a ligation reaction. The ligated DNA is then amplified by a DNA polymerase and bound to a fluorescent probed attached to the same complementary DNA. Therefore, when two proteins of interest are in close proximity, the primary and secondary antibodies are in close proximity allowing this reaction to occur (Figure 3). Under a fluorescent microscope, interaction or co-localisation between the two proteins of interest is visualised by fluorescent dots in the cell.

For this experiment, primary antibodies were CAV1 raised in rabbit and PKCα raised in mice, and the fluorescent probe was Duolink orange (554nm absorption, 579nm emission). To ensure the specificity of the assay two negative controls were used. The first was using no primary antibody to eliminate any auto-fluorescence and non-specific binding of secondary antibodies. The second negative control used primary antibodies against proteins that are not expected to interact. Since CAV1 is not localised in the nucleus it was paired with an antibody against a nuclear protein, nucleophosmin (NPM). No PLA signal was observed in either negative control, indicating that the assay is specific (Figure 10). Co-localisation between CAV1 and PKCα, observed by PLA signal, occurred only in cavin-1 expressing PC3 cells and not in GFP, cavin-2 or cavin-3 expressing cell lines (Figure 10). Intriguingly, there were two distinct subtypes of cavin-1-GFP cells, high expressors showed distinct puncta while low expressors were diffuse (arrows, Figure 10). Between low expressing and high expressing cavin-1 PC3 cells there was an observable difference in the number of PLA signals. To quantitate this difference, the number of PLA signals per cell were counted and divided by cell area calculated on image J. The mean PLA signal/μm^2 in low cavin-1 expressing PC3 cells was $6.91x10^{-2} \pm 0.79x10^{-2}$ (Figure 11). High cavin-1 expressing PC3 cells

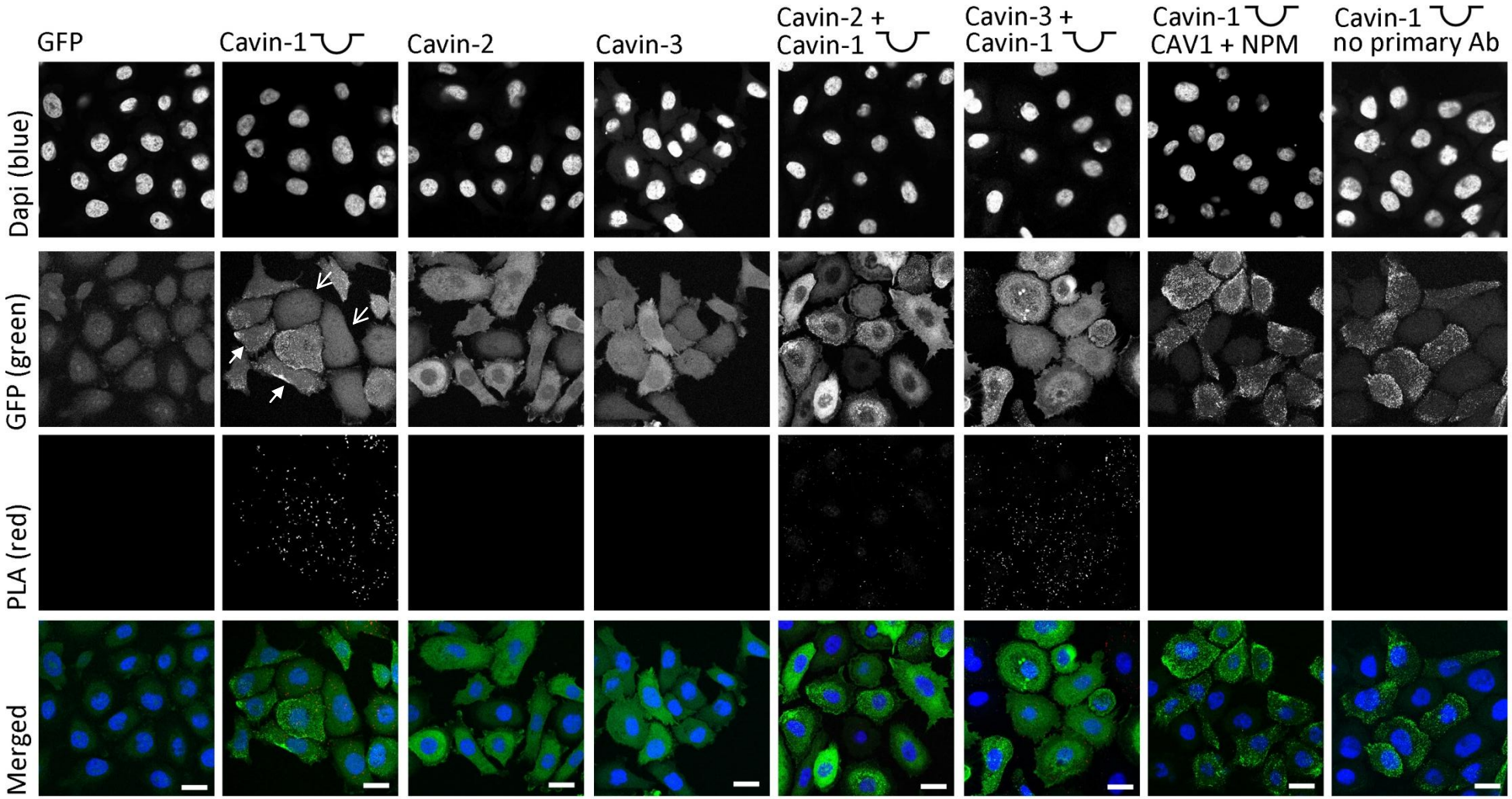

Figure 10: *PKCα and caveolin-1 co-localise only in cavin-1 expressing PC3 cells.* Confocal microscopy images of a PLA assay aiming to co-localise PKCα and caveolin-1 (CAV1) which is represented by small dots in the red channel. PKCα and CAV1 co-localise only in cavin-1 expressing PC3 cells. Introducing cavin-1 expression in cavin-2 and cavin-3 PC3 cells induces PKCα and CAV1 co-loclisation. No primary antibodies and CAV1 plus nucleophosmin (NPM) antibodies were used as negative controls. U-shaped symbol indicates the presence of caveolae in this cell line. Open arrows point to cavin-1 low expressors and closed to cavin-1 high expressors. Scale bar represents 20µm. Images are representative from at least 10 fields from 3 replicate experiments.

showed a mean PLA signal/μm^2 of $3.6x10^{-2} \pm 0.29x10^{-2}$, which was significantly decreased compared to low expressing cells ($p = 0.0005$).

Next, we wanted to see if co-expression of cavins influences PKCα co-localisation with CAV1. Since the previous co-expression model used cherry tagged proteins, it could not be used in the PLA assay because the excitation and emission wavelength overlapped with the Duolink orange PLA fluorophore. Therefore, a Flag tagged cavin-1 construct was used for transient co-expression in cavin-2 and cavin-3 PC3 cells. Transfections in both cell lines were successful, as determined by IF and western blotting of cavin-1 (Figure 12). As observed with the previous co-expressing model, cavin-1 expression changed the localisation of cavin-2 and cavin-3 into puncta that co-localised with cavin-1. Expression of cavin-1 in cavin-2 or cavin-3 expressing PC3 cells induced PLA signal between CAV1 and PKCα (Figure 10).

In cavin-2/cavin-1 co-expressing cells, $2.93x10^{-2}$ ($\pm 0.27x10^{-2}$) PLA signal/μm^2 on average were counted (Figure 11). This was similar to cavin-1 high expressors ($p = 0.0622$) but significantly lower than cavin-1 low expressors ($p < 0.0001$). Cavin-3 cells that co-expressed cavin-1 showed a mean PLA signal/μm^2 of $6.18x10^{-2}$ ($\pm 0.53x10^{-2}$). The amount of PLA signal was similar between cavin-3/cavin-1 co-expressing cells and cavin-1 low expressors ($p = 0.4505$) and significantly increased compared to cavin-1 high expressors ($p = 0.0002$). Cavin-3/cavin-1 co-expressors had significantly more co-localisation between PKCα and CAV1 than cavin-2/cavin-1 co-expressors ($p < 0.0001$).

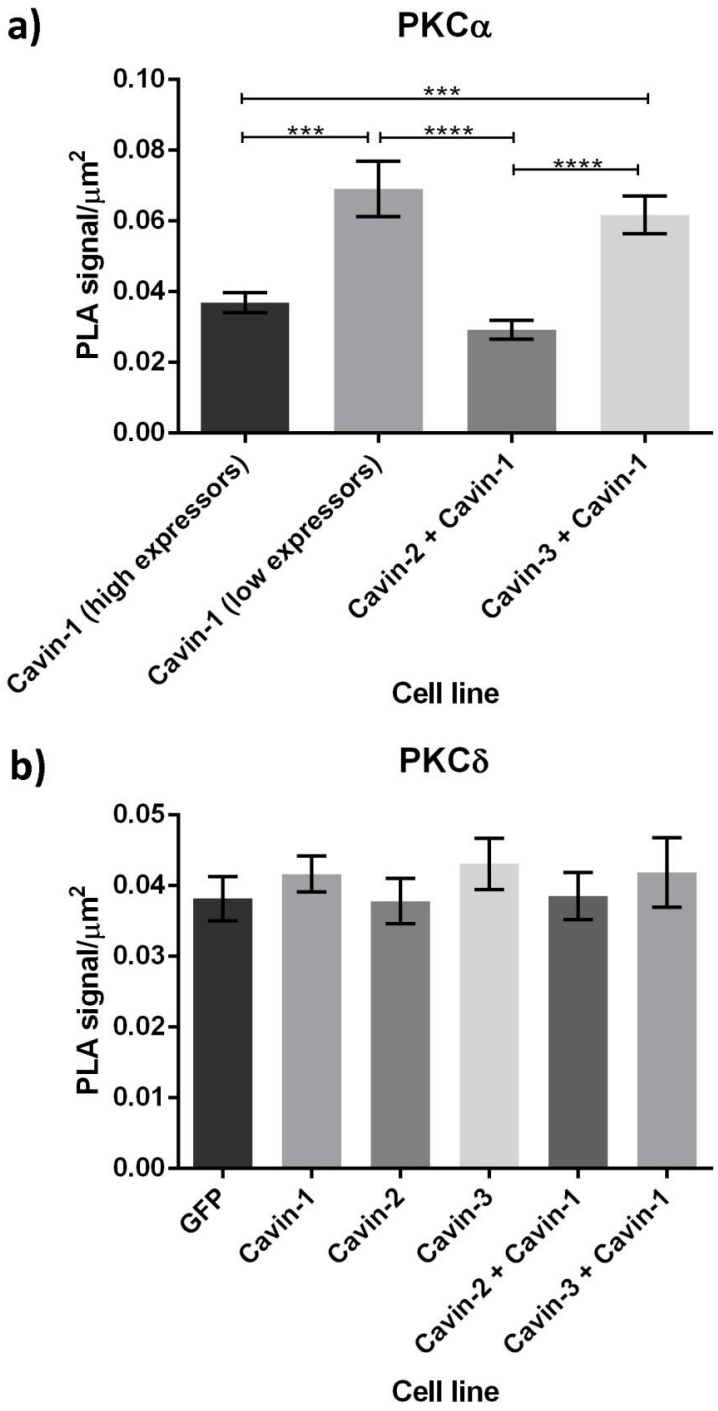

Figure 11: *PLA assay quantitation.* Results from the PLA assay (Figure 10 & 13) were quantified by counting the number of PLA signal (dots) per cell and dividing by cell area calculated on image J from 20 cells across replicate experiments. **a)** Quantitation of PKCα and CAV1 PLA assay. There was a significant decrease between cavin-1 high (distinct puncta localisation) and low expressing (diffuse) PC3 cells. Cavin-2/cavin-1 co-expressors showed a significant decrease in PKCα and CAV1 co-localisation compared to cavin-1 low expressors and cavin-3/cavin-1 co-expressors. Cavin-3/cavin-1 cells had significantly more co-localisation between PKCα and CAV1 compared to cavin-1 high expressing cells. **b)** Quantitation of PKCδ and CAV1 co-localisation. No significant differences were seen between cell lines. Data represents mean ± SEM for 20 cells from at least 2 replicate experiments.

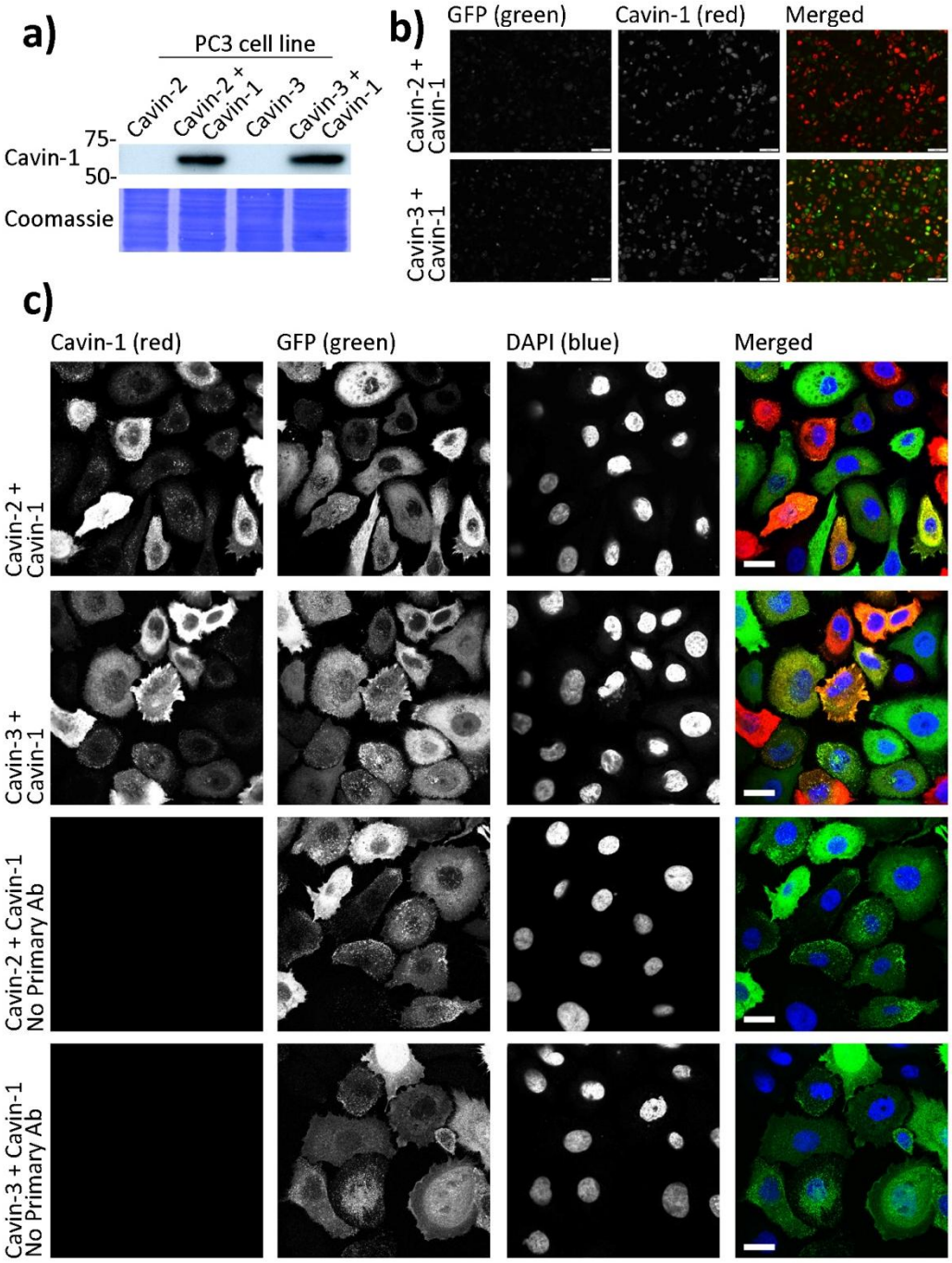

Figure 12: *Cavin-1 expression in cavin-2 and cavin-3 expressing PC3 cells alters their localisation.***a)** Cavin-1 was identified only in the whole cell lysate (WCL) (20µg) of cavin-2 and cavin-3 PC3 cells transfected with cavin-1-flag using Lipofectamine 2000. Size on blots is in kDa. **b)** Cavin-1 was detected by immunofluorescence staining in cavin-2 and cavin-3 cells transfected with cavin-1-flag by Lipofectamine 2000. Scale bar represents 100µm. **c)** Confocal microscopy images of cavin-2 and cavin-3 expressing PC3 cells transfected with cavin-1-flag using Lipofectamine 2000. Cavin-1 co-expression causes a change in localisation of cavin-2 and cavin-3 in PC3 cells and this changed expression co-localises with cavin-1. Scale bar represents 20µm.

PKCδ co-localises with caveolin-1

As cavin-3 was initially identified as a PKCδ binding protein, the co-localisation assays with PKCδ and CAV1 in the cavin expressing PC3 cells were performed. First, IF confocal microscopy was used with primary and secondary antibodies staining PKCδ and CAV1 (appendix Figure 2). Localisation of PKCδ was spread throughout the cell in highly concentrated clumps, similar to the localisation of PKCα. Again, co-localisation of CAV1 and PKCδ using this method was difficult because PKCδ is expressed widely throughout the cells.

Another PLA assay using a PKCδ primary antibody raised in mice and the same CAV1 primary antibody to was performed see if PKCδ co-localises with CAV1. To ensure the specificity of this assay, the same negative controls as with the PKCα PLA assay (no primary antibodies and CAV1 plus NPM primary antibodies) were used. No PLA signal was observed in the negative controls, indicating that the assay is specific (Figure 13). Co-localisation between PKCδ and CAV1, observed by PLA signal, occurred in all PC3 stable cell lines (Figure 13). When quantified, the mean number of PLA signals per μm^2 in the PC3 GFP expressing control cell lines was $3.82x10^{-2} \pm 0.31x10^{-2}$ (Figure 11). There were no significant differences between control and cavin-1 ($4.17x10^{-2} \pm 0.26x10^{-2}$ PLA signal/μm^2), cavin-2 ($3.78x10^{-2} \pm 0.26x10^{-2}$ PLA signal/μm^2) or cavin-3 ($4.31x10^{-2} \pm 0.36x10^{-2}$ PLA signal/μm^2) expressing cell lines. Furthermore, no significant differences were observed between high and low expressing cavin-1 PC3 cells (data not shown). Similarly, the co-localisation between PKCδ and CAV1 was unchanged when cavin-1 was co-expressed in cavin-2 ($3.85x10^{-2} \pm 0.33x10^{-2}$) or cavin-3 ($4.19x10^{-2} \pm 0.49x10^{-2}$) expressing cell lines.

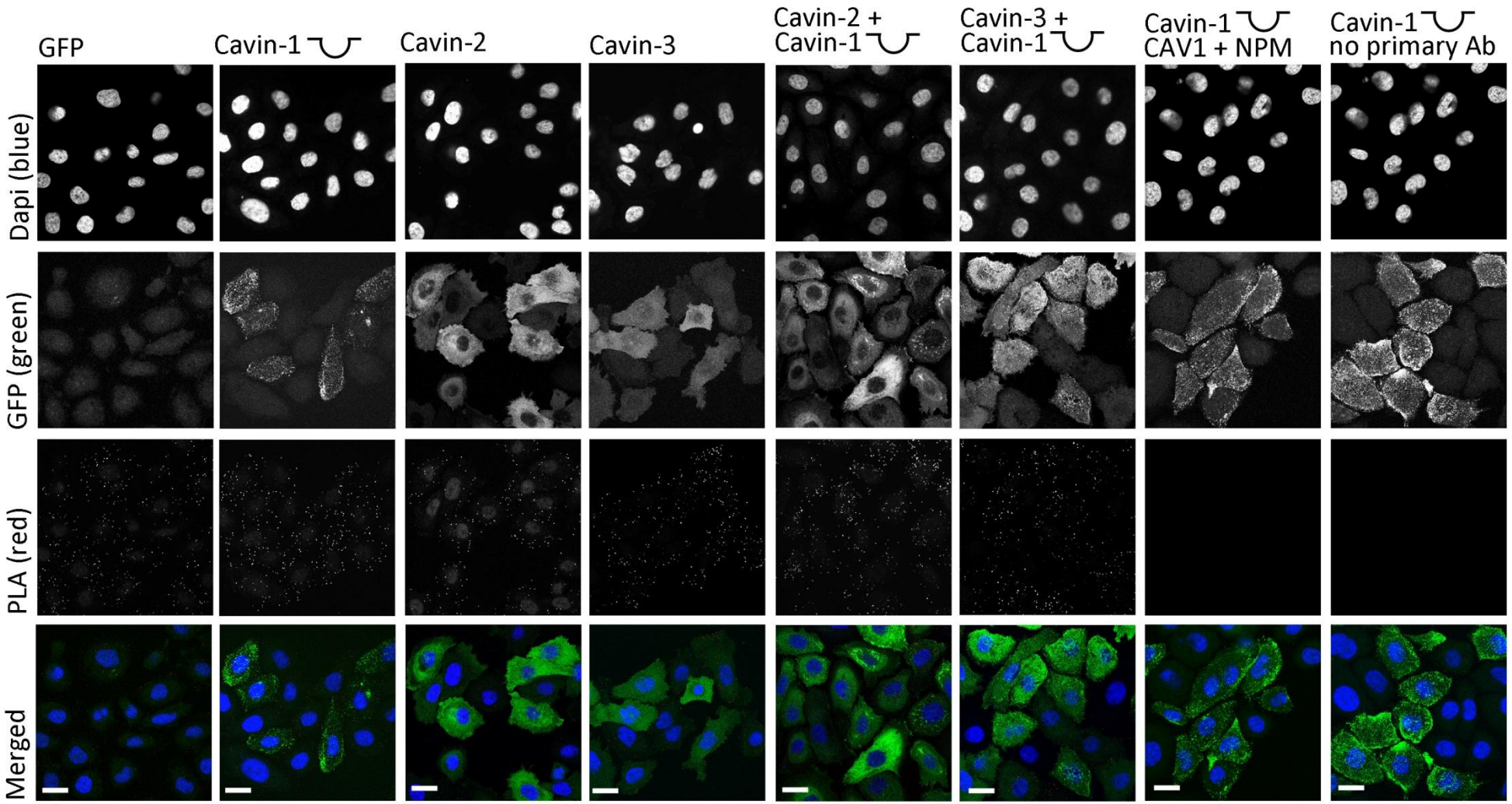

Figure 13: *PKCδ and caveolin-1 co-localise in PC3 cells.* Confocal microscopy images of a PLA assay aiming to co-localise PKCδ and caveolin-1 (CAV1) which is represented by small dots in the red channel. PKCδ and CAV1 co-localised in the GFP expressing control PC3 cells and this was unchanged upon expression of cavins. No primary antibodies and CAV1 plus nucleophosmin (NPM) antibodies were used as negative controls. U-shaped symbol indicates the presence of caveolae in this cell line. Scale bar represents 20µm. Images are representative from at least 10 fields from 2 replicate experiments.

Table 5: Summary of key findings in cavin expressing PC3 cells. Migration and adhesion are relative to GFP expressing control cells.

	GFP	Cavin-1	Cavin-2	Cavin-3
PKCα + CAV1 co-localisation	No	Yes	No	No
PKCδ + CAV1 co-localisation	Yes	Yes	Yes	Yes
Migration	-	↓	↓	↓
Adhesion to fibronectin	-	↑	Unchanged	Unchanged
Adhesion to collagen	-	↑	Unchanged	↑

Discussion

The findings of this project (summarised in Table 5) demonstrate, through analysis of cell adhesion and migration, that expression of cavins can reduce tumourigenic potential of PC3 cells. While the mechanisms behind these changes are unclear, previous studies prompted investigation of PKCα. We found that PKCα co-localises with caveolin-1 only in cavin-1 expressing cells and that this cell line shows reduced migration and increased adhesion to fibronectin and collagen. This finding was consistent with the hypothesis that the binding of cavins recruits PKCα to caveolae in cavin-1 expressing cells leading to a reduction in migration. However, the results show only correlative evidence between PKCα and reduced migration and further work is needed to demonstrate causation. Furthermore, cavin-2 and cavin-3 cells also showed reduced migration compared to the PC3 control cell line which does not involve PKCα and is independent of caveolar formation. Cavin-2 expression did not affect cell adhesion, while cavin-3 adhesion increased adhesion to collagen and fibronectin. Hence, the three cavins altered cell migration via different mechanisms.

The documented interaction of PKCα with cavins -1 and -2 (Hill et al., 2012; Mineo et al., 1998), and PKCδ with cavin-3 (Izumi et al., 1997) prompted us to investigate possible changes in localisation in response to cavin expression. Using the PLA assay, it was shown that PKCα and CAV1 co-localised only in cavin-1 expressing cells. It was previously established that PKCα was present in the detergent resistant membranes of cavin-1 expressing PC3 cells, but not in control PC3 cells (Hill et al., 2012). However, the detergent resistant membrane fraction represents the total lipid raft fraction and cannot distinguish between planar and caveolar-containing lipid rafts (Lingwood and Simons, 2007). Co-localisation of CAV1 and PKCα in cavin-1 PC3 cells shown here is the first documented and

indicates that PKCα is recruited specifically to caveolae-containing lipid rafts. However, the mechanisms behind this recruitment are still unclear and warrant further investigation. PKCα and CAV1 co-localisation was not always observed in cavin-1 puncta suggesting that in some cases, cavin-1 expression led to PKCα recruitment to non-caveolar caveolin-1 containing lipid rafts. The possibility of an extra-caveolar role for cavin-1 in PC3 cells has been previously described (Hill et al. 2012). This paper also showed that caveolin-1 is polarized at cell protrusions and caveolae at the rear of migrating PC3 cells (Hill et al., 2012). Regarding the proteomic results, PKCα may phosphorylate cell attachment proteins specifically in non-caveolar caveolin-1 domains within the cavin-1 expressing cells. This could lead to redistribution of cell attachment proteins thereby attenuating signaling pathways at the cell protrusions. Further experiments are required to test this hypothesis.

Intriguingly, there was a distinct difference in PKCα and CAV1 co-localisation dependent on whether expression of cavin-1 was diffuse (low expressing) or in distinct puncta (high expressing). There are approximately 140-150 CAV1 molecules and 1 cavin-1 molecule:3-4 CAV1 molecules per caveola (Gambin et al., 2014; Ludwig et al., 2013; Pelkmans and Zerial, 2005). Perhaps cavin-1 is over expressed in higher expressing cells causing disruption of this defined stoichiometry by increasing the number of cavin-1 molecules per caveola. This could possibly impact PKCα co-localisation with caveolin-1 by eliminating binding space for PKCα in caveolae. On the other hand, the lower expressing cavin-1 cells may not form as many caveolae as the high expressing cells. Given that cavin-1 did not always co-localise with the PLA signal this could support the hypothesis that cavin-1 expression recruits PKCα to non-caveolar caveolin-1 but does not form caveolae. Separation of these two distinct populations by fluorescence-activated cell sorting (FACS) may provide a means to further

characterise their differences. Specifically, analysis of the number of cavin-1 molecules per caveolae and possible differences in migration that could be correlated to co-localisation between PKCα and CAV1 could provide further insight.

Co-expression of cavin-1 with cavins -2 and -3 was sufficient to recruit PKCα to caveolae. As cavin-2 is a known binding partner of PKCα (Mineo et al., 1998), it was expected that co-expression of cavin-1 and cavin-2 would result in a cavin-1/2 complex that could recruit more PKCα than cavin-1 expression alone. However, based on the quantitation of PLA signals, we did not find this to be true. Co-localisation between PKCα and CAV1 was higher in low cavin-1 expressors compared to cavin-1/2 co-expressors. In high cavin-1 expressors, there was no difference compared to cavin-1/2 co-expressors. On the other hand, cavin-1/3 co-expressors showed similar PKCα and CAV1 co-localisation to cavin-1 low expressing cells but significantly more than cavin-1 high expressing cells. Possibly, the transfections resulted in a majority of high cavin-1 expressing cells and cavin-3 expression enhances the recruitment of PKCα to caveolae. However, it is difficult to make conclusions without visualising cavin-1 expression to classify cells into high or low expressors.

When investigated, PKCδ was co-localised with CAV1 in all cell lines and is therefore independent of cavin expression. This interaction provides further support for the specificity of PKCα and CAV1 interaction in cavin-1 expressing PC3 cells. To our knowledge, this co-localisation is the first documented between PKCδ and CAV1. A previous study identified PKCδ as the kinase responsible for the activation of phospholipase D (PLD)/ERK in the VEGF pathway in endothelial cells and that caveolae formation was essential in this signaling process (Cho et al., 2004). This pathway is clinically relevant in the context of angiogenesis as VEGF signals pathways involved in cell growth, migration and survival to promote tumour

vasculature (Carmeliet, 2005; Hanahan and Weinberg, 2011). Furthermore, PKCδ has a known anti-apoptotic role in response to cell death initiated by cytokine-receptors (Okhrimenko et al., 2005). Given this evidence, it is possible that PKCδ may be involved in the tumour promoting effect of CAV1 expression. However, further study is necessary to investigate this hypothesis.

A role for cavins-2 and -3 in tumour suppression has been suggested based on decreased expression commonly found in a variety of cancers. This project was the first to investigate the functions of cavins -2 and -3 in migration of prostate cancer cells. The reduced migration rate of cavin-1 PC3 cells compared to control PC3 cells was consistent with previous published results (Aung et al., 2011; Hill et al., 2012). Migration of cavin-2 and cavin-3 PC3 cells was also decreased compared to the control cell line. As PC3 cells express caveolin-1 but not cavin-1, and both proteins are required for caveola formation (Hill et al., 2008), the attenuation of migration by cavin-2 and cavin-3 was independent of caveolae formation. This finding suggests that part of the tumour suppressing functions of cavin-2 and cavin-3 is in reducing migration. Previously, it was shown that cavin-2 and cavin-3 reduce MMP9 secretion in PC3 cells however this effect was greater for cavin-1 expressing PC3 cells (Aung et al., 2011). Therefore, it is likely that novel mechanisms in addition to MMP9 secretion are responsible for decreasing migration in cavin-2 and -3 expressing PC3 cells.

The cell counting assay showed further support for a role of cavins in tumour suppression. There was a significant decrease in proliferation upon cavin-2 expression over as little as 20 hours. This result is consistent with unpublished data from the lab that shows a significant decrease in proliferation of PC3 cells expressing cavin-2 and cavin-3. Cavin-2 was originally identified as a protein expressed in serum deprived cells and named appropriately as serum-

deprivation response protein (SDPR) (Gustincich and Schneider, 1993). While cavin-2/SDPR is upregulated in serum starved cells, it becomes downregulated upon cell cycle re-entry. It is possible that cavin-2 expression in PC3 cells is sufficient to cause growth arrest and therefore a reduced proliferation rate. However, the specific pathways by which cavin-2 is upregulated in serum deprived cells and any downstream targets are unknown. Cavin-3 is also upregulated in response to serum deprivation and has been shown to influence Akt and ERK signaling, which play a role in cell cycle progression (Hernandez et al., 2013; Izumi et al., 1997). As cavin-2 and cavin-3 behave similarly, Akt and ERK signaling could be a novel mechanism of cavin-2 reduced proliferation.

Since migration and adhesion are closely linked, I investigated changes in adhesion to ECM substrates upon cavin expression in PC3 cells. As with migratory properties, there are no published reports of the effects of cavin expression on adhesion. There was a significant increase in adhesion of PC3 cells to collagen and fibronectin upon expression of cavin-1. Therefore, cavin-1 expression reduces migration but increases adhesion to ECM substrates. Cell migration requires disruption of cell adhesion at the rear and formation of adhesion complexes at the cell protrusions (Beningo et al., 2001; Zaidel-Bar et al., 2003). The formation of stable structures that enhance adhesion could prevent cycling of cell adhesions and thus slow migration in cavin-1 cells. Additionally, intiation of invasion requires tumour cells to detach from surrounding cells by downregulating cellular adhesion (Arya et al., 2006; Frixen et al., 1991). In this case, increased adhesion mediated by cavin-1 would slow this process and suppress tumour progression. Cavins-2 and -3 did not have the same effect on cell attachment. Adhesion of cavin-3 cells was significantly increased to control PC3 cells binding to collagen, suggesting that cavin-3 may influence proteins involved in attachment

to collagen but not fibronectin. Cavin-2 expression did not change adhesion to collagen or fibronectin but had a significant impact on cell migration.

Originally, it was predicted that PKCα recruitment to caveolae would correlate with PC3 cell migration. This was supported by the decrease in migration of cavin-1 PC3 cells which showed co-localisation between PKCα and CAV1. Since cavin-2 and cavin-3 expressing PC3 cells do not have caveolae for PKCα to be recruited to, it was expected that they would show similar migration to GFP control cells. However, cavin-2 and cavin-3 expression decreased migration to the same extent as cavin-1 PC3 cells. These findings suggest that while all cavins decreased migration to the same extent, the mechanisms employed by each protein are different.

Analysis of unpublished proteomics results from our lab presented some insight on potential actions of PKCα in the lipid rafts of cavin-1 PC3 cells. There was a significant increase in phosphorylation of PKC substrates involved in cell attachment in the lipid rafts of cavin-1 PC3 cells, concomitant with PKCα recruitment to lipid rafts. Bioinformatic analysis to generate PPI networks predicted interactions between integrins α6 and β4 and PKCα. Integrins α6 and β4 heretodimers are involved in the formation of hemidesmosomes, adhesive structures that link the intermediate filament cytoskeleton and ECM (Green and Jones, 1996). Integrin α6β4 hemidesmosomes bind to the laminin family of ECM proteins and their expression is associated with progression of many cancers (Rabinovitz et al., 1999). Phosphorylation of integrin α6β4 by PKCα results in mobilisation from hemidesmosomes to cell protrusions which is generally associated with decreased cellular adhesion and increased migration (Kashyap and Rabinovitz, 2012; Rabinovitz et al., 1999; Rabinovitz et al., 2004). However, there is evidence that the localisation of integrin α6β4 in lipid rafts has a

major impact on downstream signaling. One study showed that compartmentalisation of integrin α6β4 in lipid rafts was necessary for interaction with Src family kinase (SFK) which leads to intracellular signaling through known oncogenic pathways such as Ras and phosphoinositide-3 kinase (PI3K) (Gagnoux-Palacios et al., 2003). Another study showed that lipid raft localisation of integrin α6β4 was necessary for interaction with epidermal growth factor (EGF) signaling (Soung and Chung, 2011). Curcumin was shown to inhibit the interaction between EGFR and integrin α6β4 by decreasing integrin α6β4 localisation in lipid rafts. While phosphorylation and mobilisation of integrin α6β4 by PKCα is normally associated with cancer progression it is possible that phosphorylation within lipid rafts inhibits signaling pathways associated with tumourigenesis (Figure 14).

Addition of further predicted functional partners produced a network of proteins involved in cell adhesion. Of particular interest were integrins β7 and β1, which are both known to interact with integrin α6 and β4 and involved in cell attachment to the ECM (Giancotti, 2000). Plectin-1 and collagen 17α1 also have a role in cell adhesion by linking the cell, via hemidesmosomes to cytoskeletal filaments (Franzke et al., 2002; Koster et al., 2003). Ezrin, an Ezrin-radixin-moesin (ERM) family protein which are involved in cross-linking the membrane to the actin cytoskeleton (Sato et al., 1992), was predicted as a functional partner of PKCα and integrin β4. Another ERM family protein, moesin was consistently, but not significantly elevated in the lipid rafts of cavin-1 expressing cells (Table 3). As with integrin α6β4, lipid raft localisation of ERM family proteins activates signaling pathways required for tumour progression (Antelmi et al., 2013; Donatello et al., 2012). This evidence supports the hypothesis that phosphorylation of adhesion proteins, such as integrin β1, ezrin and moesin, from the lipid rafts of cavin-1 PC3 cells may facilitate relocalisation to

non-raft membranes and causing a decrease downstream signaling pathways associated with tumour promotion. However, experimental data is needed to validate a role for these predicted functional partners and PKCα and to investigate this hypothesis.

Conclusions and future directions

The results of this project established that expression of cavins can reduce the migration of aggressive prostate cancer cells, PC3 cells. Future study into the consequences and mechanisms of cavins -2 and -3 in cancer progression could provide a novel therapeutic target that could be used for both caveolin-1 positive and negative prostate cancer. A potential role for PKCα in mediating the change in migration and adhesion was investigated and found that it co-localises with CAV1 only in cavin-1 expressing cells. It was shown that cavin-1 expression increased adhesion to ECM substrates which was consistent with an increase in phosphorylation of adhesion proteins by PKC in the lipid rafts upon cavin-1 expression. Further investigation into this co-localisation, especially in the differences between cavin-1 high expressing and low expressing cell populations, and the activation of downstream pathways suggested from proteomic results may provide greater understanding into cavin-1 mediated changes in adhesion and migration. Furthermore, generation of stable cavin-1/cavin-2 and cavin-1/cavin-3 co-expressing cell lines to use in functional assays could provide further support for the hypothesis. PKCδ was co-localised with CAV1 in all cell lines, indicating that it was unchanged by cavin expression. This is the first report of PKCδ and CAV1 co-localisation in PC3 cells. As CAV1 is a known prostate tumour promoter, future study including inhibition or a knockdown model may reveal a novel role for PKCδ in prostate tumour progression.

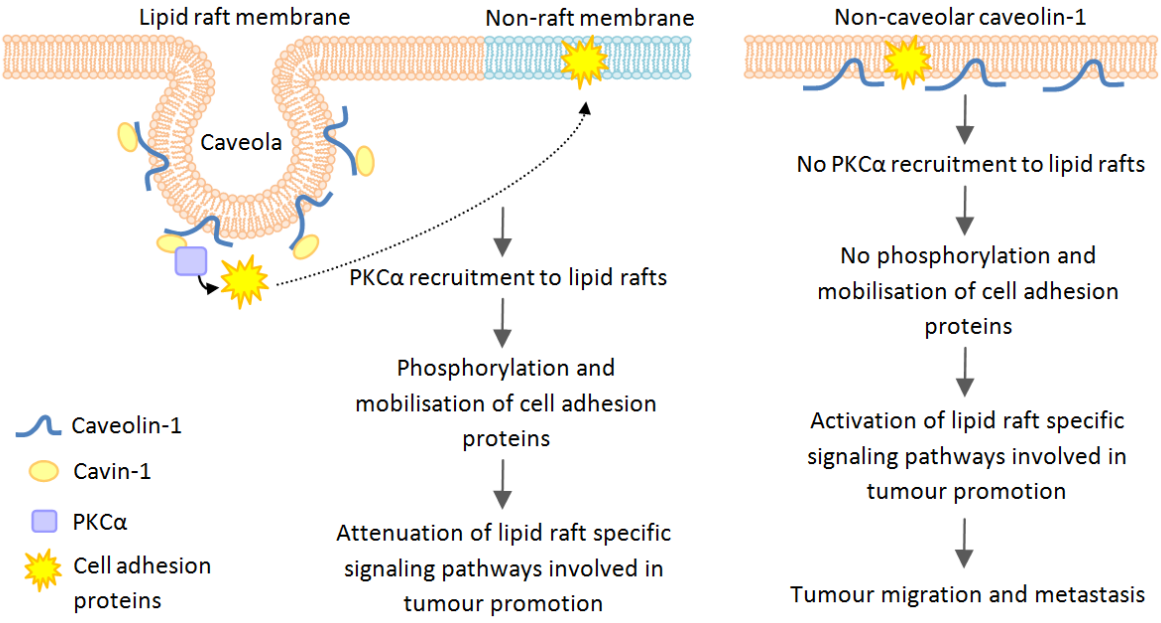

Figure 14: *Proposed mechanism of PKCα in the lipid rafts of cavin-1 expressing PC3 cells to reduce tumour progression.* Cavin-1 expression in prostate cancer PC3 cells induces caveolae formation and reduces caveolin-1 (CAV1) mediated tumour progression (Hill et al., 2008; Moon et al., 2013; Nassar et al., 2013b). The results of this project show that PKCα co-localises with CAV1 only in cavin-1 expressing cells. The actions of PKCα at caveolae are yet to be tested however proteomics and predictive bioinformatics revealed a potential role for proteins involved in cell adhesion including integrins α6β4, integrin β1, ezrin and moesin. The lipid raft localisation of these proteins is thought to be essential in oncogenic signaling pathways (Antelmi et al., 2013; Donatello et al., 2012; Gagnoux-Palacios et al., 2003; Soung and Chung, 2011). It is possible that in PC3 cells, cavin-1 expression recruits PKCα to lipid rafts where it phosphorylates cell adhesion proteins causing mobilisation to non-raft membranes. Localisation of these proteins in non-raft membranes attenuates the lipid raft specific signaling involved in tumour promotion. Therefore, when cavin-1 is not expressed, cell adhesion proteins remain in the lipid rafts to activate oncogenic signaling pathways involving tumour migration and metastasis.

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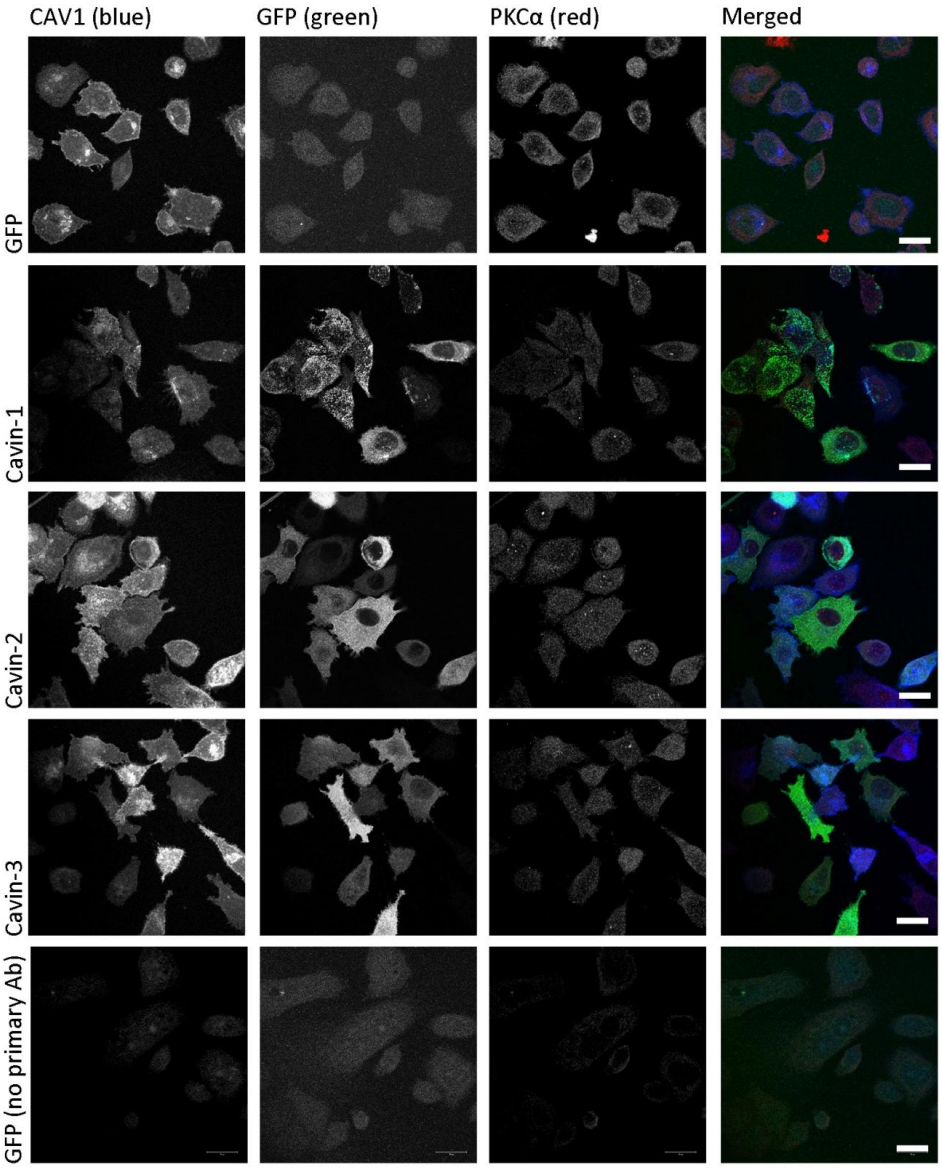
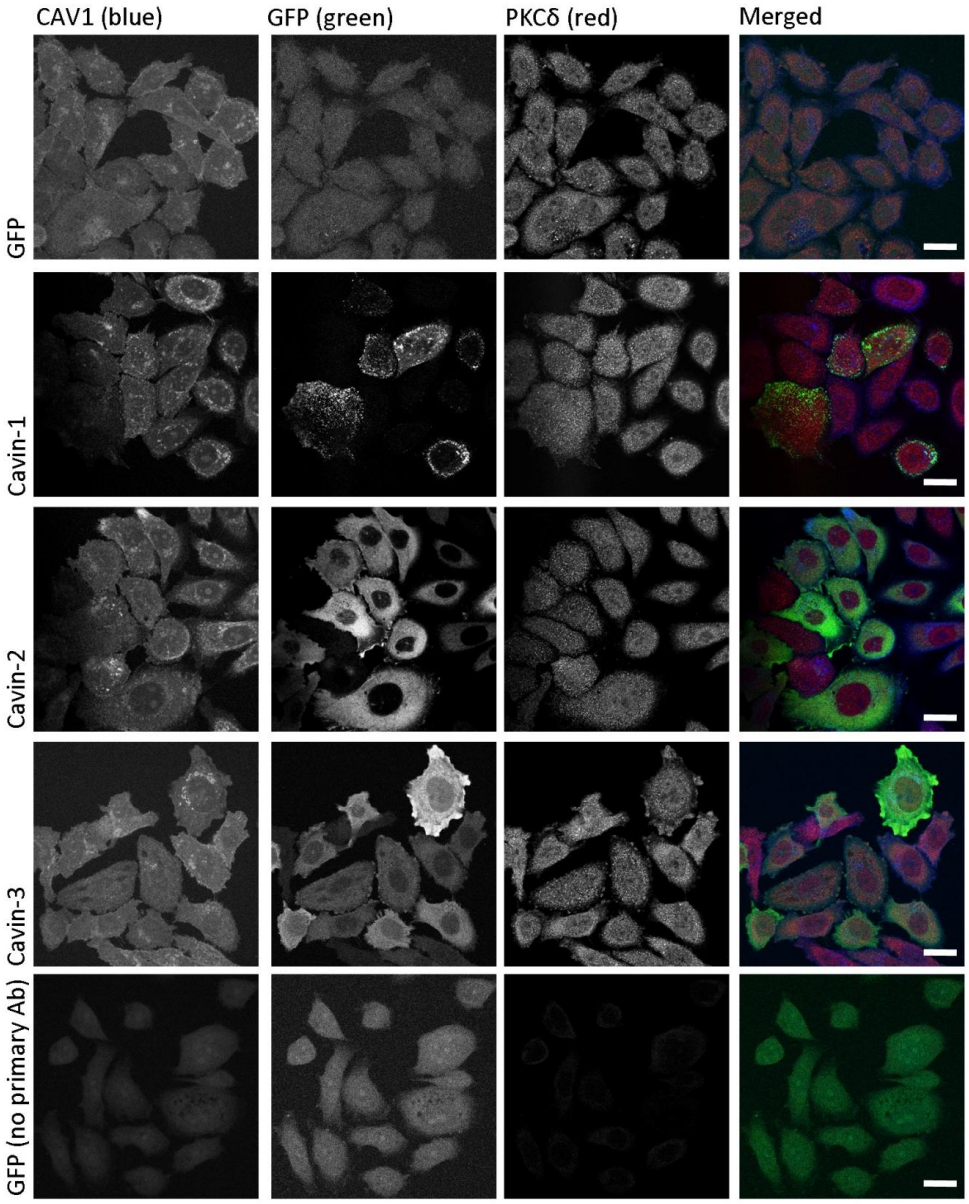
Appendicies



Appendix Figure 1: *PKCα and caveolin-1 (CAV1) localisation in PC3 cell lines.* Immunofluorescence staining of PKCα and CAV1 in PC3 cells expressing GFP, cavin-1-GFP, cavin-2-GFP and cavin-3-GFP. Incubation with no primary antibodies was used as a negative control. Scale bar represents 20µm.



Appendix Figure 2: *PKCδ and caveolin-1 (CAV1) localisation in PC3 cells.* Immunofluorescence staining of PKCδ and CAV1 in PC3 cells expressing GFP, cavin-1-GFP, cavin-2-GFP and cavin-3-GFP. Incubation with no primary antibodies was used as a negative control. Scale bar represents 20µm.