



Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

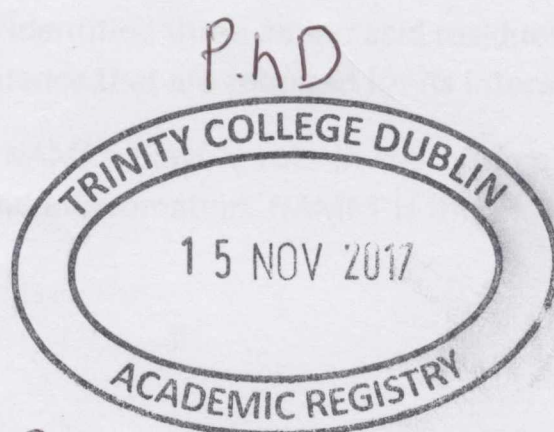
**Oesophageal adenocarcinoma cell migration and metastasis: an
investigation of the scaffolding protein RACK1 as a key player and
therapeutic target**

By Deirdre Duff



A dissertation submitted in partial fulfilment for the degree of
Doctor in Philosophy
Under the supervision of Aideen Long, Ph.D. at
Trinity College Dublin

Trinity College Dublin 2017



50763182



* 5 0 7 6 3 1 8 2 *

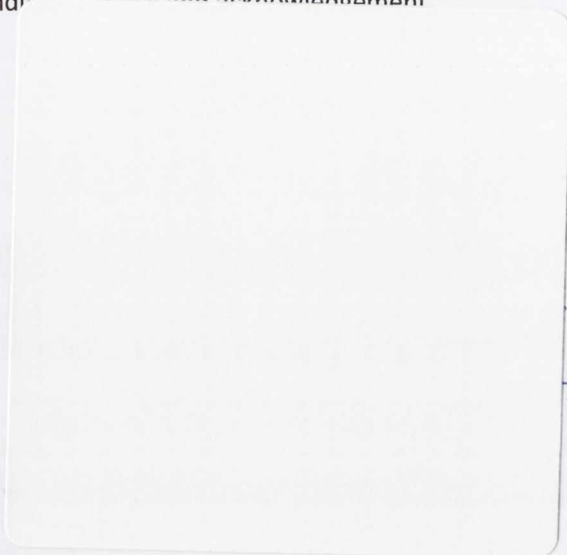
THESIS

11565

Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

I agree to deposit this thesis in the University's open access institutional repository or allow the Library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement



f

7



Thesis 11565

Q119

Summary

The incidence of oesophageal adenocarcinoma is increasing at a rapid rate in Western countries (Pennathur et al., 2013). Early oesophageal cancer is often asymptomatic and metastatic disease is common at presentation leading to poor prognosis and survival rates (Pennathur et al., 2013).

RACK1 is a 36kDa scaffolding protein that interacts with a wide variety of different molecules. It is a multifunctional adaptor protein which can contribute to numerous biological processes including cell proliferation, adhesion, migration, apoptosis and protein synthesis (Adams et al., 2011). RACK1 expression is altered in a variety of cancers including those of the breast, ovary, lung and prostate. (Adams et al., 2011, Li and Xie, 2014). However prior to this PhD study, the expression and function of RACK1 in oesophageal adenocarcinoma had not been examined.

Our group had previously identified a novel interaction between RACK1 and Non Muscle Myosin Heavy Chain IIA (NMHCIIA) in migrating T-cells. This project included an investigation into whether this interaction between RACK1 and NMHCIIA also occurs in migrating OAC cells. However a combination of immunofluorescent staining, confocal microscopy, fluorescent ratiometric imaging and FRET microscopy experiments found no evidence for a functionally significant interaction between RACK1 and NMHCIIA in migrating OAC cells. The proteins with which RACK1 interacts may vary with cell type. (Adams et al., 2011).

Interestingly, we observed that NMHCIIA localises to the leading edge lamellipodia of migrating FLO-1 cells, and also to stress fibre like structures. This indicated that NMHCIIA may play a role in FLO-1 cell migration. An siRNA approach was therefore used to investigate the functional role of NMHCIIA in FLO-1 cell biology by depleting the cells of this protein and analysing the consequences of same using cell based assays. In this way it was found that NMHCIIA depletion reduces FLO-1 cell migration, alters cell morphology and gives rise to an increased number of thick, stress fibre like structures in the cells. NMHCIIA depletion did not affect cell proliferation under the conditions tested. Thus, it appears that NMHCIIA has a specific functional role in FLO-1 cell migration and it may execute this function through its regulation of f-actin dynamics and cytoskeletal processes.

This project examined the expression and function of RACK1 in OAC cell lines. Western blotting was used to show that RACK1 is highly expressed at the protein level in OAC cell lines. RACK1 depletion, using an siRNA approach, was found to reduce FLO-1 OAC cell proliferation and two- and three- dimensional cell migration. A mass spectrometry based proteomics screen was used to study the RACK1 associated proteome, in FLO-1 OAC cells, which was found to be enriched with ribosomal proteins. A novel RACK1 interaction partner, nicotinamide phosphoribosyltransferase (NAMPT), was also identified in this screen.

Peptide array technology was subsequently used to show that RACK1 interacts directly with NAMPT. Alanine substitution arrays identified three amino acid residues (arginine 47, lysine 44 and arginine 60) in the RACK1 sequence that are required for its interaction with NAMPT.

Previous studies have shown that NAMPT plays a role in the pathophysiology of metabolic disorders associated with obesity and inflammation. NAMPT is therefore an interesting RACK1

interaction partner to study in the setting of OAC which is a disease that is strongly associated with obesity and inflammation. NAMPT is the rate-limiting enzyme in the mammalian nicotinamide adenine dinucleotide (NAD) salvage pathway (Sampath et al., 2015). NAD is an important coenzyme that is involved in cellular redox reactions and serves as a substrate for a number of different enzymes and regulatory proteins (Sampath et al., 2015). It is required for a variety of cellular processes that are essential for survival, such as metabolism and the maintenance of genomic stability (Sampath et al., 2015). NAD assays were used in this project to show that FLO-1 NAD levels decrease dramatically upon siRNA mediated NAMPT depletion from these cells. Furthermore, NAMPT depleted FLO-1 OAC cells exhibited a dramatic reduction in cell proliferation, viability and cell migration, indicating that NAMPT is of significant function importance in this cellular setting. Interestingly, our results show no evidence that RACK1 depletion affects FLO-1 NAD levels but show that RACK1 depletion results in reduced protein levels of a 70kDa NAMPT complex.

In conclusion, this project has demonstrated that NMHCIIA plays a role in FLO-1 cell migration and that this may be via an f-actin dependent mechanism. Further study, to investigate further the role of NMHCIIA in OAC cell migration (and invasion) is warranted in light of our results and such study may identify NMHCIIA as a potential therapeutic target for OAC metastasis. This project has also identified a novel, direct interaction between RACK1 and NAMPT in FLO-1 cells and has shown that both proteins play important functional roles in FLO-1 cells. Furthermore, RACK1 depletion was shown to reduce levels of a high molecular weight NAMPT complex, indicating that RACK1 may play a role in modulating NAMPT. Thus, the interaction between RACK1 and NAMPT could potentially be targeted as a therapeutic strategy for OAC and further study to investigate the functional consequences of this interaction is certainly warranted.

Acknowledgements

My heartfelt thanks go to -

My supervisor, Aideen Long, whose wisdom has guided me so well through this PhD journey. Thank you for sharing your knowledge with me, for infecting me with your enthusiasm and for rescuing me from despair on so many occasions with your wonderful optimism. I am so lucky to have had a supervisor like you.

All of my lab mates in Aideen's research group – Anne-Marie, Mike, Denise, Fran, Cormac, Joanna, Spiros, Stephen and all the students who came and went - thanks for your advice, help, chats, jokes and for just being good company! A special thanks must go to Anne-Marie; you were like my second supervisor, you have taught me so much and your advice has been invaluable. Thank you for sharing so much of your time with me and for always doing so with a smile and a cheerful laugh. A huge thanks is also due to Mike – who was always so happy to help, to answer my questions and to share his infinite Western blotting wisdom with me. Denise –you took me under your wing during my very first few days in the lab – and so patiently showed me where everything was and how things worked.

Thanks to all in the TB lab next door – you have been the best 'lab neighbours' that any scientist could wish for. Thanks also to the reading room folk and to all the other people in the TTMI who have influenced my PhD life for the better. Thank you Karima for giving the science part of my brain a rest with your Arabic lessons and for your delicious Libyan food.

Tony Davies – thanks for your high content help, for solving technological crises at any hour of the day –and for being good company when any sane folk had all gone home.

Thanks to the security staff for making life happier with your smiles and chats. And to Jacqueline O'Kelly and Sheila Walshe for helping me navigate Trinity's ordering software and invoicing systems– and always with such patience and tolerance for my stupid questions.

To the scientists outside of Trinity whose labs I was so lucky to visit. Eugene Dempsey and Kieran Wynne in UCD who were of such help to my proteomics work. Pat Kiely who welcomed me into his lab in UL and made my peptide array work possible –a big thanks to Catriona Dowling and to everyone else who helped me during my visit to the Kiely lab. My FRET work was made possible in Joel Swanson's lab in UM - I am deeply grateful for the welcome I received, the experience I gained and the amazing hospitality which Joel showed me. Thanks to Brian, Amanda, Guillermo and everyone else in Joel's lab who were not only wonderful lab mates but also amazing friends in a country where I knew no one. Thanks also to John Gormely for making my visit to UM possible.

A huge thank you to my family for always being so supportive and loving. Mammy for feeding me so well when I came home to write – even when I dropped the vegetarian bombshell – and for spoiling me rotten in my last few weeks of lab work – even when I was in Dublin you managed to surround me with freezer dinners, homemade bread and every food item I could wish for. Daddy; thanks for worrying more about my PhD more than I ever did and for all the phone calls to make sure I was both alive and ‘on track’. Mide and April for being great sisters – and ‘study buddies’!! Mary for being my second mother. Grandpa – for giving me a roof over my head when I needed it and always being so interested and supportive of my work – even when it involved my messing with ‘those maggots’. To all my friends who have been so supportive and understanding of my PhD-induced absence.

To the Fossil Free TCD gang who gave my time in Trinity a whole new dimension. I have never met a bunch of more amazing people. Climate change is scary but you guys exude hope. To the SU Presidents, Lynn Ruane and Kieran McNulty who supported us in our quest to divest the university of fossil fuels. To the College Board, CFO and Provost who ultimately answered that call for fossil fuel divestment with a resounding ‘yes’. Thank you from the bottom of my heart. Finally, my sincere thanks to Cancer Research Ireland who provided me with funding for this PhD project, without which it would not have been possible.

Abstract

Title: Oesophageal adenocarcinoma cell migration and metastasis: an investigation of the scaffolding protein RACK1 as a key player and therapeutic target

Background

The incidence of oesophageal adenocarcinoma (OAC) is increasing at a rapid rate in Western countries. Early oesophageal cancer is often asymptomatic and metastatic disease is common at presentation leading to poor prognosis and survival rates. RACK1 is a 36 kDa scaffolding protein with a seven-bladed propeller structure. RACK1 binds to a plethora of molecules including receptors, adhesion molecules, kinases and phosphatases. It is involved in the co-ordination of multiple cellular processes including cell growth, adhesion and motility. RACK1 has been implicated in a variety of cancers but it has not been previously examined in OAC.

Aims

Our group had previously identified a novel interaction between RACK1 and Non Muscle Myosin Heavy Chain IIA (NMHCIIA) in migrating T-cells. This project sought to determine if this interaction between RACK1 and NMHCIIA also occurs in migrating OAC cells. It also aimed to investigate the functional role of both RACK1 and NMHCIIA in OAC cell biology, with a particular focus on cell migration. This project aimed to examine the RACK1 associated proteome in OAC cells. It sought to identify and characterise novel RACK1 binding partner(s) within the setting of OAC and to map the sites on RACK1 which mediate its interaction with such partner(s).

Methods

Immunofluorescent staining, confocal microscopy, fluorescent ratiometric imaging and FRET microscopy were used to investigate if RACK1 and NMHCIIA interact in migrating OAC cells. siRNA technology was used to deplete RACK1, or other proteins of interest, from OAC cells, and the functional consequences of this were studied using a range of cell based assays.

A mass spectrometry based proteomics screen, of RACK1 immunoprecipitates prepared from FLO-1 cell lysates, was performed and the resultant data were analysed using PEAKS and MaxQuant mass spectrometry software. Peptide array technology was used to find evidence of direct protein-protein interactions and alanine substitution arrays were used to identify amino acid residues on the RACK1 sequence that mediate its interaction with a direct binding partner.

Results

No evidence, of a functionally important interaction between RACK1 and NMHCIIA in migrating oesophageal adenocarcinoma cells, was found.

NMHCIIA was found to localise to the leading edge of migrating FLO-1 cells and to stress fibre like structures. NMHCIIA depletion from FLO-1 cells altered cell morphology and gave rise to an increased number of thick, stress fibre like structures in the NMHCIIA depleted population.

NMHCIIA depletion also reduced FLO-1 cell migration but did not affect cell proliferation under the conditions tested.

RACK1 depletion was found to reduce FLO-1 OAC cell proliferation and two- and three-dimensional migration. The RACK1 associated proteome in FLO-1 cells is significantly enriched with ribosomal proteins. A novel RACK1 interaction partner, nicotinamide phosphoribosyltransferase (NAMPT), was identified and shown to interact directly with RACK1. Three amino acid residues (arginine 47, lysine 44 and arginine 60) on the RACK1 sequence, that mediate the RACK1-NAMPT interaction, were identified.

It was found that NAMPT depletion from FLO-1 cells dramatically reduced their nicotinamide adenine dinucleotide (NAD) levels and decreased cell proliferation, cell viability and cell migration. RACK1 depletion did not affect FLO-1 NAD levels under the conditions tested but did result in reduced protein levels of a 70kDa NAMPT complex.

Conclusions

We have found NMHCIIA localises to the leading edge of migration FLO-cells, and that NMHCIIA depletion reduces FLO-1 cell migration and alters cell morphology and stress fibre structure. These findings lead us to conclude that NMHCIIA may influence FLO-1 cell migration by regulating F-actin dynamics and the actin cytoskeleton in some way. Cell migration is tightly controlled in the healthy cell but can become dysregulated in diseases such as cancer where increased cell motility and migration can contribute to metastasis. In NMHCIIA, we have identified a protein that may be worthy of further study in the setting of OAC, as such study may yield insights into the mechanisms of migration employed by OAC cells or may identify NMHCIIA as a potential therapeutic target for this disease.

Our findings that RACK1 plays a significant functional role in OAC cells indicate that is an important player in OAC cell biology. Our finding that the RACK1-associated proteome in FLO-1 cells is enriched with ribosomal proteins leads us to conclude that RACK1 may contribute to one or more aspects of ribosomal function in this cell type.

The identification of NAMPT as a direct RACK1 interaction partner is of significant interest as it represents a novel RACK1 interaction partner. Furthermore, multiple studies have indicated that NAMPT plays a role in the pathophysiology of metabolic disorders associated with obesity and inflammation. Thus NAMPT is an interesting RACK1 interaction partner to study in the context of OAC, a disease that is strongly associated with obesity and inflammation. We have also shown that NAMPT is required by FLO-1 OAC cells to maintain NAD levels and that NAMPT depletion reduces cell proliferation, viability and migration. Thus NAMPT has an important functional role in FLO-1 cell biology and represents a potential therapeutic target for OAC. Our finding that RACK1 depletion leads to a reduction in the protein levels of a 70kDa high molecular weight NAMPT complex is intriguing and indicates that RACK1 does have a NAMPT-modulating role. The interaction between RACK1 and NAMPT that we have discovered may be important for RACK1's modulation of NAMPT and targeting this interaction may represent a potential therapeutic strategy for OAC.

Table of Contents

Declaration..... i

Summary..... ii

Acknowledgements..... iv

Abstract..... 1

Table of contents.....3

List of Figures.....9

List of Tables.....11

List of Abbreviations.....12

List of Units.....13

Chapter 1: General Introduction.....14

 1.1 Oesophageal Adenocarcinoma.....14

 1.2 Cancer cell migration and invasion.....15

 1.3 RACK1.....16

 1.3.1 RACK1 is a WD-repeat protein.....16

 1.3.2 RACK1 and cancer.....17

 1.3.3 RACK1 and cancer cell migration, invasion and metastasis.....19

 1.3.3.1 RACK1 regulates polarity and direction sensing in cancer cells.....19

 1.3.3.2 RACK1 can promote the epithelial to mesenchymal transition (EMT).....19

 1.3.3.3. The activity of Src, a key player in tumour cell migration and invasion, is modulated by RACK1.....21

 1.3.3.4. RACK1: a scaffolding protein that integrates signalling from receptors to cytoskeletal reorganization.....23

 1.3.3.5 RACK1 regulates focal adhesion kinase (FAK) activity.....25

 1.3.3.6. RACK1 and Rho GTPase signalling pathways.....26

 1.3.4 RACK1 and non-muscle myosin heavy chain IIA.....27

 1.4 Targeting interactions between RACK1 and its binding partners; a therapeutic strategy for cancer and other diseases?.....29

 1.5 Introduction to nicotinamide phosphoribosyltransferase (NAMPT).....30

 1.5.1 NAMPT and obesity.....31

 1.5.2 NAMPT and cancer.....32

 1.6 Research objectives.....33

Chapter 2: Materials and Methods.....35

 2.1 Cell culture; cell lines and growth conditions.....35

 2.2 Sub culturing/passaging of Cells.....36

 2.3 Cell counting.....37

 2.4 Medium and long term storage of cells.....38

2.5 Mycoplasma Testing.....	38
2.6 Cell Transfection.....	39
2.6.1 Reverse transfection of SKGT-4 or FLO-1 cells with siRNA.....	39
2.6.2 Forward transfection of FLO-1 cells with plasmid DNA.....	40
2.7 Scratch wound two dimensional (2D) cell migration assay.....	41
2.8 Three dimensional (3D) cell migration assay using the xCELLigence RTCA platform.....	41
2.9 Cell proliferation assay using the xCELLigence RTCA platform.....	42
2.10 Nuclear count assay to assess cell proliferation.....	43
2.11 Cell confluency assay.....	43
2.12 Methylthiazolyldiphenyl-tetrazolium (MTT) assay to assess cell viability/proliferation.....	43
2.13 Assay to quantify impact of NMHCIIA depletion on F-actin organisation.....	44
2.14 Treatment of cells with deoxycholic acid (DCA).....	44
2.15 Live cell imaging of fluorescent proteins.....	45
2.15.1 Image Acquisition.....	45
2.15.2 Image Processing.....	45
2.15.3 Ratiometric Fluorescent Microscopy.....	46
2.15.3.1 Fluorescent ratiometric imaging of RACK1-EGFP.....	46
2.15.3.2 Fluorescent ratiometric imaging of NMHCIIA-mCherry.....	46
2.15.4 Förster Resonance Energy Transfer (FRET) Sensitized Emission Microscopy.....	47
2.15.4.1 Determination of the FRET calibration parameters alpha and beta.....	48
2.16 Immunofluorescent staining and confocal microscopy.....	49
2.17 NAD Assay to measure cellular NAD concentration in NAMPT-depleted or RACK1-depleted cells.....	50
2.18 Preparation of cellular protein extracts.....	50
2.19 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE).....	50
2.20 Western blotting.....	55
2.21 RACK1 immunoprecipitation/co-immunoprecipitation.....	58
2.21.1 Protocol for RACK1 immunoprecipitation from FLO-1 cells using protein G agarose beads.....	58
2.21.2 RACK1 immunoprecipitation from FLO-1 cells using Dynabeads.....	60
2.22 Mass spectrometry based proteomics screen of RACK1 immunoprecipitates.....	61
2.22.1 PEAKS analysis of the proteomics data.....	61
2.22.2 MaxQuant analysis of proteomics data.....	61
2.23 Bacterial transformation and plasmid purification for the expansion, production and purification of pCMV-mCherry-MHC-IIA plasmid and EGFP-RACK1 plasmids.....	62
2.24 Peptide array technology and alanine substitution arrays to investigate if NAMPT interacts directly with RACK1.....	62
2.25 Statistics.....	63

Chapter 3: An investigation into the subcellular localization, and potential co-localization, of RACK1 and non-muscle myosin heavy chain IIA (NMHCIIA) in oesophageal cells across the normal to OAC cancer progression sequence. Investigating the evidence for a RACK1 – NMHCIIA interaction during oesophageal adenocarcinoma cell migration.....	64
3.1 Introduction.....	64
3.2 Objectives.....	67
3.3 Results.....	67
3.3.1 RACK1 and NMHCIIA exhibit similar perinuclear colocalisation patterns in resting oesophageal cell lines representing the non-cancerous oesophagus-Barrett's oesophagus-adenocarcinoma sequence.....	67
3.3.1.1 Analysis of RACK1 and NMHCIIA localisation/colocalisation in resting HET-1A non-cancerous oesophageal cells.....	67
3.3.1.2 Analysis of RACK1 and NMHCIIA localisation/colocalisation in resting QhTERT Barrett's metaplastic oesophageal cells.....	68
3.3.1.3 Analysis of RACK1 and NMHCIIA localisation/colocalisation in resting GohTERT Barrett's dysplastic oesophageal cells.....	69
3.3.1.4 Analysis of RACK1 and NMHCIIA localisation/colocalisation in resting SKGT-4 oesophageal adenocarcinoma cells.....	70
3.3.2. In migrating SKGT-4 cells, RACK1 and NMHCIIA exhibit perinuclear colocalisation which is similar to that observed in resting cells.....	71
3.3.3 RACK1 and NMHCIIA colocalise at the leading edge of migrating MDA-MB-231 epithelial cells.....	73
3.3.4. Identification of an OAC cell line capable of undergoing single cell polarized migration.....	74
3.3.5 Immunofluorescent staining indicates that NMHCIIA localises to the leading edge of migrating FLO-1 cells while RACK1 has a diffuse distribution throughout the cytoplasm.....	75
3.3.6 EGFP tagged RACK1 exhibits a polarized distribution pattern during live cell imaging of migrating FLO-1 cells and is most often concentrated behind the leading edge in and/or close to, the lamella.....	76
3.3.7 mCherry tagged NMHCIIA is concentrated in leading edge ruffles during live cell imaging of migrating FLO-1 cells.....	79
3.3.8 Analysis of Förster Resonance Energy Transfer (FRET) between RACK1-EGFP and NMHCIIA-mCherry in resting and migrating FLO-1 cells.....	81
3.4 Discussion.....	83
3.5 Conclusion.....	86
Chapter 4: Non Muscle Myosin Heavy Chain IIA regulates cell morphology, stress fibre structure and cell migration in FLO-1 oesophageal adenocarcinoma cells.....	87
4.1 Introduction.....	87
4.2 Objectives.....	90

4.3 Results.....	90
4.3.1 Expression of non-muscle myosin heavy chain II isoforms in SKGT-4 and FLO-1 oesophageal adenocarcinoma cells.....	90
4.3.2 NMHCIIA depletion reduces migration of FLO-1 cells but does not affect migration of SKGT- 4 cells in a wound healing assay.....	91
4.3.3 NMHCIIA colocalises with F-actin at the lamellipodial leading edge of migrating FLO-1 cells but does not colocalise with F-actin at the leading edge of migrating SKGT-4 cells.....	92
4.3.4 NMHCIIA depletion alters FLO-1 cell morphology and F-actin organisation.....	94
4.3.5 NMHCIIA depletion does not affect FLO-1 cell proliferation as assessed by MTT assay.....	95
4.4 Discussion.....	96
4.5 Conclusion.....	100

Chapter 5: An investigation into the expression and function of RACK1 in oesophageal cells.....	101
5.1 Introduction.....	101
5.2 Objectives.....	103
5.3 Results.....	104
5.3.1 RACK1 expression levels generally increase with increasing disease progression in a panel of cell lines representing the normal oesophagus - Barrett's metaplasia - Barrett's dysplasia - adenocarcinoma sequence.....	104
5.3.2 DCA suppresses cellular levels of RACK1 protein in two oesophageal adenocarcinoma cell lines (SKGT-4 and FLO-1).....	106
5.3.3 DCA does not alter the subcellular localization of RACK1 in SKGT-4 and FLO-1 oesophageal adenocarcinoma cells.....	107
5.3.4 RACK1 depletion does not affect SKGT-4 oesophageal adenocarcinoma cell proliferation as assessed by MTT assay.....	108
5.3.5 RACK1 depletion leads to a trend towards decreased SKGT-4 cell proliferation, when assessed by nuclear count and cell confluency assays, but this is not statistically significant.....	109
5.3.6 RACK1 depletion reduces FLO-1 oesophageal adenocarcinoma cell proliferation as assessed by MTT assay.....	110
5.3.7 RACK1 depletion reduces FLO-1 cell proliferation as assessed on an X-CELLigence label-free real-time cell analysis platform.....	111
5.3.8 RACK1 depletion reduces 2D cell migration into a scratch wound assay in both SKGT-4 and FLO-1 cells.....	112
5.3.9 RACK1 depletion reduces 3D FLO-1 cell migration as analysed in real time using the xCELLigence label free real time cell analysis platform.....	113
5.4 Discussion.....	114

5.5 Conclusion	122
Chapter 6: A mass spectrometry based proteomics screen of RACK1 associated proteins in oesophageal adenocarcinoma cells.....	123
6.1 Introduction.....	123
6.2 Objectives.....	125
6.3 Results.....	125
6.3.1 Isolation and mass spectrometry mediated identification of RACK1 immunoprecipitates (IPs).....	125
6.3.2 Identification of 293 proteins unique to the RACK1 IPs.....	127
6.3.3 RACK1 co-immunoprecipitating proteins are involved in ribosomal related activities, RNA & DNA regulation, cell cycle, metabolism, trafficking, integrin signalling inflammation and cytoskeletal regulation.....	129
6.3.4 MaxQuant high stringency analysis of mass spectrometry data generated from analysis of control and RACK1 IPs indicates that the RACK1 interactome in FLO-1 cells is enriched for nucleic acid binding proteins.....	135
6.3.5 Analysis of potential interaction between RACK1 and vigilin (HDLBP) in FLO-1 cells.....	138
6.3.5.1 Efforts to verify that RACK1 and vigilin co-immunoprecipitate from FLO-1 lysates.....	138
6.3.5.2 Analysis of RACK1 and vigilin localization and potential colocalization in FLO-1 cells.....	139
6.3.6 RACK1 and nicotinamide phosphoribosyltransferase (NAMPT) co-immunoprecipitate from FLO-1 cells.....	140
6.4 Discussion.....	141
6.5 Conclusion.....	148
Chapter 7: RACK1 interacts with Nicotinamide Phosphoribosyltransferase (NAMPT) in FLO-1 Oesophageal Adenocarcinoma Cells.....	149
7.1 Introduction.....	149
7.2 Objectives.....	151
7.3 Results.....	152
7.3.1 NAMPT interacts directly with RACK1.	152
7.3.2 NAMPT interacts with arginine 47 in the linker region between WD1 and WD2 of RACK1 and is also very likely to interact with lysine 44 in WD1 and arginine 60 in the linker region between WD1 and WD2.....	154
7.3.3 NAMPT is present in the cytoplasm and nucleus of FLO-1 cells.....	157
7.3.4 NAMPT depletion reduces FLO-1 cell proliferation and viability.....	158
7.3.5 NAMPT depletion reduces FLO-1 cell migration.....	159
7.3.6 A second, higher molecular weight form of NAMPT is observed in FLO-1 cell lysates	

analysed by SDS-PAGE.....	161
7.3.7 RACK1 depletion results in decreased levels of high molecular weight NAMPT....	162
7.3.8 The effects of IL-1b stimulation on NAMPT protein levels in FLO-1 cells.....	163
7.3.9 NAMPT depletion dramatically reduces NAD levels in FLO-1 cells.....	165
7.3.10 RACK1 depletion does not affect NAD levels and the NAD/NADH ratio in FLO-1 cells.....	165
7.4 Discussion.....	166
7.5 Conclusion.....	171
Chapter 8: General Discussion.....	172
References.....	185
Appendix 1: siRNAs and plasmids.....	206
Appendix 2: Antibodies.....	208
Appendix 3: Appendix 3: Supplementary Data for Chapter 6.....	209

List of Figures

Figure 1.1 RACK1 regulates migration and invasion of cancer cells via a range of mechanisms.	23
Figure 1.2. RACK1 is a scaffolding protein that integrates signalling from receptors to cytoskeletal reorganization.....	25
Figure 2.1. Diagram of a haemocytometer grid.....	36
Figure 3.1: Subcellular localisation of RACK1 and NMHCIIA in HET-1A non-cancerous oesophageal cells.....	68
Figure 3.2: Subcellular localisation of RACK1 and NMHCIIA in resting QhTERT Barrett's metaplastic oesophageal cells.....	69
Figure 3.3: Subcellular localisation of RACK1 and NMHCIIA in resting GohTERT Barrett's dysplastic oesophageal cells.....	70
Figure 3.4: Subcellular localisation of RACK1 and NMHCIIA in resting SKGT-4 oesophageal adenocarcinoma cells.....	71
Figure 3.5: Subcellular localisation of RACK1 and NMHCIIA in scratch wounded, migrating SKGT-4 oesophageal adenocarcinoma cells.....	72
Figure 3.6: RACK1 and NMHCIIA colocalise at the leading edge of migrating MDA-MB-231 cells.....	74
Figure 3.7: Immunofluorescent staining and confocal microscopy was used to examine RACK1 and NMHCIIA colocalisation in polarised, migrating FLO-1 cells.....	76
Figure 3.8: : EGFP tagged RACK1 exhibits a cytoplasmic distribution pattern, with some accumulation in the lamella and cell rear, during live cell imaging of migrating FLO-1 cells...	78
Figure 3.9: mCherry tagged NMHCIIA localises to the leading edge and to ruffling lamellipodia in live, migrating FLO-1 cells.....	80
Figure 3.10: No significant FRET signal occurs between RACK1-EGFP and NMHCIIA-mCherry in FLO-1 cells.....	82
Figure 4.1: Expression of the three NMHCII heavy chain isoforms in FLO-1 and SKGT-4 oesophageal adenocarcinoma cells.....	91
Figure 4.2: NMHCIIA depletion reduces FLO-1 cell migration but does not reduce SKGT-4 cell migration.....	92
Figure 4.3. NMHCIIA co-localises with F-actin at the lamellipodial leading edge of migrating FLO-1, but not SKGT-4, cells.....	93
Figure 4.4: NMHCIIA depletion alters FLO-1 cell morphology and F-actin organisation.....	95
Figure 4.5: NMHCIIA depletion does not affect FLO-1 cell proliferation as assessed by MTT assay.....	96

Figure 5.1 RACK1 expression levels generally increase with increasing disease progression in a panel of cell lines representing the normal oesophagus→Barrett’s metaplasia→Barrett’s dysplasia→ adenocarcinoma sequence.....	105
Figure 5.2: DCA suppresses RACK1 protein levels in SKGT-4 and FLO-1 oesophageal adenocarcinoma cells.....	107
Figure 5.3. RACK1 has a cytoplasmic subcellular localization in SKGT-4 and FLO-1 cells which is not altered by DCA.....	108
Figure 5.4: Impact of RACK1-depletion on SKGT-4 oesophageal adenocarcinoma cell proliferation.....	109
Figure 5.5. RACK1 depletion reduces FLO-1 oesophageal adenocarcinoma cell proliferation as assessed by MTT assay.....	110
Figure 5.6 RACK1 depletion reduces FLO-1 cell proliferation as assessed on an X-CELLigence label-free real-time cell analysis platform.....	111
Figure 5.7. RACK1 depletion reduces 2D migration of both SKGT-4 and FLO-1 oesophageal adenocarcinoma cells.....	112
Figure 5.8 RACK1 depletion reduces 3D migration in FLO-1 cells as assessed by X-CELLigence label free real time cell analysis platform.....	114
Figure 6.1: Detection of RACK1 in control versus RACK1-specific IPs by mass spectrometry.....	126
Figure 6.2: Venn diagram of control IP and RACK1 IP proteomic lists.....	127
Figure 6.3: Protein-protein interaction network of RACK1 co-immunoprecipitating proteins.....	128
Figure 6.4: Gene Ontology (Biological Processes) analysis of RACK1 co-immunoprecipitating proteins.....	130
Figure 6.5: Results of Gene Ontology (Biological Processes) analysis of RACK1 co-immunoprecipitating proteins in pie chart format.....	131
Figure 6.6: Reactome analysis of RACK1 co-immunoprecipitating proteins.....	132
Figure 6.7: Results of Reactome analysis of RACK1 co-immunoprecipitating proteins in pie chart format.....	133
Figure 6.8 PANTHER DB pathway analysis of RACK1 co-immunoprecipitating proteins.....	134
Figure 6.9: Proteomics approach to identifying the GNB2L1 (RACK1) interact me.....	136
Figure 6.10: Proteomics approach to identifying the GNB2L1 (RACK1) interact me.....	137
Figure 6.11. Investigating if vigilin co-immunoprecipitates with RACK1 in FLO-1 cell lysates.....	139
Figure 6.12: RACK1 and vigilin in FLO-1 cells.....	140
Figure 6.13: NAMPT co-immunoprecipitates with RACK1 from FLO-1 cells.....	141
Figure 7.1: Identification of NAMPT as a direct RACK1 interaction partner.....	153
Figure 7.2: Identification of NAMPT binding sites on RACK1.	156
Figure 7.3 NAMPT is present in the cytoplasm and nucleus of FLO-1 cells.....	158
Figure 7.4: NAMPT depletion reduces proliferation and viability of FLO-1 cells.....	158

Figure 7.5: NAMPT depletion reduces 2D and 3D migration of FLO-1 cells.	160
Figure 7.6: Detection of 70kDa NAMPT by Western blotting is influenced by the sample buffer used when preparing lysates for SDS PAGE.	162
Figure 7.7: RACK1 depletion results in decreased levels of high molecular weight NAMPT...163	
Figure 7.8: The impact of IL-1 β stimulation on NAMPT protein levels in FLO-1 cells.....	164
Figure 7.9: NAMPT depletion reduces NAD levels in FLO-1 cells.....	165
Figure 7.10: RACK1 depletion has no significant effect on NAD concentration in FLO-1 cells..	166
Appendix 1 Figure 1: Plasmid map for pCMV-mCherry-MHC-IIA.....	207

List of Tables

Table 2.1. Cell culture media and growth conditions.....	36
Appendix 1 Table 1: siRNAs and DNA plasmids.....	206
Appendix 2; Table 1. Antibodies used during the PhD project.....	208
Appendix 3: Table 1: PEAKS analysis of data generated by mass spectrometry analysis of RACK1 IP and Control IP. This table lists proteins present in the <u>Control IP</u>	209
Appendix 3: Table 2: PEAKS analysis of data generated by mass spectrometry analysis of RACK1 IP and Control IP. This table lists proteins present in the <u>RACK1 IP</u>	215
Appendix 3: Table 3: Lists from Venn diagram (Chapter 6, figure 6.2).....	226
Appendix 3: Table 4: MaxQuant/Perseus analysis of data generated by mass spectrometry analysis of RACK1 IPs.....	230
Appendix 3: Table 5: MaxQuant/Perseus analysis of data generated by mass spectrometry analysis of RACK1 IPs.....	231

List of Abbreviations

▪ BEBM	bronchial epithelial basal medium:
▪ BEGM	bronchial epithelial growth medium:
▪ BMI	Body Mass Index
▪ CXCR4	chemokine receptor cysteine (C)-X-C receptor
▪ DCA	deoxycholic acid
▪ DMEM	Dulbecco's modified eagle's medium
▪ EMT	epithelial to mesenchymal transition
▪ eNAMPT	extracellular NAMPT
▪ ERK1/2	extracellular signal-regulated protein kinase 1/2
▪ FAK	focal adhesion kinase
▪ FCS	foetal calf serum
▪ GFR	growth factor receptor
▪ GH	glycine-histidine
▪ GORD	gastro-oesophageal reflux disease
▪ G $\beta\gamma$	heterotrimeric G protein $\beta\gamma$
▪ HDLBP	high-density lipoprotein-binding protein (HDLBP).
▪ HGF	hepatocyte growth factor
▪ hnRNP K	heterogeneous ribonucleoprotein K
▪ HUVECs	human umbilical vein endothelial cells
▪ IGF-1	Insulin-like Growth Factor-1
▪ IGF-1R	Insulin-like Growth Factor 1 Receptor
▪ iNAMPT	intracellular NAMPT
▪ MARTs	mono-ADP ribosyltransferases
▪ MDR	multi-drug resistant
▪ MMP	matrix metalloprotease
▪ MTT	methylthiazolyldiphenyl-tetrazolium
▪ NAD	nicotinamide adenine dinucleotide
▪ NMHCIIA	non muscle myosin heavy chain IIA
▪ NMHCIIA	non-muscle heavy chain IIC
▪ NMHCIB	non-muscle heavy chain IIB (NMHCIB)
▪ NSCLC	non-small cell lung cancer
▪ N-terminus	amino-terminus
▪ OAC	oesophageal adenocarcinoma
▪ PARPs	poly (ADP-ribose) polymerases
▪ PBEF	pre-B-cell colony-enhancing factor
▪ P-gp	P-glycoprotein
▪ PKC	Protein Kinase C

- PP2A protein phosphatase 2A
- RCC renal cell carcinoma
- Rho Ras homologous
- RPMI Roswell Park Memorial Institute medium
- SCC squamous cell carcinoma
- VHL von Hippel–Lindau
- WD-repeat tryptophan-aspartic acid repeat

List of Units

- μl microliter
- μm micrometer
- μM micromolar
- g gram
- mg microgram
- ml milliliter
- mM millimolar
- ng nanogram
- nM nanomolar

Chapter 1. General Introduction

1.1 Oesophageal Adenocarcinoma

Oesophageal cancer is the sixth most common cause of cancer related death and the eight most common cancer in the world (Pennathur et al., 2013). Despite recent advances in treatment, prognosis for oesophageal cancer is poor, with the overall 5-year survival of patients with this malignancy ranging from 15% to 25% (Pennathur et al., 2013).

There are two main histological types of oesophageal carcinoma; namely oesophageal squamous cell carcinoma (SCC) and oesophageal adenocarcinoma (OAC). On a global level, SCC has the greatest incidence. However, the incidence of OAC is increasing rapidly in the Western world and OAC is now more common than SCC in countries such as the USA, Australia and some western European countries (Pennathur et al., 2013).

OAC usually develops from a pre-cancerous lesion called Barrett's oesophagus; a metaplastic condition whereby the normal squamous epithelium of the distal oesophagus is replaced by an intestinal-type columnar epithelium (de Almeida et al., 1997). Population based studies have found that people with Barrett's oesophagus have a ten times greater risk of developing OAC than people without the condition (Lepage et al., 2013). However, as OAC is relatively rare, only a minority of patients with Barrett's oesophagus actually developed OAC (Lepage et al., 2013). Other important risk factors for OAC include obesity, symptomatic gastro-oesophageal reflux disease (GORD), tobacco use, male gender and low fruit and vegetable intake (Pennathur et al., 2013, Pohl et al., 2013).

Chronic gastro-oesophageal reflux disease is one of the strongest risk factors for OAC (Pennathur et al., 2013). The components of gastro-oesophageal refluxate include gastric acid, bile acids and other harmful chemicals which damage the oesophagus and promote inflammation (Duggan et al., 2010, Jenkins et al., 2006, Jenkins et al., 2004). Chronic exposure to this refluxate can lead to the development of Barrett's oesophagus which, in some patients, can develop further into adenocarcinoma (McQuaid et al., 2011, Pennathur et al., 2013).

Obesity is also an important risk factor for OAC (Thrift et al., 2014). The link between obesity and OAC may be partly mechanical. Central adiposity promotes gastro-oesophageal reflux disease by increasing intragastric pressure, increasing transient lower oesophageal relaxation and reducing lower oesophageal sphincter pressure (Beddy et al., 2010). However visceral adipose tissue is also metabolically active and can promote inflammation and metabolic syndrome (Picardo et al., 2012, Xu et al., 2003). Evidence is accumulating which suggests that intra-abdominal visceral adipose tissue may promote OAC carcinogenesis by releasing adipokines, proinflammatory cytokines and growth factors which can drive cellular proliferation, reduce apoptosis and trigger neoplastic transformation in the oesophageal epithelium (Lynam-Lennon et al., 2014) (O'Sullivan et al., 2014, Thompson et al., 2010).

Oesophageal cancer is a notoriously invasive cancer, with 20%-30% of patients already having distant metastasis on diagnosis (Napier et al., 2014). This aggressive cancer can spread by a variety of routes, which include direct extension, haematogenous metastasis and lymphatic spread (Napier et al., 2014). Local extension is aided by the fact that the oesophageal wall has no serosa. Without an anatomical barrier to restrict it, the primary tumour can extend quickly into the surrounding structures of the thorax (Napier et al., 2014). The oesophagus's extensive lymphatic drainage system also facilitates lymphatic spread of the cancer while haematogenous spread to the liver, lungs, bones, adrenal glands, kidneys and brain, is more common with advanced stages of the disease (Napier et al., 2014).

1.2 Cancer cell migration and invasion

Aberrant regulation of cell migration contributes to the progression of many diseases, including cancer cell invasion and metastasis (Yamaguchi and Condeelis, 2007). The process of metastasis involves a number of steps. These steps include local cancer cell infiltration or invasion into adjacent tissue, trans endothelial migration of cancer cells into vessels of the vasculature (this is called intravasation), survival in the circulatory system, exit of carcinoma cells from the circulation (extravasation) and proliferation in competent organs, leading to colonization of distal sites (van Zijl et al., 2011).

The ability of a cancer cell to migrate and invade enables it to change position within tissues and break free from the primary tumour, thus spreading the disease. It also allows tumour cells to enter lymphatic and blood vessels for dissemination into the circulation, thus enabling the cancer to colonise distant organs (Polacheck et al., 2013). Cell migration is a dynamic, complex process which requires multiple, interdependent steps. The cytoskeleton must be reorganized to form leading edge protrusions, mechanical forces must be generated and the cell tail must be retracted and detached from the extracellular matrix (Yamaguchi and Condeelis, 2007). The mechanisms of cell migration used by migrating cancer cells are broadly quite similar to those used by healthy cells during normal physiological processes such as wound healing, immune surveillance and embryonic morphogenesis (Friedl and Wolf, 2003).

When migration is stimulated, a cell generally polarises and generates cell protrusions in the direction of migration. These protrusions are usually driven by actin polymerization and they vary in nature depending on the cell type; some cells generate broad lamellipodia while others generate spike-like filopodia (Ridley et al., 2003). Transmembrane receptors linked to the actin cytoskeleton stabilize these protrusions by mediating their adhesion to the extracellular matrix or to adjacent cells. These adhesions act as traction sites for cell movement as the cells progresses forward over them. At the cell rear, adhesions are disassembled, allowing the cell to detach. The collective migration of cell sheets share some of the features of single cell migration but in the case of the former, polarization extends across the sheet (Ridley et al., 2003).

The precise details of cell migration depend on the cell type and the cell's environment. For example, somatic cells migrating in vivo exhibit large single protrusions and highly directional cell migration. Conversely, on planar substrates these cells exhibit multiple small protrusions. Cancer cells can respond to environmental changes by modifying their morphology and nature of migration (Ridley et al., 2003).

Metastasis accounts for approximately 90% of all deaths from cancer (Leber and Efferth, 2009). As outlined above, cancer cell migration and invasion constitute the initial step in the metastatic process (Leber and Efferth, 2009). While we have learnt much about these complex processes, there is still much to be discovered about the precise molecular regulation of cancer migration and invasion. New insights into the molecular processes which drive and regulate migration and invasion are necessary for a deeper understanding of carcinogenesis and may pave the way for new, more specific cancer therapeutics.

1.3 RACK1

RACK1 (Receptor for activated C kinase 1) is a 36kDa scaffolding protein. It was originally characterised as a scaffold responsible for regulating the subcellular localisation of Protein Kinase C (PKC) isoforms (Ron et al., 1994, Ron et al., 1995). However RACK1 is now known to bind a wide variety of different molecules and it is well recognized as a multifaceted scaffolding protein involved in numerous biological processes including cell proliferation, adhesion, migration and survival, apoptosis, protein synthesis, virus infection, neural development, angiogenesis and circadian rhythm (Berns et al., 2000, Daniels et al., 2008, Gandin et al., 2013b, Kiely et al., 2002, Mamidipudi and Cartwright, 2009, Robles et al., 2010, Wehner et al., 2011).

1.3.1 RACK1 is a WD-repeat protein

RACK1 is a member of the tryptophan-aspartic acid repeat (WD-repeat) family of proteins and has significant homology to the β subunit of G-proteins (Adams et al., 2011). WD-repeat proteins are an ancient, highly conserved family of proteins found in prokaryotes and all eukaryotes. WD repeats are sequences of approximately 44-66 amino acids that end in a signature WD dipeptide or its equivalent. They usually also have a glycine-histidine (GH) dipeptide positioned 11-24 residues from the N-terminus and often have an aspartic acid residue positioned 6 residues before the WD dipeptide. However none of these features are absolutely conserved and it is their collective critical mass instead of the absolute conservation of any particular amino acid that gives the sequence its identity as a WD repeat (Adams et al., 2011). WD repeat proteins have at least four of these WD repeat sequences which generate a WD-domain. These domains form a circular β -propeller structure, characterised by propeller blades arranged radially around a central axis (Adams et al., 2011, Sondek et al., 1996, Wall et al., 1995).

WD repeat proteins have a wide variety of roles, however they share a common function in scaffolding protein complexes, usually with numerous and competing partners, thus acting as hubs for the spatiotemporal coordination of cellular signalling across many pathways and networks. WD repeat proteins do not have any enzymatic activity but they do undergo post-translational modification and some of them, including RACK1, form both homodimers and heterodimers (Adams et al., 2011).

WD repeat proteins play a role in nearly every signalling pathway and thus they play an important role in a variety of human diseases (LiD, 2001, Stirnimann et al., 2010). Small changes in the expression of WD repeat proteins can have serious impacts on the assembly of protein complexes and on important signalling pathways (Ron et al., 2013).

RACK1 has a seven bladed WD-repeat propeller and has significant homology to the β subunit of G-proteins. As is the case with other WD repeat proteins, RACK1's propeller blades serve as binding sites for multiple interaction partners and enable it to act as a scaffolding protein which nucleates key signalling complexes. RACK1 also has important roles in shuttling proteins within the cell, anchoring proteins at specific subcellular locations and regulating protein stability and activity (Adams et al., 2011).

1.3.2 RACK1 and cancer

As discussed above, RACK1 participates in multiple signalling pathways and is implicated in a wide range of cellular functions and processes including transcription and protein synthesis, cell proliferation, survival, apoptosis, adhesion and migration. RACK1's roles in such processes are very important for normal, healthy cellular functions. The expression and activity of RACK1, and its associated molecules, is normally kept under tight regulation. However, if this tight regulation is lost, such as in a cancer setting, RACK1 can become implicated in carcinogenesis, or in some rarer cases, in tumour suppression (Ron et al., 2013). As will be discussed below, whether RACK1 promotes or suppresses carcinogenesis appears to depend on cancer type and on the particular cellular setting in which RACK1 is operating.

Increased RACK1 expression has been observed in studies of patient samples from a variety of cancers including breast carcinoma, non-small-cell lung cancer, pulmonary adenocarcinoma, hepatocellular carcinoma, oesophageal squamous cell carcinoma and glioma (Cao et al., 2010b, Guo et al., 2013, Hu et al., 2013, Peng et al., 2013, Ruan et al., 2012, Shi et al., 2012, Zhong et al., 2013). In contrast, studies in samples from gastric cancer patients have found that RACK1 expression is decreased in this malignancy (Chen et al., 2015, Deng et al., 2012).

RACK1 can promote or suppress carcinogenesis through a wide variety of different mechanisms which vary depending on the cell and cancer type and subtype. For example, RACK1 interacts with MKK7 in hepatocellular carcinoma cells and promotes proliferation by enhancing MKK7/JNK activity (Guo et al., 2013). In oesophageal SCC cells, RACK1 appears to drive cancer cell growth and migration through activation of the hedgehog signalling pathway (Hu et al., 2013). RACK1 interacts with the IGF-IR in a variety of cell types and facilitates

migration of breast and prostate tumour cells through a mutually exclusive interaction with $\beta 1$ integrin or Protein Phosphatase 2A (Kiely et al., 2008). Conversely, RACK1 appears to have a tumour suppressive function in colon cancer cells where its inhibition of Src delays cell cycle progression, suppresses colonic growth and induces apoptosis (Mamidipudi and Cartwright, 2009).

RACK1 acts at important points during transcription and translation. In particular, it acts at the ribosomal small subunit and impacts upon nuclear translocation and regulation of chromatin and transcriptional complexes (Ron et al., 2013). RACK1 is a component of the small subunit of the eukaryotic ribosome; it is located at the back of the 40S head region and it interacts with the ribosomal RNA (Link et al., 1999, Sengupta et al., 2004). In humans, RACK1 promotes translation by recruiting active PKC and eIF6 which stimulates subunit association via PKC-mediated eIF6 phosphorylation (Ceci et al., 2003, Li and Xie, 2014). The ribosomal function of RACK1 enables it to alter gene translation and signal transduction in response to various stimuli (Li and Xie, 2014). In some cancer settings, RACK1's function as a ribosomal protein can promote proliferation and survival of cancer cells. A study in hepatocellular carcinoma found that increased RACK1 expression promoted tumour growth and that this effect depended on RACK1 being localized to the ribosome. This study demonstrated that ribosomal RACK1 couples with PKC β II which promotes phosphorylation of eukaryotic initiation factor 4E resulting in the preferential translation of factors which drive cell growth and survival, such as cyclin D1, MYC, Survivin, and BCL-2 (Ruan et al., 2012).

The vast number of proteins with which RACK1 interacts is likely to account for its contrasting roles in different cancer settings. It is likely that the particular cohort of binding partners with which RACK1 interacts at any particular moment, in any particular disease or cell setting, will determine whether RACK1 acts as a tumour promoter or suppressor. Thus it is important that we consider cell and cancer type when examining RACK1 function.

The subcellular localisation of RACK1 is also an important determinant of its cellular function as it interacts with different proteins at different locations within the cell (Adams et al., 2011). Aberrant signalling, cellular stimuli or protein expression in cancer may alter RACK1 localisation which in turn may influence the function which RACK1 plays in cancer cells. For example, RACK1 normally has a diffuse cytoplasmic localisation in prostate cancer cells but it accumulates in the membrane compartment when expression of Trop-2 is upregulated. This shift in RACK1 localisation increases the association between RACK1 and $\beta 1$ integrin which results in increased $\beta 1$ integrin-RACK1-focal adhesion kinase (FAK)-Src signalling and loss of cell adhesion (Trerotola et al., 2012). Thus alterations in RACK1 subcellular localisation can have important impacts on its ability to promote or suppress carcinogenic processes and should thus be considered when examining the role of RACK1 in disease.

Thorough examination of RACK1 in a variety of cancer settings should enable us to increase our understanding of this multi-functional protein and may ultimately lead to the development of therapeutic strategies which hinder its pro-tumourigenic functions or promote its tumour suppressive functions.

1.3.3 RACK1 and cancer cell migration, invasion and metastasis

As discussed in section 1.2, cancer cell migration, invasion and metastasis represent important targets for cancer therapeutic strategies and research to further our understanding of the mechanisms which control these processes is crucial. Mounting evidence suggests that RACK1 can regulate cell migration, invasion and metastasis through multiple mechanisms. Such evidence is summarized in the paragraphs below.

1.3.3.1 RACK1 regulates polarity and direction sensing in cancer cells.

The regulation of cell polarity is fundamental to the process of cell migration (reviewed by (Etienne-Manneville, 2008) and (Cramer, 2010)). While cancer cells often lose apical-basolateral polarity, it is probable that polarization and directional migration toward chemotactic stimuli still play a role during cancer cell invasion and metastasis (Serrels et al., 2010). Cell polarization requires the differential localization of protein complexes in cells (Etienne-Manneville, 2008) and research has demonstrated a role for RACK1 in the construction and functioning of such complexes. To illustrate, RACK1 acts as a molecular bridge in a 'direction-sensing' pathway that links the integrin effector FAK to the recruitment of PDE4D5, a cAMP-degrading phosphodiesterase (Serrels et al., 2010). It facilitates the formation of a FAK-RACK1-PDE4D5 complex which is recruited to newly formed integrin adhesions and promotes cell polarity. This FAK-RACK1-PDE4D5 complex recruits specific components of the cAMP messenger system to nascent adhesions and the leading edge of polarized cells and is required for squamous cell carcinoma cells to sense direction and respond to directional cues (Serrels et al., 2010). This complex functions by signalling, via the guanine nucleotide exchange factor EPAC, to its small GTPase target Rap1 (Serrels et al., 2011). This FAK-RACK1-PDE4D5 complex is also essential for the construction of actin-based protrusive structures at the edges of polarized or spreading cells (Serrels et al., 2010).

In leukaemia cells, RACK1 interacts with heterotrimeric G protein $\beta\gamma$ ($G\beta\gamma$) subunits and functions as a negative regulator of directed cell migration (Chen et al., 2008). This study shows that when Jurkat and HL60 cells polarize, in response to chemoattractant, RACK1 interacts with $G\beta\gamma$ and translocates to the leading edge. RACK1 negatively regulates migration in these cells by preventing $G\beta\gamma$ stimulation of phosphoinositide 3-kinases and phospholipase C.

1.3.3.2 RACK1 can promote the epithelial to mesenchymal transition (EMT).

In many cancers, cell motility and migration is stimulated, and invasiveness enhanced, when a process known as the epithelial to mesenchymal transition (EMT) occurs (Savagner, 2015). As cells undergo EMT, they lose their epithelial characteristics, such as their polarity and epithelial

cell-cell contact structures, and instead acquire a mesenchymal phenotype (Savagner, 2015). They thus acquire a migratory behaviour which allows them to migrate away from their epithelial cell neighbours and invade into surrounding tissue (Xu et al., 2009). A number of studies now indicate that RACK1 can contribute to cancer cell migration and invasion by enhancing the epithelial to mesenchymal transition (Lv et al., 2016, Shi et al., 2012, Wang et al., 2015a). A study in glioma cells found that siRNA-mediated depletion of RACK1 suppressed cell migration and invasion by inhibiting expression of a number of EMT markers, namely, N-Cadherin, ZEB1, MMP-2, MMP-9 and Integrin- β 1 (Lv et al., 2016). A recent study of oesophageal squamous cell carcinoma patient tissue reported a positive correlation between RACK1 expression and vimentin, and an inverse correlation between RACK1 expression and E-cadherin. Furthermore, when the authors depleted RACK1 from oesophageal SCC cells, levels of vimentin were reduced, E-cadherin increased and cell migration and invasion was inhibited (Wang et al., 2015a).

The hedgehog signalling pathway contributes to EMT and enhanced cell migration and invasion in a number of cancers (Katoh and Katoh, 2008). In some cases, RACK1 contributes to EMT through activation of this pathway. For example, a study in non-small cell lung cancer (NSCLC) found that RACK1 interacted with and activated Smoothened to mediate Gli1-dependent transcription and hence activate sonic hedgehog signalling. Furthermore, when RACK1 was silenced, metastasis was dramatically inhibited by blocking the sonic hedgehog signalling pathway (Shi et al., 2012). Similarly, in oesophageal SCC RACK1 has been shown to promote cell migration via the hedgehog signalling pathway with upregulated expression of Gli1 and Patch (Hu et al., 2013).

Matrix degradation enzymes such as the proteolytic matrix metalloproteases (MMPs) promote EMT by degrading the basal membrane and extracellular matrix, thus alleviating the physical constraint that otherwise hinders cell motility in vivo and hence aiding tumour infiltration and metastasis (Kalluri and Weinberg, 2009). A number of studies now indicate that RACK1 can play a role in regulating MMP expression and/or activity. As mentioned above, a study in glioma cells found that depletion of RACK1 inhibited a variety of EMT markers which included MMP-2 and MMP-9 (Lv et al., 2016). Furthermore, a study in oral SCC cells found that RACK1 depletion by siRNA decreased the invasion, migration and adhesion capability of the tumour cells and this was associated with down-regulation of MMP-2, MMP-9 (and also EGFR, pEGFR, HER2) which they observed in these cells (Li et al., 2012b).

CD147 (extracellular matrix metallo-proteinase inducer) is a highly glycosylated transmembrane protein and an important stimulator of matrix metalloproteinases (MMPs) (Sun and Hemler, 2001). CD147 expression has been examined in breast carcinoma where RACK1 has been shown to promote invasion both in vitro and in vivo (Cao et al., 2010a). Interestingly, when breast carcinoma cells are stably transfected with RACK1 (so that they overexpress it) their levels of CD147 significantly increase. These RACK1-overexpressing transfectants also exhibit much higher MMP2 activity in comparison to parental cells (Cao et al., 2010a). Similarly, in pulmonary adenocarcinoma, tumour invasiveness is associated with

RACK1 expression and there is a significant correlation between RACK1 and CD147 expression both in vitro and in vivo (Zhong et al., 2013). Thus RACK1 is involved in multiple cell signalling pathways that regulate EMT across a broad range of tissues/cancers.

1.3.3.3. The activity of Src, a key player in tumour cell migration and invasion, is modulated by RACK1

Src is a non-receptor tyrosine kinase which can regulate several important signalling pathways which influence cancer migration, invasion and angiogenesis (as well as survival and proliferation) (Creedon and Brunton, 2012). RACK1 is a Src substrate (Chang et al., 2002). The phosphorylation of RACK1 by this kinase is necessary for Src interaction with RACK1 via its SH2 domain (Chang et al., 2002). Upon RACK1 binding, Src activity is inhibited. Thus RACK1 is a negative regulator of Src activity (Chang et al., 1998, Chang et al., 2001, Chang et al., 2002) and this contributes to the ability of RACK1 to act as a negative regulator of invasion in some cancer types. For example, a study by Swaminathan and colleagues reported that the inhibition of Src by RACK1 was partly responsible for its ability to reduce the invasive potential of colon cancer cells (Swaminathan and Cartwright, 2012). Epithelial cell-cell adhesion is mediated by E-cadherin and catenins. In cancer, aberrant Src activity can disrupt such adhesions and allow cancer cells to invade and metastasize. However, in colon cancer cells (HT29 and WiDr), RACK1 stabilises cell-cell contacts, in part through its inhibition of Src-mediated E-cadherin phosphorylation and consequent E-cadherin endocytosis, a key step in EMT (Swaminathan and Cartwright, 2012).

In contrast to colon cancer, RACK1-mediated regulation of Src activity appears to promote migration in other tumour types. In a study conducted in two neuroblastoma cell lines, namely SK-N-SH and SK-N-BE, high RACK1 expression was associated with increased migration (and proliferation) and increased phosphorylation of Src at Tyr416. Thus in neuroblastoma, RACK1 appears to promote migration through activation of Src activity (Lu et al., 2012). A study in glioma U87 and CHG-5 cell lines and xenograft tissue found that RACK1 expression was significantly higher in glioma tissue and cells in comparison to normal brain tissue (Peng et al., 2013). Furthermore, siRNA mediated RACK1 depletion reduced invasion and proliferation of glioma cell lines while also promoting their apoptosis. RACK1 depletion also resulted in a marked decrease in the activity of the Src/Akt signalling pathway, indicating that RACK1 may promote glioma cell invasion (as well as cell proliferation and survival) through modulation of Src activity (Peng et al., 2013).

A recent study in multidrug resistant breast cancer cells has demonstrated that RACK1 promotes invasion by acting as a scaffold that facilitates the binding of P-glycoprotein to Anxa2 and Src (Yang et al., 2016). P-glycoprotein is a well-known multidrug-efflux transporter which increases the invasion potential of drug resistant cells (Yang et al., 2016). This study showed that RACK1 regulates the activity of P-glycoprotein and hence facilitates adriamycin-induced P-glycoprotein-mediated phosphorylation of Anxa2 and Erk1/2. RACK1 silencing disrupted the P-

glycoprotein-Anxa2-Src protein complex in multidrug resistant breast cancer cells and significantly reduced cell invasion (Yang et al., 2016).

The conflicting anti- and pro- invasion properties associated with modulation of Src activity by RACK1 highlight the fact that RACK1 function can vary hugely depending on the cell or cancer type in which it is expressed. RACK1 function and interaction with Src also depends on stimuli received by the cell. For example, PKC activation promotes association of RACK1 with Src (Chang et al., 2001) while Insulin-like Growth Factor-1 (IGF-I) stimulation has been shown to disrupt the interaction between RACK1 and Src in epithelial cancer cells and fibroblasts (Kiely et al., 2005). Such stimulus-dependent interaction between RACK1 and Src could enable RACK1 to regulate local Src activation. Furthermore, it is known that RACK1 can shuttle its interacting partners to certain intracellular locations (Adams et al., 2011). Therefore, it is plausible to suggest that RACK1 may bind Src and shuttle it to particular subcellular locations before releasing it (and thus enabling its activation) at its site of action. Such a mechanism could possibly explain the apparently positive RACK1-mediated regulation of Src observed in the neuroblastoma and glioma studies above.

A number of other RACK1-Src mediated mechanisms that influence cell migration have been reported in non-tumorigenic cells. While this section specifically focuses on the role of RACK1 in cancer cell migration and invasion, nevertheless, it may be worthwhile to briefly mention such mechanisms as they may, or may not (given the sometimes very cell-specific functions of RACK1) also be relevant in some tumour cell types. Briefly, RACK1 promotes efficient migration of HEK-293 cells where its role as a key regulator of migration and adhesion dynamics is mediated through its modulation of Src activity and its resultant modulation of paxillin dynamics at early adhesions (Doan and Huttenlocher, 2007). Another study, this time in CHO-K1 cells, found that RACK1 localises to nascent focal complexes in paxillin-rich areas of protrusion and regulates integrin-mediated adhesion, protrusion and chemotactic migration through its Src binding site (Cox et al., 2003).

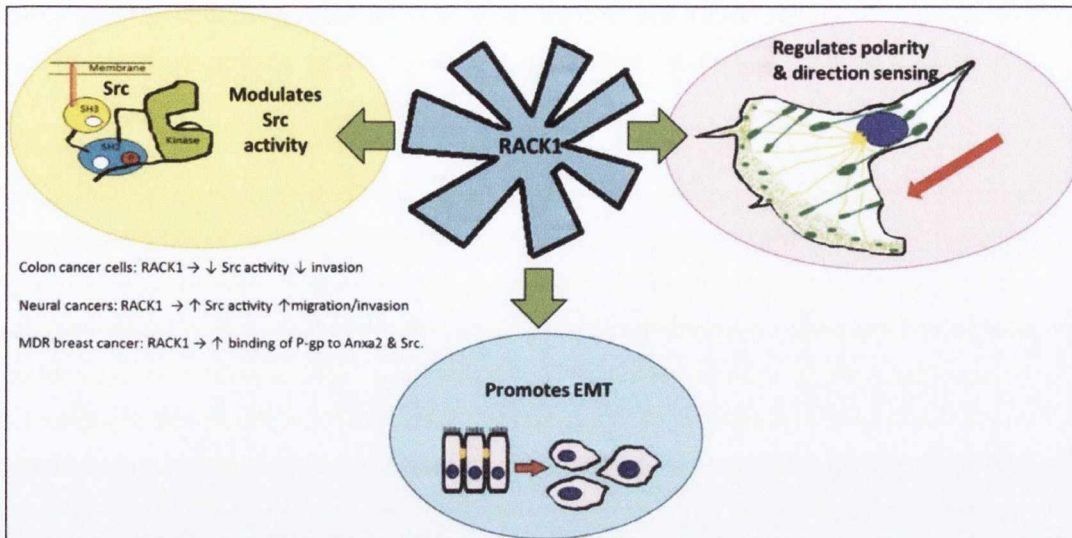


Figure 1.1 RACK1 regulates migration and invasion of cancer cells via a range of mechanisms. These mechanisms can vary with cell type. Three such mechanisms are illustrated above; namely RACK1-mediated modulation of Src kinase activity, regulation of cell polarity and direction sensing, and promotion of the epithelial to mesenchymal transition. EMT = epithelial to mesenchymal transition. MDR = multi-drug resistant. P-gp = P-glycoprotein.

1.3.3.4. RACK1: a scaffolding protein that integrates signalling from receptors to cytoskeletal reorganization.

RACK1 can promote tumour cell migration by using its scaffolding ability to bridge together different signalling pathways and integrate signalling between them (Adams et al., 2011). For example, RACK1 integrates signalling between the Insulin-like Growth Factor 1 Receptor (IGF-1R) and $\beta 1$ integrin by nucleating a signalling complex which contains both receptors as well as other signalling molecules which promote migration. Such RACK1-mediated integration of IGF-1R and integrin signalling promotes migration of a variety of tumour cells (Hermanto et al., 2002, Kiely et al., 2006, Kiely et al., 2005). In breast (MCF-7) and prostate (PC3 and DU145) cancer cells RACK1 promotes IGF-1R stimulated migration through its mutually exclusive association with either $\beta 1$ integrin or the serine-threonine phosphatase protein phosphatase 2A (PP2A). IGF-1 stimulation promotes the release of PP2A from a RACK-1-IGF-1R complex. This enables $\beta 1$ integrin to be recruited into an IGF-1R-RACK1- $\beta 1$ integrin complex and cell migration and proliferation is stimulated (Kiely et al., 2006, Kiely et al., 2008). A recent study in breast cancer cells also suggested a pro-carcinogenic role for the RACK1/PP2A interaction and reported that disruption of this interaction reduced cell adhesion, proliferation, migration and invasion (Kiely et al., 2016). Interestingly, a study in non-small cell lung cancer, found that RACK1 mediates the assembly of a RACK1-PP2A-Akt signalling complex in response to EphB3 activation which results in reduced Akt phosphorylation and inhibition of cell migration (Li et al., 2012a).

Interaction between RACK1 and β 1 integrin can promote prostate cancer invasion and metastasis as indicated by a recent study on the role of Trop-2, a transmembrane glycoprotein, which is upregulated in this malignancy (Trerotola et al., 2012). Trop-2 upregulation was reported to increase interaction between RACK1 and β 1 integrin which in turn increased activation of Src and FAK and inhibited cell adhesion to fibronectin. Prostate cancer cells may utilise this mechanism to detach and invade into the surrounding extracellular matrix, thus promoting their metastasis (Trerotola et al., 2012). Interestingly, a study in H9c2 rat cardiomyocytes has observed that RACK1 does not interact with the IGF-1R in these non-transformed cells (O'Donovan et al., 2007). Furthermore, IGF-1 promotes recruitment of Src to RACK1 as well as recruitment of PKC α , and PKC ϵ , to RACK1. In these cells overexpression of RACK1 increased migration but its responses to IGF-I are independent of an association with the IGF-1R (O'Donovan et al., 2007). Therefore the make-up of the RACK1 scaffolding complex and its effects on IGF-1 signalling can be different in normal and cancer cells. Indeed this provides an interesting example of how the function of RACK1 can vary depending on cell type or whether it is present in normal or cancer cells.

Abnormal IGF-1 signalling is associated with the development of very invasive metastatic renal cell carcinoma (Cardillo et al., 2013). In this cancer, RACK1 interacts with the IGF-1R and promotes IGF-1 induced cell invasiveness which is associated with increased IGF-1R kinase activity and increased MMP-2 expression and activation (He et al., 2011). Interestingly, RACK1 also interacts with the von Hippel–Lindau (VHL) tumour suppressor whose loss is associated with renal cell carcinoma (RCC) pathogenesis (Baldewijns et al., 2010, He et al., 2011). RACK1 interaction with pVHL disrupts the association between RACK1 and IGF-1R thereby suppressing RACK1-mediated IGF-1-induced renal cell carcinoma cell invasiveness. Thus RACK1 acts as a direct mediator between pVHL loss of function and increased IGF-1R signalling in this cancer setting (He et al., 2011).

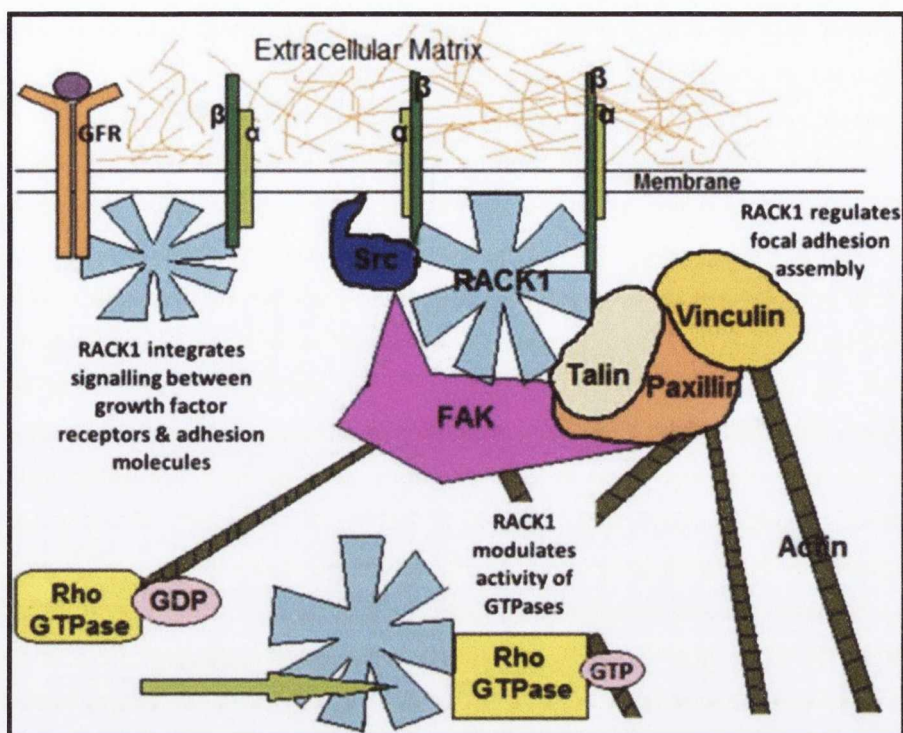


Figure 1.2. RACK1 is a scaffolding protein that integrates signalling from receptors to cytoskeletal reorganization. GFR = growth factor receptor. α = integrin α chain. β = integrin β chain.

1.3.3.5 RACK1 regulates focal adhesion kinase (FAK) activity.

FAK regulates critical processes in both normal and tumour cells through its kinase activity and function as a scaffold (Lee et al., 2015). It regulates carcinogenesis and metastatic potential through highly coordinated signalling networks that coordinate a wide range of cellular processes including cell migration, invasion and adhesion (Lee et al., 2015). FAK is recruited early to the leading edge of the cell as new focal adhesion complexes are assembled. Meanwhile, at the trailing edge, focal adhesion complexes must be dismantled to facilitate migration. This focal adhesion assembly and disassembly must be precisely coordinated for efficient cell migration and is highly dependent on cross talk between growth factor and adhesion (mainly integrin) signalling pathways (Dwane et al., 2014, Mitra et al., 2005). RACK1 plays a crucial role in cell adhesion and migration by regulating FAK activity and focal adhesion assembly (Kiely et al., 2009, Adams et al., 2011). RACK1 is critical for the function of FAK as it scaffolds it for activation by IGF-1 and adhesion signalling (Adams et al., 2011, Kiely et al., 2009). When RACK1 expression is suppressed, FAK activity is disrupted which interferes with cell adhesion and cell spreading, two crucial processes required for cell migration (Kiely et al.,

2005, Kiely et al., 2009, Onishi et al., 2007). Phosphorylation of RACK1 on Tyr-52 by c-Abl mediates the interaction between RACK1 and FAK (Kiely et al., 2009). Thus RACK1 acts as an important convergence point for adhesion and growth factor signalling and is a key regulator of the signalling events necessary to initiate cell migration (Adams et al., 2011). Furthermore, as is discussed in 1.3.3.1 above, a RACK1/FAK complex, together with PDE4D5, regulates cell spreading and direction sensing and is thus important for establishing cell polarity (Serrels et al., 2010, Serrels et al., 2011). It is likely that RACK1 regulates migration and invasion through FAK in a variety of ways which vary depending on the cancer setting. A recent study in nasopharyngeal carcinoma reported that RACK1 promoted invasion and metastasis by activating FAK and Akt (Peng et al., 2016). The phosphoinositide 3-kinase (PI3K)/Akt signalling pathway is highly implicated in nasopharyngeal carcinoma and this study suggests that regulation of the PI3K/Akt/FAK pathway may underlie the contribution of RACK1 to this disease (Peng et al., 2016).

Angiogenesis is critical for primary tumour growth but also contributes to tumour invasion and metastasis (Bielenberg and Zetter, 2015). RACK1 has been found to be induced during tumour angiogenesis (Berns et al., 2000) and a study by Dave and colleagues has found that RACK1 can promote angiogenesis by enhancing endothelial sprout formation through an interaction with FAK and the intermediate filament protein, vimentin, in three-dimensional collagen matrices (Dave et al., 2013). In this study, RACK1 and vimentin were found to complex in invading endothelial cells where they regulate FAK. In response to stimulation by sphingosine 1-phosphate (which is required for invasion) and growth factors, RACK1, vimentin and FAK formed an intramolecular complex in invading endothelial cultures. Furthermore, RACK1 depletion decreased the association between FAK and vimentin, indicating that RACK1 is important for stabilising their interaction during angiogenic sprouting (Dave et al., 2013).

1.3.3.6. RACK1 and Rho GTPase signalling pathways

In order for cancer cells to migrate and invade, significant changes to the cell cytoskeleton must be made. Cell shape must be altered, actin polymerisation and depolymerisation must occur and integrin-mediated membrane attachment to the substrate must be modified (Ridley et al., 2003). Rearrangements of the actin cytoskeleton are necessary to allow such modifications and processes to occur (Ridley et al., 2003). A number of proteins belonging to the Ras homologous (Rho) family of small GTPases function as crucial regulators of the actin cytoskeleton (Spiering and Hodgson, 2011). Rho GTPases are normally highly regulated, and like other low molecular weight GTPases of the Ras family, they cycle between an active, GTP-bound and an inactive, GDP-bound state (Malliri and Collard, 2003). Therefore deregulation or dysfunction of Rho pathways in cancer cells can lead to aberrant cytoskeletal modulation and hence create the potential for aberrant cell motility, migration and invasion (Malliri and Collard, 2003, Schmitz et al., 2000).

A number of studies have demonstrated that RACK1 can regulate cancer cell migration and invasion via modulation of some of the Rho GTPases themselves or of other proteins which function in Rho GTPase signalling pathways (Cao et al., 2010a, Cao et al., 2011, Wu et al., 2013). A study in breast carcinoma cells found that RACK1-induced cell migration could be dramatically inhibited by the addition of a Rho-kinase inhibitor (Cao et al., 2010a). A subsequent study demonstrated that RACK1 interacts with RhoA and promotes breast carcinoma migration and metastasis by activating the RhoA/Rho kinase pathway in breast cancer cell lines and in patient samples (Cao et al., 2011). The level of active, GTP-bound RhoA significantly increased in breast carcinoma cells stably transfected with RACK1 (in comparison to parental control cells) while the level of total RhoA remained unchanged. Furthermore, the level of GTP-bound RhoA was significantly decreased in breast carcinoma cells treated with siRNA targeting RACK1 (Cao et al., 2011). Thus RACK1 appears to promote cell migration by enhancing the activation of RhoA.

RACK1 may also influence cancer cell migration through its influence on the signalling pathways associated with other Rho GTPases such as Rac1. Overexpression of RACK1 in a mouse hepatocellular carcinoma cell line increased expression of Rac1, Jnk1 and Gsn and promoted cell migration and invasion in comparison to control cells (Wu et al., 2013). Treatment with LY294002, an inhibitor of PI3K, decreased these RACK1-induced phenomena (Wu et al., 2013). Interestingly, RACK1 has been found to interact with PI3K p110 alpha in breast carcinoma cell lines (Cao et al., 2010a). PI3K p110 is the catalytic subunit of PI3K which generates phosphatidylinositol-3, 4, 5-trisphosphate, PI(3,4,5)P3, which can in turn activate multiple downstream signalling pathways (Vara et al., 2004). Thus, over-expressed RACK1 may act as a scaffold which binds and activates PI3K which then affects activation of Rac1. Pre-treatment of these RACK1-overexpressing mouse hepatocellular carcinoma cells with an inhibitor of Rac1 (NSC33766) also decreased the RACK1-induced upregulation of Gsn (Wu et al., 2013). Gsn is an important actin-regulatory protein which can both nucleate and sever actin and which may play an important role in regulating cell motility (Deng et al., 2015). Furthermore, Gsn has been shown to induce invasion of epithelial cells which rely on the Ras-Rac signalling pathway, with Ras activated through PI3K (De Corte et al., 2002). Thus, RACK1 may promote cell migration in mouse hepatocellular carcinoma cells by regulating Gsn through a Rack1/PI3K/Rac1 pathway.

1.3.4 RACK1 and non-muscle myosin heavy chain IIA (NMHCIIA)

As illustrated in section 1.2 and section 1.3.3 above, cell migration is a complex process involving a plethora of proteins which regulate the cytoskeleton and enable it drive the cell forward. Research, which improves our understanding of how these numerous proteins are coordinated in such a way that enables them to regulate cytoskeletal dynamics, could greatly improve our understanding of the cell migration process. RACK1, as an adaptor protein, may

contribute to this coordination; it may link signalling proteins to the cytoskeleton, shuttle proteins involved in migration to their site of action within the cell and/or anchor such proteins at their required location. The mechanics of cell migration require that contractile forces are generated within the cell and that the cell polarises, rearranging various components of the cytoskeleton (Devreotes and Horwitz, 2015). One family of proteins which contribute to such physical processes and help to regulate the mechanics of cell migration is non muscle myosin II (Heissler and Manstein, 2013).

Non-muscle myosin II proteins are actin binding motor proteins with actin cross-linking and contractile properties. Their ability to catalyse the conversion of chemical energy into directed movement and force enables them serve as important regulatory components of the cytoskeleton and they are proteins with critical roles in the control of cell adhesion, cell migration and tissue architecture (Heissler and Manstein, 2013) (Vicente-Manzanares et al., 2009). Non muscle myosin II is a hetero-multimeric protein complex composed of two 230kDa heavy chains, two 20kDa regulatory light chains and two 17kDa essential light chains. Three mammalian isoforms of non-muscle myosin II have been identified, namely non muscle myosin IIA, non-muscle myosin IIB and non-muscle myosin IIC. These isoforms have both overlapping and distinct functions (Heissler and Manstein, 2013).

Research from our group has previously discovered a novel interaction, between non-muscle myosin heavy chain IIA (NMHCIIA) and RACK1, in T lymphocytes. This research demonstrated that RACK1 translocates to the leading edge of migrating T-lymphocytes in association with NMHCIIA. This indicates that RACK1 may control the subcellular localisation of this non muscle myosin II isoform during cell migration (Christine Rudolph, unpublished data).

A small number of studies have begun to demonstrate an association between NMHCIIA and cancer migration, invasion and metastasis (Ouderkirk and Krendel, 2014). In tissue from oesophageal SCC patients, increased NMHCIIA expression is associated with increased metastatic lymph nodes, poorer cancer differentiation, and advanced tumour stage. Meanwhile, NMHCIIA depletion in oesophageal SCC cell lines increases cell–matrix adhesion and reduces cell migration (Xia et al., 2012). NMHCIIA depletion from MCF-7 breast carcinoma cell lines promotes cell aggregation and inhibits invasion (Derycke et al., 2011). A study in MDA-MB-231 breast cancer cells demonstrated that NMHCIIA plays an important role in matrix remodelling and its depletion also reduces migration of this cell type (Hindman et al., 2015, Betapudi et al., 2006). In contrast, NMHCIIA depleted A549 lung carcinoma cells exhibit a more motile morphology, with decreased amounts of stress fibres and focal adhesions, increased cellular protrusions and an increased migration speed (Sandquist et al., 2006). Meanwhile, a study in squamous cell carcinoma reported a positive association between NMHCIIA depletion and metastasis and suggested that NMHCIIA acts as a tumour suppressor by regulating p53 stabilisation and nuclear retention (Schramek et al., 2014). Thus, whether NMHCIIA promotes or suppresses cancer migration, invasion and metastasis is the subject of conflicting reports and more research is needed to improve our understanding of same.

1.4 Targeting interactions between RACK1 and its binding partners; a therapeutic strategy for cancer and other diseases?

The abundance and near ubiquitous expression of RACK1 coupled with its involvement in a plethora of cellular and physiological processes renders the unspecific therapeutic targeting of this protein unsuitable as a strategy to treat cancer or other diseases. However, the possibility of targeting specific interactions between RACK1 and particular RACK1 binding partners may offer promising therapeutic potential.

As outlined in the preceding sections, RACK1 interacts with a host of different interaction partners; such partners include kinases, phosphatases, phosphodiesterases, ion channels, membrane receptors, G proteins, IP3 receptors, apoptosis-related molecules and structural proteins associated with the ribosome. RACK1 mediates and controls cellular processes through its interaction with these partners and its influence on them can be broadly classified into four categories; modifying the activity of its partners, changing intermolecular interaction (enhancing association/dissociation), modulating the stability of binding proteins and shuttling partners from one subcellular location to another (Li and Xie, 2014).

Like other scaffolding proteins, the expression of RACK1 is normally tightly controlled in the healthy cell (Adams et al., 2011). This is important because scaffolding proteins such as RACK1 exert major impacts on the cell's signalling networks. For example, when a signalling protein binds to RACK1, the local concentration of that signalling protein is increased. In many diseases or disorders associated with RACK1, abnormal RACK1 signalling, which underlies the pathology, occurs as a result of either increased or decreased RACK1 expression. It is likely that sub-optimal RACK1 protein levels interferes with its compartmentalisation and, as a result, impedes the formation of signalling complexes with correct stoichiometry at distinct locations within the cell (Ron et al., 2013). Thus it is likely that even a small deviation from the normal expression levels of RACK1 can have major consequences for signalling networks and the cellular processes that they control (Adams et al., 2011).

Taken the above into consideration, the therapeutic potential of RACK1 may be realized by developing small molecules which target specific RACK1 binding partners and selectively disrupt their interaction with it (Ron et al., 2013). Some progress has already been made in this regard. For example, cell permeable peptides which function to hinder the interaction between a specific subset of RACK1 and its binding partner PDE4D5 have successfully inhibited metastasis and direction sensing of cancer cells without adversely affecting the many other critical cellular functions of RACK1 (Serrels et al., 2010, Serrels et al., 2011, Smith et al., 2007).

Co-crystal structures of a RACK1-like, 7-bladed beta-propeller protein (WDR5) with molecules that were designed to disrupt binding of a single interaction partner have also been developed (Karatas et al., 2012, Senisterra et al., 2013). Such work paves the way for molecules which could inhibit RACK1's interaction with specific client proteins and which could potentially be used to target specific actions of RACK1 which contribute to cancer or other diseases (Ron et al., 2013).

As we identify new RACK1 interaction partners and improve our knowledge of the interaction between RACK1 and already identified partners, we may discover important interactions which we could specifically target for therapeutic benefit.

1.5 Introduction to nicotinamide phosphoribosyltransferase (NAMPT)

As discussed in section 1.1, OAC is associated with obesity (Thrift et al., 2014). Furthermore, meta-analysis has shown that central adiposity, independent of body mass index (BMI), is associated with oesophageal inflammation, metaplasia and OAC (Singh et al., 2013). Metabolic changes related to central adiposity may contribute to dysfunctional adipose tissue inducing chronic inflammation, insulin resistance and abnormal production of adipokines (Dalamaga and Christodoulatos, 2017).

Nicotinamide phosphoribosyltransferase (NAMPT), also known as visfatin and pre-B-cell colony-enhancing factor (PBEF), is a multifaceted protein which is believed to act as both an enzyme and a cytokine. Evidence is accumulating to suggest that NAMPT can modulate important processes involved in the pathogenesis of obesity and related diseases and disorders through its influence on the oxidative stress response, inflammation, lipid and glucose metabolism, insulin resistance and cellular growth and apoptosis (Garten et al., 2015). Thus it is not surprising that NAMPT has been associated with a number of metabolic disorders associated with obesity and inflammation (Chang et al., 2011, Garten et al., 2015). Furthermore, NAMPT plays an important role in cancer cell metabolism and is overexpressed in a wide range of cancer tissues (Garten et al., 2015).

NAMPT is expressed in a wide variety of organs, tissues and cells, suggesting that it has pleiotropic functions in human physiology (Garten et al., 2015). NAMPT occurs both intracellularly (iNAMPT) and extracellularly NAMPT (eNAMPT). iNAMPT is widely expressed and has been well characterised as an NAD biosynthetic enzyme (Bi and Che, 2010). eNAMPT has been detected in human and mouse circulation, in human cerebrospinal fluid and seminal plasma as well as in the supernatant of multiple cell types including adipocytes, hepatocytes, leucocytes, neurons, cardiomyocytes, amniotic epithelial cells, lipopolysaccharide-activated monocytes and pancreatic β cells (Garten et al., 2015). There is still some debate and uncertainty regarding the precise functions of eNAMPT but potential pro-inflammatory, NAD biosynthetic, insulin-mimetic and growth factor functions have been ascribed to it (Kieswich et al., 2016, Moschen et al., 2007) (Yoon et al., 2015) (Dahl et al., 2012, Garten et al., 2015). There is now significant evidence that eNAMPT is a cytokine that binds to a presently unknown extracellular receptor (Grolla et al., 2015). There is still some debate on eNAMPT's potential role as pro-inflammatory molecule. However many studies have now demonstrated pro-inflammatory effects of eNAMPT which include production of cytokines such as TNF- α , IL-6, IL-8, IL-1 β , induction of inducible nitric oxide synthase, activation of extracellular signal-regulated protein kinase 1/2 (ERK1/2) and nuclear factor NF- κ B activation (Garten et al., 2015).

Working as a biosynthetic enzyme, NAMPT catalyses the generation of nicotinamide mononucleotide from nicotinamide and 5'-phosphoribosyl-1-pyrophosphate. This makes NAMPT the rate-limiting enzyme in the mammalian NAD salvage pathway (Sampath et al., 2015). NAD is an important coenzyme which is involved in cellular redox reactions and serves as a substrate for a number of different enzymes and regulatory proteins (Sampath et al., 2015). It is a cofactor and second messenger for a variety of cellular processes that are essential for survival, such as metabolism and the maintenance of genomic stability (Sampath et al., 2015). By way of its NAD biosynthesis ability, NAMPT is one of the regulators of the intracellular NAD pool and it influences the action of NAD-dependent enzymes. Through its influence on such enzymes, which include sirtuins and poly (ADP-ribose) polymerases, NAMPT regulates cellular metabolism, mitochondrial biogenesis and adaptive responses to oxidative, proteotoxic, genotoxic and inflammatory stress. It is likely that NAMPT also influences the function of mono-ADP-ribosyl transferases and ADP-ribosyl cyclases as they also use NAD. These enzymes are involved in a number of physiological processes including DNA repair and calcium signalling (Garten et al., 2015). NAMPT activity has been shown to augment cell proliferation and tip the balance towards cell survival following a genotoxic insult (Gallí et al., 2010).

1.5.1 NAMPT and obesity

The correlation between NAMPT and obesity is the subject of conflicting reports; some of which may be due to differences between study populations, sample handling and NAMPT detection methods. However, a meta-analysis investigating the association between eNAMPT and obesity parameters, found that, in general, eNAMPT levels are increased in obese individuals (Chang et al., 2011). NAMPT expression is higher in visceral adipose tissue in comparison to subcutaneous adipose tissue (Garten et al., 2015).

A number of metabolic factors which are elevated in obese patients have been shown to influence the expression and production and/or release of NAMPT (Garten et al., 2015). Glucose and oxidized LDL stimulate NAMPT gene and protein expression as well as the release of NAMPT in human adipocytes via the PI3-kinase–AKT pathway (Haider et al., 2006a, Haider et al., 2006b, Chen et al., 2013). When glucose is administered to humans, eNAMPT levels increase (Friebe et al., 2011). An in vitro study has demonstrated that the expression of NAMPT mRNA increases during adipogenesis (Kralisch et al., 2005) while another study has found that hypoxia upregulates NAMPT mRNA expression in adipocytes through a HIF1 α -dependent mechanism (Segawa et al., 2006).

A number of single nucleotide polymorphisms in the NAMPT gene are associated with obesity and obesity related comorbidities such as coronary artery diseases (Blakemore et al., 2009, Saddi-Rosa et al., 2013).

1.5.2 NAMPT and cancer

Increased expression of NAMPT has been associated with the development of multiple cancers including cancers of the prostate, ovaries, breast and stomach, melanoma, myeloma, endometrial carcinomas, colorectal cancer and astrocytomas (Garten et al., 2015).

Cancer cells have high energy demands and, due to the Warburg effect, they have a high capacity for glucose uptake and increased rates of glycolysis (Palsson-McDermott and O'Neill, 2013). As a result of this altered metabolic phenotype, cancer cells require increased amounts of NAD. NAD-dependent enzymes, such as poly (ADP-ribose) polymerases (PARPs), mono-ADP ribosyltransferases (MARTs) and sirtuins, which are required for DNA repair, genome stability and proliferation, are also highly active in cancer cells (Schuster et al., 2015, Sampath et al., 2015). Cancer cells are highly reliant on the NAMPT dependent salvage pathway for NAD production and thus NAMPT is an attractive therapeutic target for cancer (Sampath et al., 2015). The specific NAMPT inhibitor, FK866 has been evaluated in a wide range of cancers including solid tumours and leukaemias, both in vitro and in nude mouse xenografts, and was capable of attenuating tumour growth (Garten et al., 2015). A study in cancer cells and tumour xenografts demonstrated that NAMPT inhibition with FK866 leads to the attenuation of glycolysis due to the reduced availability of NAD. This study reported that the blockade of glycolysis at the glyceraldehyde 3-phosphate dehydrogenase step was the central metabolic basis of NAMPT inhibition responsible for ATP depletion, metabolic perturbation, and subsequent tumour growth inhibition (Tan et al., 2013). In hepatocarcinoma cells, FK866 can induce delayed energy stress which stimulates activation of AMPK α and downregulation of mTOR signalling which are associated with increased tumour cell death. Noncancerous hepatocytes are less sensitive to FK866 than cancerous cells (Garten et al., 2015).

NAMPT may contribute to cancer progression through multiple mechanisms. NAMPT modulates resting monocytes, polarising them towards a tumour-supporting M2 macrophage phenotype; using this mechanism NAMPT induces an immunosuppressive and tumour-promoting microenvironment in chronic lymphocytic leukaemia (Audrito et al., 2015). As mentioned above, NAMPT tips the balance towards cell survival following a genotoxic insult and could thus promote survival of mutated tumour cells (Gallí et al., 2010). NAMPT overexpression increases the activity of NAD-dependent SIRT6 and protect cells from death due to PARP overexpression. Thus NAMPT may contribute to stress resistance in cancer and protect cells from stress induced PARP-mediated cell death (Bi and Che, 2010). A study in human umbilical vein endothelial cells (HUVECs) reported that NAMPT up-regulates gene expression and protein production of VEGF and MMP2 and MMP9 through MAPK/PI3K-Akt/VEGF signalling pathways. Thus NAMPT may play an important role in angiogenesis, a process which is critical for tumour growth and expansion (Adya et al., 2008b). A study in prostate cancer cell lines also demonstrated that NAMPT promotes the expression and activity of MMP-2/9. These proteases play an important role in the breakdown of the extracellular

matrix and thus these studies suggest a potential role for NAMPT in cancer cell migration, invasion and metastasis (Patel et al., 2010, Dalamaga, 2012).

1.6 Research objectives

The incidence of OAC is on the increase in Western countries. Early oesophageal cancer is often asymptomatic and hence metastatic disease is common at presentation leading to poor prognosis and survival rates (Pennathur et al., 2013). Thus, metastasis is an important target in oesophageal cancer treatment.

RACK1 is a 36 kDa scaffolding protein containing seven internal Trp-Asp (WD) repeats forming a seven-bladed propeller structure. It interacts with a plethora of molecules including receptors, adhesion molecules, kinases and phosphatases (Li and Xie, 2014). Thus RACK1 is involved in the co-ordination of multiple cellular processes including cell growth, adhesion and motility. RACK1 expression is altered in a wide variety of cancers and mounting evidence suggests that this scaffolding protein plays important roles in the development and progression of many cancers (Adams et al., 2011, Li and Xie, 2014). However, the role of RACK1 in OAC has not been previously examined. Therefore, this PhD project aimed to investigate the potential role of RACK1 in this malignancy. My host laboratory had previously identified a novel interaction between RACK1 and non-muscle myosin heavy chain IIA in migrating T cells. Therefore, this project also aimed to investigate if such an interaction also occurs in OAC cells.

Specifically, the objectives of this PhD project were to;

- 1.) Examine the localisation, and potential co-localisation, of RACK1 and NMHCIIA in oesophageal cells across the cancer progression sequence from non-cancerous oesophagus to OAC. Determine if RACK1 and non-muscle myosin heavy chain IIA interact in migrating OAC cells and determine if this interaction is of functional significance to migration. My host laboratory had previously identified a novel interaction between RACK1 and non-muscle myosin heavy chain IIA in migrating T cells. I was therefore interested to see if this interaction also occurs in OAC cells.
- 2.) Examine the function of NMHCIIA in OAC cell biology, with a particular focus on investigating if NMHCIIA plays a role in OAC cell migration.
- 3.) Examine the expression, localisation and function of RACK1 in OAC cells. The role of RACK1 in OAC cell migration was of particular interest to this study.
- 4.) Examine and characterise the RACK1 associated proteome in OAC cells.
- 5.) Identify a RACK1 binding partner of particular interest. Determine if RACK1 interacts directly with that partner. Map the sites on RACK1 which mediate its interaction with that

binding partner. Investigate the functional implications of the interaction between RACK1 and such a binding partner. As OAC is strongly associated with obesity, the potential interaction of RACK1 with obesity-associated proteins was of particular interest.

Chapter 2. Materials and Methods

2.1 Cell culture; cell lines and growth conditions

Cell lines were cultured in the media described below in table 2.1. All cell lines used were grown in an incubator at 37°C in a 5% CO₂ humidified atmosphere. Cell culture media, phosphate buffered saline (PBS), trypsin, Hanks’ balanced salt solution (HBSS), trypsin and soybean trypsin inhibitor were all warmed to 37 °C prior to performing cell culture.

FLO-1 cells were bought from Culture Collections, Public Health England. SKGT-4 and OACM5.1C cells were kind gifts from Dr Gisella Vaas, Trinity College Dublin. OE33 cells were bought from European Collection of Cell Cultures. QhTERT and GohTERT cells were kind gifts from Dr Rabinovitch (University of Washington, WA, USA).

HET-1A cells were bought from American Type Culture Collection (LGC-standards, Middlesex, UK). HEEC cells were obtained from 3H Biomedical (Uppsala, Sweden). MDA-MB-231 cells were a kind gift from Dr Kathy Gately.

Cell Line	Base medium	Supplements added to prepare complete medium	Media Supplier
FLO-1	RPMI 1640 with UltraGlutamine or DMEM with UltraGlutamine	10% FCS	Lonza/Biosciences for FCS
SKGT-4	RPMI 1640 with UltraGlutamine	10% FCS	Lonza/Biosciences for FCS
OE33	RPMI 1640 with UltraGlutamine	10% FCS	Lonza/Biosciences for FCS
OACM5.1 C	RPMI 1640 with UltraGlutamine	10% FCS	Lonza/Biosciences for FCS
QhTERT	BEBM	BEGM SingleQuots™ growth supplement kit + 5% FCS	Lonza/Biosciences for FCS
GohTERT	BEBM	BEGM SingleQuots™ growth supplement kit + 5% FCS	Lonza/Biosciences for FCS

HET1A	BEBM	BEGM SingleQuots™ growth supplement kit except GA (gentamycin/amphotericin).	Lonza
HEEC	BEBM	BEGM SingleQuots™ growth supplement kit except GA (gentamycin/amphotericin).	Lonza
MDA-MB-231	RPMI 1640 with UltraGlutamine	10% FCS	Lonza/Biosciences for FCS

Table 2.1. Cell culture media and growth conditions

2.2 Sub culturing/passaging of Cells

- (1) The laminar flow hood was cleaned with 70% industrial methylated spirits (IMS) prior to performing cell culture. The surfaces of all materials (bottles, pipette tip boxes, etc.) to be used in the hood were also sprayed with IMS to avoid contamination. Gloved hands were also sprayed. Hands were kept gloved at all times during cell culture work, both for personal protection and to protect cells from infection.
- (2) The flask of cells to be cultured was examined under an inverted microscope to check for contamination and assess cell health and confluency. In general, cells were always split before they reached greater than 80% confluency.
- (3) Old media was removed from the cell culture flask containing the cells. Cells were washed twice with PBS. In the case of HET-1A, HEEC and QhTERT cells, HBSS was used instead of PBS.
- (4) Cells were incubated with trypsin (1ml of trypsin-versene per T75 flask) for approximately 3 minutes. The flask was then checked under an inverted microscope to check that cells were detached from the flask bottom.
- (5) 5mls of complete medium (for 1ml trypsin in a T75ml flask) were added to inhibit the trypsin. In the case of HET-1A, HEEC and QhTERT cells, 1ml of soybean trypsin inhibitor was used instead of the 5mls of complete medium.

(6) The cell suspension was transferred to a sterile 15ml conical centrifuge tube and centrifuged for 3 minutes at 1200 rpm in an Eppendorf 5810 R centrifuge. The supernatant was then carefully discarded, so as not to disturb the cell pellet. The pellet was then broken up by tapping the tube and the cells were re-suspended in 3ml of fresh complete cell culture medium.

(7) Cells were then counted (using a haemocytometer as described in detail in 2.3 below) and seeded for an experiment or were they split by adding 0.5ml (if a splitting ratio of 1/6 was required) or 1ml (if a splitting ratio of 1/3 as required) of the suspended cells into a T75 flask containing 10ml of fresh medium.

2.3 Cell counting

It is usually necessary to seed a precise number of cells into wells when setting up an experiment using cell lines. This ensures consistency between experiments. Cell numbers were therefore estimated using a haemocytometer. Steps 1 and 2 below described the protocol that was used when estimating cell numbers in this way.

(1) Cells were re-suspended in 3 ml of complete medium. 20 μ l of cell suspension was taken and added to 180 μ l of Trypan Blue dye in a 96 well plate. This was mixed well and 10 μ l was then added to the haemocytometer. Cells in the 4 squares (labelled A,B,C,D in figure 2.1 below) were counted. This was done for both the bottom and top grid of the haemocytometer.

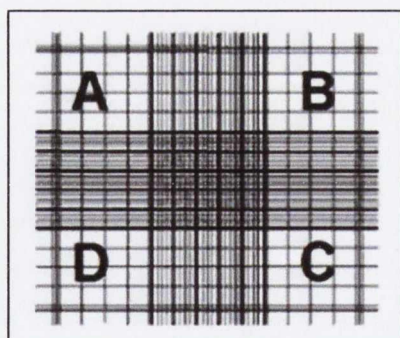


Figure 2.1. Diagram of a haemocytometer grid. The areas used to determine cell number are marked as A, B, C and D.

(2) The average number of cells per square was calculated by adding up the cells in the 8 squares and dividing by 8. The number of cells in 1 ml of suspension was then equal to the 'Average number of cells x 10^5 /ml'.

The following formula was used to calculate the volume of cell suspension required to seed a desired number of cells in a desired volume of media.

$$\text{Number of cells you want/ml} = \frac{\text{Volume of cell suspension}}{\text{Number of cell you have/ml} \times \text{Volume of cells you want}}$$

2.4 Medium and long term storage of cells.

(1) Stocks of cells were maintained by cyro-preservation. Following trypsination and centrifugation of cells as described above in 2.2, cell pellets were re-suspended in FCS with 10% dimethyl sulfoxide (DMSO) and placed in a - 80°C freezer for medium term storage. Gradual cooling of the cells (whereby temperature was reduced by approximately 1°C per minute) to -80°C was achieved using a "Mr. Frosty" controlled rate cryo-freezing container. For long term storage cells were then transferred to liquid nitrogen.

(2) After storage, cells were resuscitated as follows. Cells were defrosted rapidly in a 37°C incubator or water bath. Cells were then added to a sterile 15ml conical centrifuge tube containing 5ml complete medium and centrifuged for 3 minutes at 1200 rpm in an Eppendorf 5810 R centrifuge. The supernatant was gently discarded, the cell pellet was then re-suspended in fresh complete medium and the cells were seeded into a tissue culture flask. Following overnight incubation at 37°C, the newly resuscitated cells were washed with PBS to remove dead cells, debris and any remaining traces of DMSO.

2.5 Mycoplasma Testing

Cells were tested for mycoplasma infection using a commercially available kit (MycoAlert™ PLUS Mycoplasma Detection Kit, Lonza, Walkersville, USA). The MycoAlert™ PLUS Assay is a biochemical test that exploits the activity of certain mycoplasmal enzymes. The presence of these enzymes allows the sensitive detection of contaminating mycoplasma in a test sample. Viable mycoplasma are lysed and the enzymes react with the MycoAlert™ PLUS Substrate which catalyses the conversion of ADP to ATP. By measuring the level of ATP in a sample both before and after the addition of the MycoAlert™ PLUS Substrate, a ratio can be obtained which is indicative of the presence or absence of mycoplasma.

Cell samples to be tested for mycoplasma infection were prepared according to the kit manufacturers' detailed instructions. MycoAlert™ PLUS Reagent and MycoAlert™ PLUS Substrate were reconstituted in assay buffer and equilibrated for time 15 minutes at room

temperature. MycoAlert™ PLUS Reagent was added to the samples and incubated for 5 minutes. Luminescence was measured and recorded as reading A. MycoAlert™ PLUS Substrate was then added to the samples, incubated for 10 minutes and luminescence was measured again and recorded as reading B. The ratio of reading B/reading A was calculated and used to determine whether the sample cell culture was contaminated by mycoplasma. If mycoplasma enzymes are not present, reading B should show no increase over reading A. If mycoplasma are present, reaction of mycoplasmal enzymes with the MycoAlert™ PLUS Substrate should lead to elevated ATP levels and an increase in reading B. Therefore a reading of < 1 was interpreted as negative for mycoplasma, a reading of 1 to 1.2 was interpreted as border line and a reading of > 1.2 was interpreted as positive for mycoplasma contamination.

2.6 Cell Transfection

2.6.1 Reverse transfection of SKGT-4 or FLO-1 cells with siRNA

(1) SMARTpool siRNA was bought from Dharmacon. See Appendix 1; Table 1 for details of each siRNA used during this PhD project. The siRNA was resuspended according to the manufacturer's instructions. Briefly, the tube containing the siRNA was centrifuged to ensure that the siRNA pellet was collected at the bottom of the tube. RNase-free 1X siRNA Buffer was then added to the siRNA pellet to give a stock concentration of 20µM. The siRNA/siRNA buffer was pipetted up and down 3-5 times, avoiding the introduction of bubbles, and then placed in an orbital mixer/shaker for 30 minutes at room temperature. Tube containing the siRNA was centrifuged briefly to ensure that the solution was collected at the bottom of the tube. Aliquots of this 20µM stock were then made and stored at - 20°C.

(2) Cells were washed, trypsinized and counted as described above in 2.2. A suspension of cells at a concentration of 80 000 cells/ml was then prepared in fresh complete medium.

(3) A six well tissue culture plate was propped up at an angle in the laminal flow hood on a 10ml serological pipettes. 250 µl of Opti-MEM reduced-serum medium was added to the lowest portion of each tilted well. 3.75µl of 20µM siRNA stock was added to the 200 µl of Opti-MEM in the well. (4) The plate was tilted back and forward gently to mix the siRNA and Opti-MEM. It was then propped back up on the 10ml pipette and 7.5µl of Lipofectamine RNAiMAX (Invitrogen) was added to each well to be transfected. The plate was again tilted back and forth gently to mix the reagents and then propped back up on the 10ml pipette and left to incubate at room temperature for exactly 5 minutes.

(5) 2250µl of the cell suspension prepared in (2) above was added to the Opti-MEM/siRNA/lipofectamine mixture, the plate was tilted gently to mix and then incubated in the 37° C incubator. This gave a final concentration of 180 000 cells/well with 30µM siRNA. Experiments using the transfected cells were performed at 48-96 hours post transfection at which point cell lysates were also simultaneously prepared to assess % depletion (knockdown efficiency) of the target protein.

Note: In some cases siRNA transfection was also performed in 12 well plates. In these cases the same protocol as that described above was used and quantities were scaled down accordingly.

2.6.2 Forward transfection of FLO-1 cells with plasmid DNA

Note: See Appendix 1; Table 1 and Appendix 1; figure 1 for details of each DNA plasmid used during this PhD project.

Day 1: Cells were seeded at a density of 25, 000 cells/well in 24-well cell culture plates.

Day 2: For each well to be transfected, 1µg of plasmid DNA was added to 50µl Opti-MEM reduced serum medium (Tube A). For each well to be transfected, 3µl of Eugene HD Transfecting reagent (Promega) was added to 50µl of Opti-MEM (Tube B). Tube A and Tube B were left to incubate at room temperature for 5 minutes. The contents of Tube A and Tube B were then combined and left to incubate together for 20 minutes. (Complex C).

Media was removed from cells in the 24well plate seeded on Day 1 and replaced with 500µl fresh serum free media/well. 100ul of Complex C was then gently added in a dropwise fashion to each well to be transfected. The plate of cells was then placed back into a 37C ° incubator.

Day 3: At 24hours post transfection the cells were washed with PBS and the serum free cell culture media was replaced with complete media.

Note 1: In the case of transfection with pCMV-mCherry-NMHCIIA plasmid DNA transfection efficiency using the above protocol was poor. The protocol was therefore modified such that 2µg (instead of 1µg) of plasmid DNA and 6µl Eugene HD (instead of 3µl) were added per well.

Note 2: In the case of a co-transfection of cells with two plasmids, 1µg of DNA for each plasmid was added per well; i.e. 2 µg of DNA in total was added per well. In this situation 6µl of Eugene HD was added per well.

In the case of co-transfection with pCMV-mCherry-NMHCIIA and a second plasmid, 2µg of pCMV-mCherry-NMHCIIA DNA + 1µg of the second plasmid was added to give a total of 3µg of plasmid DNA per well. In this case, 9µl of Eugene HD was used per well.

2.7 Scratch wound two dimensional (2D) cell migration assay

SKGT-4 or FLO-1 cells were seeded at a density of 50,000 cells/well in 96 well plates and allowed to form fully confluent monolayers. A single scratch wound was then created in the middle of each well using a p200 pipette tip. Cells were then gently washed once and new complete media was added to each well. In the case of scratch wound assays to assess the impact of RACK1 or NMHCIIA depletion in FLO-1 cells, the new complete media that was added post wounding also contained 50ng/ml HGF to help promote cell migration. The scratch wounds were imaged immediately after wounding (time zero) using the Cellavista imaging platform. Subsequent images of the scratch wounded wells were taken at 10 hours, 14 hours and 24 hours post wounding. The size of each wound at each time point was quantified using the 'plaque assay' tool included in the Cellavista software (Roche, Basel, Switzerland). The area of wound which had been filled by migrating cells was then calculated by subtracting the size of the wound at a given post-wounding time point from the original wound area recorded immediately after wounding at time zero.

2.8 Three dimensional (3D) cell migration assay using the xCELLigence real time cell analysis (RTCA) platform

Cell migration was monitored in real-time in xCELLigence CIM-Plate-16 using the xCELLigence RTCA platform according to manufacturer's instructions. Briefly;

(1) FLO-1 cells (which had been transfected 48 hours previously with the appropriate siRNA – e.g. siControl or siRACK1 or siNAMPT) were serum starved for four hours prior to setting up the experiment.

(2) Each side of the upper chamber of the CIM plate was coated with 1µg/µl of fibronectin.

(3) The X-CELLigence RTCA CIM plate cell migration assay was then set up according to the manufacturer's instructions. Briefly, 160µl of complete media was added to each lower chamber of the CIM plate, while avoiding bubbles and ensuring that a clearly defined meniscus

was formed in each well. The upper and lower chambers were then locked together and 30 μ l of serum free media was added to the upper chamber.

(4) The CIM-Plate was then loaded onto the RTCA DP Analyzer and allowed to equilibrate for an hour. A background measurement was then performed using the xCelligence software. This measured the background impedance of cell culture media and the data obtained were subsequently used as reference impedance for calculating Cell Index values.

(5) Suspensions of FLO-1 cells at a concentration of 200,000cells/ml were prepared in serum free media. 100 μ l of cell suspension was added to each well of the CIM plate upper chamber to give 20000 cells per well. The CIM plate was then left for 30 minutes at room temperature to allow the cells to settle down to the upper side of the membrane located in the bottom of the upper chamber.

(6) The CIM plate was then loaded into the RTCA DP Analyzer. The xCELLigence system then monitored cell migration by measuring the impedance values of each well every 15 minutes for a period of 60 hours. Impedance values were expressed as Cell Index values.

2.9 Cell proliferation assay using the xCELLigence real time cell analysis (RTCA) platform

Cell proliferation was monitored in real-time in xCELLigence E- Plates using the xCELLigence RTCA platform according to manufacturer's instructions. Briefly;

(1) 100 μ l of complete cell culture media was added to each well of the E-Plate. The E-plate was then left in the tissue culture hood for 30 minutes at room temperature to ensure that the culture media and E-Plate surface achieved equilibrium. A background measurement was then performed using the xCelligence software. This measured the background impedance of cell culture media and the data obtained were subsequently used as reference impedance values for calculating Cell Index values.

(2) A cell suspension of FLO-1 cells (which has been transfected 48 hours previously with the appropriate siRNA) was prepared at a concentration of 200,000 cells/ml in complete medium.

(3) 100 μ l of cell suspension was added to each well of the E-Plate so as to give 20,000 cells per well. The E plate was then left for 30 minutes at room temperature to allow the cells to settle to the bottom of the wells.

(4) The E-Plate was then loaded into the RTCA DP Analyzer. The xCELLigence system then monitored cell proliferation by measuring the impedance values of each well every 15 minutes

for a period of 80 hours. Impedance values were expressed as Cell Index values. Measurements taken for the first 6-8 hours were more reflective of cell adhesion than cell proliferation. Therefore, the rate of cell proliferation was determined by analysing the slope of the line between the 8 and 48 hour interval.

2.10 Nuclear count assay to assess cell proliferation.

SKGT-4 cells were transfected with appropriate siRNA (e.g. siControl or siRACK1). At approximately 24 hours post transfection cells were re-seeded at a density of 6000 cells/well into two 96 well plates; one plate was labelled 'T42 hour cell count' and the other was labelled 'T96 hour cell count'.

At 42 hours post transfection, cell permeable Hoechst 33342 dye (Invitrogen) diluted 1/10,000 in cell culture media was added to cells in the plate labelled 'T42 hour cell count'. The plate was incubated at 37° for 30 minutes and was then imaged using the 'Nuclear Count Assay' application of the Cellavista cell imaging system (Roche, Basel, Switzerland). Whole well images were obtained for each well and cell number was determined by counting the number of Hoechst stained nuclei. At 96 hours post transfection, cell numbers in the plate labelled 'T96 hour cell count' were determined using the same method. The resultant data was then used to determine the increase in cell number between 42 and 96 hours post transfection.

2.11 Cell confluency assay

SKGT-4 cells were transfected with appropriate siRNA (e.g. siControl or siRACK1). At approximately 24 hours post transfection cells were re-seeded at a density of 6000 cells/well in a 96 well plate. At 72 hours post transfection bright field whole well images of the wells were taken using the Cellavista cell imaging system (Roche, Basel, Switzerland). % cell confluency was then measured by measuring the % surface area of tissue culture plates that was covered by the cells using the 'Confluency' application included in the Cellavista software.

2.12 Methylthiazolyldiphenyl-tetrazolium (MTT) assay to assess cell viability/proliferation

SKGT-4 or FLO-1 cells were transfected with appropriate siRNA in 12 well plates at a density of 38,000 cells/well.

Methylthiazolyldiphenyl-tetrazolium (MTT) powder was purchased from Sigma (Cat. No M5655).

A 5mg/ml MTT solution was prepared in PBS and filter sterilized in the laminal flow hood.

At 65-72 hours post transfection, cells were washed gently with PBS. 1ml of fresh complete medium was then added to each well along with 200µl of 5 mg/ml MTT in PBS. The cells were then placed back into the 37°C incubator but were checked regularly under an inverted microscope for the appearance of purple crystals which occurred at approximately 1-3 hours after MTT addition. At this point cell culture containing MTT reagent was carefully removed and cells were solubilized by adding 1ml of DMSO per well. This produced a purple colour in the wells which was quantified by measuring absorbance at 570nm using an ELx800 Absorbance Plate Reader (BioTek).

2.13 Assay to quantify impact of NMHCIIA depletion on F-actin organisation

FLO-1 cells were transfected with anti-NMHCIIA siRNA or control siRNA. 24 hours later cells were reseeded into 96 well plates. At 84 hours post transfection cells were washed twice with PBS and then fixed with 4% PFA in PBS for 15minutes at room temperature. Cells were then permeabilized for 10 minutes with 0.3% Triton X-100 in PBS, blocked for 30 minutes with 3% BSA in PBS and then incubated with phalloidin-TRITC (which stains F-actin) and a rabbit anti-NMHCIIA primary antibody (1/100 dilution) in 3% BSA in PBS overnight at 4°C in the dark. Cells were then washed twice x 5 minutes with PBS-0.01% Tween and were then incubated with an AlexaFluor goat anti-rabbit 488 fluorescently-labelled secondary antibody at a 1/500 dilution for two hours at room temperature in the dark. Cells were then washed twice x 5 minutes with PBS-0.01% Tween and wells were then filled with PBS. The 96 well plate containing the stained cells was then imaged using the InCell 1000 High Content Cell Analysis platform.

Cells with 4 or more 'very thick stress fibres' were counted and expressed as a percentage of total cells in the field of view under analysis. 'Very thick stress fibres' were defined as those stress fibres whose width exceeded an arbitrarily set length measured using ImageJ (NIH free download).

2.14 Treatment of cells with deoxycholic acid (DCA)

DCA was purchased from Sigma and was solubilised in DMSO (Sigma). Exposure of SKGT-4 and FLO-1 cells to DCA was performed in RPMI media with 10% foetal calf serum supplementation throughout the time course under study as this produced a more physiologically relevant setting than that which could be achieved under conditions of serum starvation. The concentrations and time periods used when treating cells with DCA are indicated in figure legends. Matched time point controls treated with vehicle (DMSO) were also conducted to normalise data for time or vehicle effects.

2.15 Live cell imaging of fluorescent proteins

2.15.1 Image Acquisition

During live cell imaging cells were maintained at 37°C on a heated stage in 35mm MatTek dishes (MatTek Corporation) in Ringer's buffer (155 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 2 mM NaH₂PO₄, 10 mM glucose and 10 mM HEPES at pH 7.2) supplemented with 10% FCS Serum and 2mM GlutaMAX (Life Technologies).

Images were acquired on a Nikon Eclipse TE-300 inverted fluorescence microscope with a 60X 1.4 PlanApo oil immersion objective lens, excitation and emission filters in filter wheels, dichroic mirrors that allowed simultaneous detection of multiple fluorophores, a temperature-controlled stage, shutters for both phase-contrast and epifluorescence illumination and a cooled digital charge-coupled camera (Quantix; Photometrics). The source of epifluorescent light was a 75 W mercury arc lamp.

All of the above were controlled with MetaMorph image processing software (Molecular Devices).

2.15.2 Image Processing

MetaMorph software (Molecular Devices, Sunnyvale, CA) was used for image processing. Fluorescent images were background bias-subtracted and shading-corrected using the "Correct Shade" tool in MetaMorph, which performs the shading correction as $\text{Corrected Image} = (\text{Max value of Shade Image}) * (\text{Acquired Image} - \text{Background}) / (\text{Shade Image} - \text{Background})$.

(Note; In the equation above MetaMorph the single term 'background' is used by MetaMorph to refer to 'bias' or 'background bias')

The background bias image was a 20-frame average of the camera bias, taken in identical conditions as for imaging, but with the excitation light blocked. The shade image was collected from a 20-frame average of images of a glass slide impregnated with green or red fluorescent dye.

Background bias corrections corrects for dark current of camera while shading correction controls for variation in illumination across an image plane.

In the case of some mCherry images, an additional shading correction was required to control for additional variation in illumination across the image plane when imaging glass MatTek

chambers at 580nm excitation. This correction was performed as (Acquired image) - (Shade image taken in presence of a MatTek chamber).

Background noise was removed from images by using the thresholding function of MetaMorph to remove signal below a manually chosen threshold.

2.15.3 Ratiometric Fluorescent Microscopy

2.15.3.1 Fluorescent ratiometric imaging of RACK1-EGFP

Live cells co-expressing RACK1-EGFP and free mCherry were imaged as described in 2.15.1 above. Chimeric EGFP should localise according to the protein (RACK1) which it is linked to while free mCherry serves as a general marker of soluble cytosol. Images of free mCherry were collected with 580 nm excitation and 630nm emission. Images of chimeric EGFP were collected with 485nm nm excitation and 525nm emission. Phase contrast images were also collected.

Images were corrected as described in 2.15.2 above. The corrected EGFP and mCherry images were then added and a manual threshold was applied to the ADD image. This threshold was then used to create a binary mask which was then taken as a 'logical AND' with each of the corrected EGFP and mCherry images.

Finally, ratio images of RACK-EGFP were obtained by dividing the fully corrected EGFP images by the corresponding mCherry images. Such ratio images report the localisation of the RACK1-EGFP chimera normalized to cell volume and thus corrected for variation in optical path length due to cell shape.

A pseudocolour scale was applied to the ratio images using imageJ where low ratio values (and hence low RACK1 concentrations) were represented by cool colours and high ratio values (and hence high RACK1 concentration) were represented by warm colours. Specifically, 'lookup table '16_colors' in ImageJ (NIH, free download) was the pseudocolour scale which was used. The pseudo coloured ratio images were then overlaid on corresponding phase contrast images to aid identification of subcellular locations/structures with high or low RACK1 concentration.

2.15.3.2 Fluorescent ratiometric imaging of NMHCIIA-mCherry

Live cells co expressing NMHCIIA-mCherry and free EGFP were imaged as described in section 2.15.1. Chimeric mCherry should localise according to the protein (NMHCIIA) to which it is linked while free EGFP serves as a general marker of soluble cytosol. Images of chimeric mCherry were collected with 580 nm excitation and 630nm emission. Images of free EGFP

were collected with 485nm excitation and 525nm emission. Phase contrast images were also collected.

Images were corrected as described in section 2.15.2. The corrected EGFP and mCherry images were then added and a manual threshold was applied to the ADD image. This threshold was then used to create a binary mask which was then taken as a 'logical AND' with each of the corrected EGFP and mCherry images.

Lastly, ratio images of NMHCIIA-mCherry were obtained by dividing the fully corrected NMHCIIA-mCherry images by the corresponding free EGFP images. Such ratio images report the localisation of the NMHCIIA-mCherry chimera normalized to cell volume and thus corrected for variation in optical path length due to cell shape.

A pseudo colour scale was applied to the ratio images using imageJ where low ratio values (and hence low NMHCIIA concentrations) were represented by cool colours and high ratio values (and hence high NMHCIIA concentration) were represented by warm colours. Specifically, 'lookup table '16_colors' in ImageJ was the pseudocolour scale which was used. The pseudo coloured ratio images were then overlaid on corresponding phase contrast images to aid identification of subcellular locations/structures with high or low NMHCIIA concentration.

2.15.4 Förster Resonance Energy Transfer (FRET) Sensitized Emission Microscopy

EGFP-RACK1 and NMHCIIA-mCherry were co expressed in FLO-1 cells and served as FRET donor and acceptor, respectively. Cells, which has successfully co-expressed both recombinant proteins, were identified and imaged as described in section 2.15.1 Four images were acquired for each cell, namely;

Phase-contrast

IA was collected at the acceptor excitation wavelength and the acceptor emission wavelength; i.e. it was collected with 580 nm excitation and 630nm emission.

ID was collected at the donor excitation and the donor emission; i.e. it was collected with 485nm excitation and 525nm emission.

IF was collected at the donor excitation wavelength and the acceptor emission wavelength; i.e. it is collected at 485nm excitation and 630 nm emission.

Images were corrected as described in 2.15.2. MetaMorph was then used to further process the images for FRET determination as follows. Firstly, the corrected ID and IA images were

added and a manual threshold was applied to the ADD image. Next, a single binary mask was created using this threshold. The binary mask was then taken as a logical AND with each of the corrected IA, ID and IF images. The resultant masked images were then used to generate corrected FRET images using the equation below;

$$\text{Corrected FRET image} = I_f(100) - ((\alpha * I_A) + (\beta * I_D)) * 1000$$

(equation 1; see below for determination of parameters alpha and beta)

Corrected FRET images were pseudo coloured for display in figures using the '16_colour lookup table' in imageJ look up tables. This lookup table incorporates a pseudocolour scale which uses cool colours to represent regions of low FRET signal and warm colours to represent regions of high FRET signal. The pseudo coloured FRET images were then overlaid on corresponding phase contrast images to aid identification of subcellular locations/structures with low or high FRET signal.

2.15.4.1 Determination of the FRET calibration parameters alpha and beta

The FRET signal can be contaminated in two significant ways which must be corrected for when producing FRET images. Donor fluorophore emission into the acceptor channel can occur which contaminates the FRET signal and may be termed 'spectral bleed through'. The FRET signal is also contaminated by 'cross-excitation' whereby direct excitation of the acceptor fluorophore by donor excitation occurs. Both phenomena were corrected for by conducting two additional calibration experiments along with the actual FRET experiments. Such correction methods has been previously described by others (Elder et al., 2009) (Hoc and Phillips, 1997). Specifically, cross excitation was determined by collecting IF and IA images from 25 control cells expressing free mCherry only (i.e. acceptor fluorophore) only. These images were corrected for bias, shade and background as described in 2.15.2. Approximately 20 control cells were imaged and the corrected and averaged IF and IA images were then used to calculate the calibration parameter alpha according to equation 2 below;

$$\alpha = I_F / I_A \text{ (equation 2)}$$

In my experimental system a value of 0.0481 was determined for alpha.

Spectral bleed through was determined by collecting IF and ID images from control cells which expressed free EGFP (i.e. donor fluorophore) only. These images were corrected for bias, shade and background as described in 2.15.2. Approximately 20 control cells were imaged and

the corrected and averaged values for IF and ID and were then used to calculate the calibration parameter beta according to equation 3 below

$\text{beta} = \text{IF}/\text{ID}$ (equation 3)

In my experimental system a value of 0.0515 was determined for beta.

Alpha and beta were then used in equation 1 above to correct for bleed through and cross excitation and thus calculate the true FRET contribution (termed corrected FRET in equation 1) in the acceptor channel.

2.16 Immunofluorescent staining and confocal microscopy

Cells were grown on sterile glass coverslips placed into the bottom of 24 well cell culture plates.

Cells were washed, fixed for 15 minutes with 4% paraformaldehyde in PBS, washed X 3 with PBS, permeabilised for 10 minutes with 0.3% Triton X-100 in PBS, blocked for 30 minutes with 3% BSA in PBS before incubation with primary antibody in 3% BSA overnight at 4°C or for two hours at room temperature. Coverslips were then washed X 2 for 5 minutes in PBS with 0.01% Tween and primary antibodies were detected with appropriate Alexa-Flour fluorescently labelled secondary antibodies which were incubated for 1 hour at room temperature in the dark. Controls with secondary antibody only were also employed. In some cases, cells were also stained with Hoechst nuclear stain (1/5000). The cells were visualised using a Zeiss LSM confocal microscope. Antibodies used are detailed in Appendix 2; Table1.

In the case of NAMPT immunofluorescent staining, an additional Antigen Retrieval step was employed after fixation and prior to cell permeablization. Antigen retrieval as performed as follows;

- (i) Antigen retrieval buffer (100 mM Tris, 5% [w/v] urea, pH 9.5) was pre-heated to 95°C. This was done by heating the buffer in a heat-proof glass slide-staining tray in an oven set to 95°C.
- (ii) Using a broad-tipped forceps, the coverslips of fixed cells were carefully placed into the hot antigen retrieval buffer, making note of which side of the coverslips the cells are on. The tray of buffer and coverslips was placed back into the oven and heated at 95°C for 10 minutes.
- (iii) The coverslips were removed from the antigen retrieval buffer and immersed, with the side containing the cells facing up, in PBS, in 6-well tissue culture plates.
- (iv) Cells were washed in PBS three times for 5 minutes.

2.17 NAD Assay to measure cellular NAD concentration in NAMPT-depleted or RACK1-depleted cells.

Cells were transfected with control siRNA, anti-RACK1 siRNA or anti-NAMPT siRNA. Cells were harvested at 72 hours post transfection. One 50% confluent well of cells from a six well cell culture plate was harvested for each cell population to be tested.

The NAD concentration in each population were then measured using a commercially available NAD/NADH Colorimetric Assay Kit (ab65348) supplied by Abcam according to the manufacturer's detailed protocol instruction booklet.

2.18 Preparation of cellular protein extracts

Cellular protein extracts were prepared by washing cells twice with ice cold PBS followed by incubation at 4 °C for 20 minutes in cell lysis buffer composed of 10 mM Tris-HCl, pH 7.4, 150 mM NaCl and 1% Igepal CA-630 (Nonidet P-40) supplemented with freshly added Sigma protease inhibitor cocktail (P8340) (1/100 dilution) and Sigma phosphatase inhibitor cocktail 2 (P5726) and Sigma phosphatase inhibitor cocktail 3 (P0044) both used at a 1/500 dilution.

Following incubation, nuclear and cellular debris were removed by microcentrifugation at 10,000 rpm for 5 minutes at 4 °C. The supernatant was carefully removed, taking care not to disturb the pellet of debris, and added to a clean Eppendorf tube. It was then prepared for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) or stored at – 20 °C.

2.19 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE)

(1) Protein concentrations were determined by BCA assay (Pierce) according to the manufacturer's protocol.

(2) Protein samples were then prepared for sodium dodecyl gel electrophoresis by boiling for 5 minutes in sample buffer; two different sample buffers were employed during this PhD project and were prepared according to the protocols below:

SDS-PAGE sample buffer (1X)

Combine: 1.51 g Trizma
 20 ml Glycerol
 35 ml dH₂O

Adjust the pH to 6.8 with HCl and then add:

4g SDS powder (Sigma catalogue L-5750) (Wear a face mask and take caution when weighing)

0.002g bromophenol blue (Sigma catalogue B-0126)

Add distilled water to 180ml. Store at room temperature.

Important: Add fresh DTT to 100mM final concentration immediately before use!

Laemmli Sample Buffer (5X)

(60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue)

Note: Wear a mask weighing SDS and handle β-mercaptoethanol in a ventilated fume hood.

Required reagents:

4ml 1.5M Tris-HCL pH 6.8

10ml glycerol

5ml β-mercaptoethanol

2g SDS

1ml bromophenol blue

Add 1 ml of 1% bromophenol blue to 4 ml of 1.5 M Tris-Cl pH 6.8.

Add 10ml of glycerol and mix.

Add 2 g of SDS and mix (the SDS will take a few minutes to dissolve).

Add 5 ml of β-mercaptoethanol and mix.

Aliquot and store at -20°C.

(3) The protocol below was used to resolve proteins by SDS PAGE. It was composed by Dr Michael Freeley and is based on the Atto gel electrophoresis system.

- i. Rinse electrophoresis plates with ethanol
- ii. Attach rubber gaskets to notched plates, add the plain glass plate and fasten the green clips to each side.
- iii. Add a comb between the plates and mark the glass 1 cm down from the bottom of the comb
- iv. Remove comb and prepare 10 % acrylamide *resolving gel* in the following order as shown

Component	Volume
dH ₂ O	7.9 ml
30 % Acrylamide	6.7 ml
1.5 M Tris-HCl (pH 8.8)	5.0 ml
10 % w/v SDS	200 µl
10 % w/v APS	200 µl
TEMED	8 µl

(the above resolving gel is enough for two complete gel plates)

- v. Quickly mix the resolving gel solution and pour between the glass plates up to the 1 cm mark
- vi. Overlay the resolving gel with 1 ml of isobutanol or isopropanol
- vii. Allow the gel to set for approximately 40 minutes at room temperature

- viii. Remove isobutanol/isopropanol and wash the top of the gel with dH₂O
- ix. Remove any excess water with paper towel
- x. Prepare *stacking gel* in the following order as shown

Component	Volume
dH ₂ O	3.4 ml
30 % Acrylamide	0.83 ml
1.0 M Tris-HCl (pH 6.8)	0.63 ml
10 % w/v SDS	50 µl
10 % w/v APS	50 µl
TEMED	5 µl

- xi. Mix the stacking gel quickly and pipette between glass plates until solution reaches the top of the plate
- xii. Add combs (be careful to exclude any air bubbles from stacking gel)
- xiii. Allow stacking gel to set for approximately 40 minutes at room temperature
- xiv. Remove the green clips and rubber gaskets and place the glass plates into the electrophoresis rig (the shorter, notched glass plates should be facing each other on the inside of the electrophoresis rig). Fasten the plates with the clear plastic holders
- xv. Prepare 1X SDS-PAGE running buffer (= 80 mls of 5X SDS-PAGE running buffer and 320 mls dH₂O) and pour the running buffer into the electrophoresis rig and between the glass plates in the centre of the electrophoresis rig.
- xvi. Remove combs carefully (you can mark the bottom of wells with a marker before you remove the combs as it makes them easier to see), straighten any crooked wells with a gel-loading tip and wash each well by pipetting up and down.

- xvii. Load protein samples (approx. 20 μ l) and molecular weight markers (approx. 5 – 10 μ l) (both of these must be boiled previously for 5 mins) into the wells.
- xviii. Attach the leads and run gel at 200V and 40 mAmps until the blue dye reaches the bottom the gel (approximately 1.5 hours).

Solutions required

30 % acrylamide (Sigma catalogue A3699)

Store at 4 degrees

10 % w/v ammonium persulphate (10 % APS) (Sigma catalogue A3678)

Dissolve 0.1g APS in 1 ml dH₂O

Store at 4 degrees

10 % APS must be prepared fresh weekly!

1.5 M Tris-HCl (pH 8.8) (Sigma catalogue T-1503)

Dissolve 90.825g Trizma in 400 ml dH₂O

Adjust pH to 8.8 with HCl and add dH₂O to 500 ml

Store at room temperature

1.0 M Tris-HCl (pH 6.8) (Sigma catalogue T-1503)

Dissolve 60.55g Trizma in 400 ml dH₂O

Adjust pH to 6.8 with HCl and add dH₂O to 500 ml

Store at room temperature

10 % w/v SDS (Sigma catalogue L-5750)

Dissolve 10g of SDS in 100 ml dH₂O

Wear a face mask when weighing out as the powder creates lots of dust and is toxic (Consult MSDS)

TEMED (Sigma catalogue T-9281)

Store in the fume-hood as TEMED is pungent. Add TEMED to the resolving/stacking gel in the fume-hood

Isobutanol (Sigma catalogue 58448)

Store in the fume-hood or in solvent cupboard

5X SDS-PAGE running buffer

15.1 g TRIZMA (Sigma catalogue T-1503)

94 g Glycine (Sigma catalogue G-8898)

50 mls of 10 % w/v SDS

dH₂O to 1 litre

Dilute to 1X before use with dH₂O (=80 mls of 5X buffer and 320 mls dH₂O)

Molecular weight markers (Sigma catalogue SDS-6H)

Reconstitute powder with SDS-PAGE sample buffer according to the instructions and aliquot 5-10 uL into tubes and store at -20 degrees

2.20 Western blotting

The following protocol was used for Western blotting. It is adapted from a protocol written by Dr Michael Freeley and is based on the Atto semi-dry technique.

- (1) While the SDS-PAGE gel is running cut 4 sets of filter paper (5 sheets in each set) and 2 pieces of PVDF membrane (6.5 cm x 9.2 cm for both filter paper and PVDF)
- (2) Activate the PVDF membrane by soaking in methanol for 30 seconds and then place it in Western blot transfer buffer
- (3) When the SDS-PAGE gel has run, prise apart the plates, remove the stacking gel with a scalpel and discard the stacking gel
- (4) On the Atto Western blot transfer system, soak 1 set of filter papers in Western blot transfer buffer and place on the bottom of the rig
- (5) Place the activated PVDF membrane on top of the filter papers and add more Western blot transfer buffer on top
- (6) Take the SDS-PAGE gel, immerse it in Western blot transfer buffer briefly and place it on top of the PVDF membrane (exclude any air bubbles)
- (7) Complete the sandwich by adding 1 set of filter papers (previously soaked in Western blot transfer buffer) on top of the SDS-PAGE gel
- (8) Remove any excess transfer buffer on the bottom of the rig with paper towel
- (9) Lower the top piece of the transfer system onto the sandwich, connect the leads and run at 100 V and 100 mAmps per gel sandwich for 1 hour (the system can accommodate two gel sandwiches)

The following procedure to detect antigens on the PVDF membrane with antibodies is described below and is a general protocol. Each antibody may have specific incubation conditions and buffers so consult the antibody data sheet as they may not be the same as described below.

- (10) Remove filter papers on top of gel sandwich and also remove SDS-PAGE gel and discard it
- (11) Cut off molecular weight marker lane with a scalpel and incubate with Coomassie stain solution for 5 minutes. Destain with Coomassie destaining solution.
- (12) Mark any relevant lanes on the PVDF membrane with a pencil and incubate with Western blot blocking buffer for 1 hour at room temperature on a shaker. *From this point on, do not let the PVDF membrane go dry!*
- (13) Wash the PVDF membrane three times with Western blot wash buffer (5 minutes

washes) at room temperature

- (14) Incubate PVDF membrane with primary antibody (diluted according to manufacturer's instructions) in Western blot blocking buffer overnight at 4 degrees with gentle agitation
- (15) Wash the PVDF membrane three times with Western blot wash buffer (5 minutes washes) at room temperature
- (16) Incubate PVDF membrane with HRP-labelled secondary antibody (diluted according to manufacturer's instructions) in Western blot blocking buffer for 1-2 hours at room temperature with gentle agitation
- (17) Wash the PVDF membrane three times with Western blot wash buffer (5 minutes washes) at room temperature
- (18) Prepare fresh HRP development solution: (100mM Tris-HCl, pH 8.5, 0.009% H₂O₂, 1.2mM luminol, 1.9mM IBPA).
- (19) Add a few mls of HRP development solution to the membrane for 1-2 minutes and then image the blot using the 'Chemiluminescence' application on a FUSION gel imaging system (Vilber Lourmat).
- (20) Densitometry can be performed on blots, if required, using ImageJ image analysis software (free download, NIH).

Reagents required:

Western blot transfer buffer

48 mM Trizma Base, 39 mM Glycine, 0.04 w/v SDS and 20 % Methanol

5.8 g Trizma base (Sigma catalogue T-1503)

2.9 g Glycine (Sigma catalogue G-8898)

0.37 g of SDS powder (Sigma catalogue L-5750) or 8 mls 10 % w/v SDS

200 ml Methanol

dH₂O to 1 L

Coomassie stain solution

2.5 g Coomassie brilliant blue G-250 (Bio-Rad; catalogue number 161-0406)

450 ml dH₂O

450 ml Methanol

100 ml glacial acetic acid

Store at room temperature

Coomassie destaining solution

450 ml dH₂O

450 ml Methanol

100 ml glacial acetic acid

Store at room temperature

Western blot blocking buffer

5 % w/v milk powder (Marvel) in Western blot wash buffer (TBS-T)

Western blot wash buffer

1X TBS with 0.1% v/v Tween-20 (TBS-T)

To make 10X TBS:

24.2g Trizma base (Sigma catalogue T-1503)

80g NaCl (Sigma catalogue S-7653)

900 ml dH₂O

pH solution to 7.6 with HCl

Add dH₂O to 1L.

Store at room temperature and dilute to 1X with dH₂O before use

2.21 RACK1 immunoprecipitation/co-immunoprecipitation

Except for where it is otherwise indicated, the protocol described below in 2.21.1 was used for RACK1 immunoprecipitation (IP) experiments throughout my PhD project.

2.21.1 Protocol for RACK1 immunoprecipitation from FLO-1 cells using protein G agarose beads

RACK1 cell lysis buffer: 10 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% Igepal CA-630 (Nonidet P-40) plus freshly added sigma protease inhibitor cocktail (1/100) plus sigma phosphatase inhibitor cocktail 2 (1/500) plus sigma phosphatase inhibitor cocktail 3 (1/500).

RACK1 IP buffer: 1 mM EGTA, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1% deoxycholate plus freshly added sigma protease inhibitor cocktail (1/100) plus sigma phosphatase inhibitor cocktail 2 (1/500) plus sigma phosphatase inhibitor cocktail 3 (1/500).

Cell lysis:

1. Wash cells x2 with ice cold PBS
2. Add appropriate amount of ice cold RACK1 cell lysis buffer to flask of cells and incubate flask on ice on a rocker for 20mins.
3. Scrape cells from flask bottom using cell scraper and transfer to 1.5 ml Eppendorf tube. Centrifuge at 10000rpm in microcentrifuge @4°C for 5minutes to remove unlysed cells, nuclei and detergent-insoluble material. Remove supernatant to a clean tube and proceed to co-IP.

Immunoprecipitation:

1. Determine protein concentration of cell lysate obtained above using BCA assay.
2. Add the following to a 1.5ml Eppendorf tube and then incubate tubes overnight on a rotating wheel at 4°C;
 - Cell lysate to give 700ug of protein
 - 3ug anti-RACK1 IgM (BD transduction laboratories) or control anti-mouse IgM (IgM Isotype Control from murine myeloma from Sigma)
 - 5 µg goat anti-mouse IgM Fab fragment (Jackson ImmunoResearch)
 - 30ul (packed volume) protein G agarose beads
 - 500ul RACK1 IP buffer
3. Recover complexes by microcentrifugation (1000xg, 2mins @4°C). Carefully remove and discard supernatant using a syringe (syringe allows all of the supernatant to be removed while avoiding loss of beads).

4. Wash complexes with 1ml of RACK1 IP buffer and rotate for 5mins @ 4°C. Transfer to fresh tubes. Centrifuge at 1000xg for 1min @ 4°C and remove supernatant without removing beads using a syringe. Repeat two more times but do not change tubes during the second and third wash.

If analyzing the complexes by SDS PAGE and Western blotting go to step 5 W.B. at this point. If analyzing the IPs by mass spectrometry proceed to 5 M.S.

5. **M.S.** Wash complexes with 1ml of ice-cold PBS and rotate for 5mins @ 4°C. Centrifuge at 1000xg for 1min @ 4°C and remove supernatant with syringe without removing beads. Repeat one more time. Proceed to on bead digestion in preparation for mass spectrometry.

5. **W.B.** Re-suspend bead complexes in 2X SDS-PAGE sample buffer and heat for 5 mins at 100°C (to break the antibody and antigen from the beads). Vortex the samples

6. **W.B.** Remove beads by centrifugation (10,000 rpm, 1 min, 4°C). Save the supernatant and transfer it to a new tube. Load the supernatant onto an SDS-PAGE gel.

2.21.2 RACK1 immunoprecipitation from FLO-1 cells using Dynabeads

This alternative method for RACK1 immunoprecipitation was occasionally used during my PhD project. A Dynabeads® Protein G Immunoprecipitation kit (Life Technologies; Cat. no. 10007D) was employed according to the manufacturer's instructions with the following alternation; incubation of the cell lysate with the Dynabead-Antibody complex was performed for 2 hours at 4 °C in contrast to the 10 minutes at room temperature which was prescribed by the manufacturer.

Cell lysate containing 700 µg of protein was used per Dynabead IP. Each IP used 3µg of anti-RACK1 IgM (BD transduction laboratories) or control anti-mouse IgM (IgM Isotype Control from murine myeloma from Sigma) and 5 µg goat anti-mouse IgM Fab fragment (Jackson ImmunoResearch).

2.22 Mass spectrometry based proteomics screen of RACK1 immunoprecipitates.

On bead digestion of the RACK1 IPs to be analysed was performed using the method described by Turriziani *et al.*, 2014. Sample clean-up was performed using c18 ziptips (Millipore) and the samples were then run on a Thermo Scientific Q 28 Exactive mass spectrometer connected to a Dionex Ultimate 3000 RSLCnano chromatography system. Tryptic peptides were resuspended in 0.1% formic acid. Each sample was loaded onto a fused silica emitter (75 μ m ID, pulled using a laser puller (Sutter Instruments P2000 Novato, CA, USA)), packed with ReproCil Pur C18 (1.9 μ m) reverse phase packing material (Dr Maisch, Ammerbuch-Entringen, Germany) and was separated by an increasing acetonitrile gradient over 30 minutes at a flow rate of 250 nL/min. The mass spectrometer was operated in positive ion mode with a capillary temperature of 320 °C, and with a potential of 2200V applied to the frit. All data was acquired with the mass spectrometer operating in automatic data dependent switching mode. A high resolution (70,000) MS scan (300-1600 m/z) was performed using the Q Exactive to select the 8 most intense ions prior to MS/MS analysis using HCD.

All solvents used were LCMS grade.

2.22.1 PEAKS analysis of the proteomics data

The raw data was de novo sequenced and searched against the Homosapien subset of the Uniprot Swissprot database using the search engine PEAKS Studio 7, for peptides cleaved with trypsin.

Each peptide used for protein identification met specific PEAKS parameters, i.e. only peptide scores that corresponded to a false discovery rate (FDR) of $\leq 1\%$ were accepted from the Peaks database search. The Peaks De Novo results were filtered using an average local confidence (ALC) of $\geq 50\%$ and a peptide score ($-10\lg P$) of ≥ 15 .

2.22.2 MaxQuant analysis of proteomics data

The raw data was searched against the Homo sapiens subset of the Uniprot Swissprot database using the search engine MaxQuant for peptides cleaved with trypsin. Each peptide

used for protein identification met specific MaxQuant parameters, i.e. only peptide scores that corresponded to a false discovery rate (FDR) of 0.01 were accepted from the MaxQuant database search. The protein intensity of each identified protein was used for relative quantitation; as described by Cox and Mann, 2008.

2.23 Bacterial transformation and plasmid purification for the expansion, production and purification of pCMV-mCherry-MHC-IIA plasmid and EGFP-RACK1 plasmids.

1. Competent *E. Coli* were transformed with the desired plasmid (pCMV-mCherry-MHC-IIA plasmid or EGFP-RACK1; both of which contained a Kanamycin resistant gene). Briefly, cells were thawed on ice for 20-30 minutes and approximately 2µl of plasmid DNA (approximately 50ng) was added to 50µl of competent cells. The competent cell/DNA mixture was incubated on ice for 20-30 mins. Cells were heat shocked at 42°C for 45 seconds and then placed back on ice for 2 minutes. 250-1000µl of SOC media (without antibiotic) was added to the cells and they were grow in a 37°C shaking incubator for 45 min. 50µl of the transformation mixture was then plated onto a pre-warmed (37°C) LB agar plate containing 50 µg/mL Kanamycin. The rest of the mixture was plated onto a second a pre-warmed (37°C) LB agar plate containing 50 µg/mL Kanamycin. Both plates were then incubated overnight at 37 °C.
2. Single colonies picked from the agar plates were then used to inoculated starter cultures, and bacteria were grown up, harvested and their plasmid DNA extracted and purified using a QIAfilter plasmid midi kit (Quigen; Cat No 12243) according to the manufacturer's detailed protocol.

2.24 Peptide array technology and alanine substitution arrays to investigate if NAMPT interacts directly with RACK1.

Peptide arrays of RACK1 on nitrocellulose were generated as previously described (Kramer and Schneider-Mergener, 1998). Scanning libraries of overlapping 18-mer peptides covering the entire RACK1 sequence were produced by automatic SPOT synthesis and synthesized on Whatman 50 cellulose using Fmoc (9-fluorenylmethyloxycarbonyl) chemistry with the AutoSpot-Robot ASS 222 (Intavis Bioanalytical Instruments).

The interaction of NAMPT with the RACK1 protein array was investigated by overlaying the nitrocellulose membranes with recombinant NAMPT-GST protein. This was performed as follows. The membrane was first activated by placing it in 97% ethanol for 5 minutes, followed by equilibration in TBS with 0.1% Tween for 10 minutes. Membranes were then blocked in 0.1% BSA-TBS for 10 minutes. The test membrane was then incubated with 3.3 µg/ml of recombinant-NAMPT-GST protein (Biorbyt) in 0.1% BSA-TBS overnight at 4°C. A control array was incubated with 0.1% BSA-TBS only. Bound recombinant NAMPT protein was then detected as follows. The membranes were then washed X 3 for 5 minutes in TBS with 0.1% Tween and were then incubated with a rabbit anti-NAMPT antibody (Proteintech) at a 1/2000 dilution for 2 hours at room temperature. Membranes were then washed X 3 for 5 minutes in TBS with 0.1% Tween and incubated with an anti-rabbit secondary antibody coupled with LI-COR dye (1/10,000 dilution) in 0.1% BSA-TBS for one hour at room temperature. Membranes were then imaged on an Odyssey Imaging System.

Once candidate binding regions for NAMPT on the full-length RACK1 array had been identified, specific alanine scanning substitution arrays were generated for the relevant sequences using the same synthesis procedure. The progeny peptide arrays were synthesized in 18-mer format in such a way that each of the 18 amino acids of the RACK1 sequence were sequentially substituted with alanine (or aspartic acid where the wild-type sequence exhibited alanine). The arrays were probed with 3.3µg/ml NAMPT-GST using the same protocol as that described above.

A decrease in NAMPT binding to the peptides after alanine substitution is indicative of decreased binding of NAMPT to RACK1. The binding of NAMPT to each alanine-substituted RACK1 peptide was quantified by densitometry and presented as a percentage of the control "parent" or wildtype sequence.

2.25 Statistics

Statistical analysis during this PhD project was carried out using Prism v6.0, Prism v7.0 and InStat (all from GraphPad Software, CA, USA). A P-value ≤ 0.05 was considered significant: *P ≤ 0.05 , **P ≤ 0.01 and *** ≤ 0.001 .

The difference between a test and control group of cells was analysed using a two-tailed unpaired Student's t-test. The difference in band intensity (as calculated by densitometry) between two different Western blot bands was analysed using a Student's paired t-test.).

Chapter 3: An investigation into the subcellular localization, and potential co-localization, of RACK1 and non-muscle myosin heavy chain IIA (NMHCIIA) in oesophageal cells across the normal to OAC cancer progression sequence. Investigating the evidence for a RACK1 – NMHCIIA interaction during oesophageal adenocarcinoma cell migration.

3.1 Introduction

RACK1 plays a role in regulating the migration and/or spreading of a variety of cells, as was illustrated in chapter 1. In such cell types, the subcellular location of RACK1 can provide clues regarding its contribution to cell migration and movement. For example, in scratch wounded SCC cells, RACK1 localises to the leading edge of cells at the wound front where its role in scaffolding a FAK-RACK1-PDE4D5 complex is required for wound induced polarisation (Serrels et al., 2010).

Indeed, investigating the subcellular localisation of proteins is of huge importance as it provides valuable information on protein function. Knowledge of the subcellular localisation of a protein provides us with clues regarding its potential interaction partners and helps us to elucidate the interaction networks and signalling pathways in which it participates (Nakai et al 2000, Stadler et al 2013). Furthermore, the subcellular localization of certain proteins can become altered in diseases such as cancer and identification of aberrantly localized proteins can indicate that such proteins may be involved in disease causation or progression. For example, heterogeneous ribonucleoprotein K (hnRNP K) is associated with poor prognosis of colorectal cancer and it has aberrant subcellular localisation in this disease; in normal colon cancer cells hnRNP K is exclusively nuclear but in colorectal cancer cells it localizes to both the cytoplasm and the nucleus (Carpenter et al, 2006). We can learn a lot about a protein's function, and its potential role in disease, by studying its subcellular localisation. We can also gain insights into a protein's function by analysing its interaction partners. This is particularly important in the case of RACK1, whose function in any particular setting is very dependent on the particular proteins with which it is interacting in that time and space (Adams et al., 2011). As discussed in chapter 1, RACK1 has been implicated in a diverse range of cellular functions which vary depending on the proteins with which it interacts and on the cell type in which it is expressed. This diversity is reflected in its subcellular localisation patterns which are cell-type specific and are influenced by its binding partners and cellular stimuli. Indeed, analysis of

RACK1 in a variety of different cell contexts has resulted in the detection of RACK1 localisation in a wide range of subcellular compartments including the cytosol, nucleus, endoplasmic reticulum and stress granules (Kiely Adams *et al.* and Arimoto *et al.*, 2008). No known organelle localisation motifs or nuclear entry or export signals are contained within the RACK1 sequence. Furthermore, there is no conclusive evidence to suggest that post-translational modification of RACK1 controls its subcellular localisation (Adams Kiely *et al.*). Thus, the particular set of interaction partners with which RACK1 associates at any one time, and in any one cell type, is likely to have a strong influence on its subcellular localisation and movement around the cell (Adams *et al.*, 2011). For example, RACK1's interaction with 14-3-3 mediates RACK1's translocation to the nucleus following activation of the cAMP/PKA pathway (Neasta *et al.*, 2012). The identification of new RACK1 interaction partners can provide us with valuable new insights into the function of RACK1 itself.

Cell migration requires that cells project their plasma membrane forward at the front, or leading edge of the cell. Cells can extend four different kinds of plasma membrane protrusions at the leading edge; namely lamellipodia, blebs, filopodia and invadopodia (Ridley, 2011). The term 'lamellipodium' was first termed by Michael Abercrombie in 1970 to describe the thin sheet-like region at the leading edge of migrating cells in culture (Abercrombie *et al.*, 1970). Actin polymerisation is responsible for driving forward protrusion of the plasma membrane in the highly dynamic lamellipodium. A more stable region known as the lamella is found behind the lamellipodium; the lamella is believed to contribute to migration by linking the actin network to myosin II-mediated contractility and substrate adhesion (Ridley, 2011, Ponti *et al.*, 2004). Filopodia, are long, thin and contain actin bundled in parallel filaments. They are exploratory extensions from the plasma membrane which explore the cell's surroundings and provide guidance (Ridley, 2011).

As is the case with lamellipodia, in filopodia the plasma membrane is pushed forward by actin polymerization. In invadopodia, actin polymerisation coupled with the action of matrix metalloproteases cuts out a route for cells through the extracellular matrix. Membrane blebs propel the plasma membrane forward using a combination of actomyosin-based contractility and reversible detachment of the membrane from the cortical actin cytoskeleton (Ridley *et al.*, 2003). Sometimes, these different types of protrusions can coexist at the leading edge and, *in vivo*, they are likely to act in concert to move cells through complex environments.

The protrusion process relies on the complex coordination of a wide variety of signalling proteins and regulators of cytoskeletal dynamics (Ridley, 2011). It is possible that RACK1, which can coordinate and integrate multiple signalling pathways by means of its function as a scaffolding protein, could potentially play a role in such coordination. Multiple studies have

demonstrated the importance of RACK1 for cell migration and several studies have also provided evidence for an indirect involvement for this scaffolding protein in the molecular mechanisms that regulate actin cytoskeletal dynamics, through its interaction with kinases, such as Src, PKC or FAK whose activities control microfilament dynamics (Buensuceso et al., 2001, Frame et al., 2002, Larsson, 2006, Li and Xie, 2014, Miller et al., 2004, Parsons et al., 2000, Serrels et al., 2010). RACK1 also interacts with other cytoskeletal regulators such as 14-3-3 ζ which regulates cytoskeleton dynamics by interacting with actin-filament-associated proteins such as cofilin (Gohla and Bokoch, 2002). Furthermore, a recent study has just shown that RACK1 can interact directly with β -actin which raises the possibility that RACK1 might also play a direct role in controlling cytoskeletal dynamics (Neasta et al., 2016). Using its scaffolding properties, it is possible that RACK1 binding to the actin cytoskeleton may nucleate proteins which can then regulate cytoskeletal dynamics. Alternatively, RACK1 binding to F-actin might work to competitively displace or block the binding of molecules, such as Arp2/3 or cofilin, which control actin filament assembly or disassembly (Neasta et al., 2016).

It is also possible that RACK1 may use its shuttling ability to shuttle cytoskeletal regulatory proteins to plasma membrane protrusions at the leading edge. A study conducted by our group has discovered a novel interaction, between NMHCIIA and RACK1, in T lymphocytes. This study demonstrated that RACK1 translocates to the lamellipodial leading edge of migrating T-lymphocytes in association with NMHCIIA, indicating that RACK1 may control the subcellular localisation of NMHCIIA during cell migration (Christine Rudolph, unpublished data).

NMHCIIA belongs to the myosin superfamily of motor proteins that play important roles in cellular processes that involve force of translocation. Myosin molecules can walk along, produce tension on or propel the sliding of actin filaments. They also act indirectly through actin to bring adhesion related molecules, such as integrins, or signalling proteins into contact with each other (Vicente-Manzanares et al., 2009).

Non-muscle myosin II proteins are found in all non-muscle eukaryotic cells (despite their name, they are also present in muscle cells) and their contractile properties and ability to bind and cross-link actin makes them important regulatory components of the cytoskeleton. They play critical roles in the control of cell adhesion, cell migration and tissue architecture (Heissler and Manstein, 2013) (Vicente-Manzanares et al., 2009). Like muscle myosin II, non-muscle myosin II molecules consist of three pairs of peptides: two heavy chains, two regulatory light chains, that regulate myosin II activity, and two essential light chains that stabilize the heavy chain structure. Three mammalian isoforms of non-muscle myosin II have been identified, namely non muscle myosin IIA (or NMHCIIA) non-muscle myosin IIB and non-muscle myosin IIC (Heissler and Manstein, 2013).

A small number of studies have suggested that NMHCIIA may contribute to the migration and/or invasion of certain types of cancer cells. However the evidence on the role of NMHCIIA in cancer is far from comprehensive and includes some conflicting or paradoxical reports (Ouderkirk and Krendel, 2014).

3.2 Objectives

Our group has previously identified a novel interaction between RACK1 and NMHCIIA at the leading edge of migrating T-cells. Here we sought to gain an insight into the potential roles of RACK1 and NMHCIIA in OAC by investigating the subcellular localisation (and potential colocalisation) of RACK1 and NMHCIIA in oesophageal cells.

We also sought to determine if the localisation and/or colocalisation of RACK1 and NMHCIIA is of particular relevance to cancer and to cancer progression in the context of OAC. We therefore compared RACK1 and NMHCIIA localisation patterns across the cancer progression sequence from normal oesophageal epithelial cells to OAC.

We also sought to investigate if RACK1 and NMHCIIA interact in migrating OAC cells in a functionally important manner.

3.3 Results

3.3.1 RACK1 and NMHCIIA exhibit similar perinuclear colocalisation patterns in resting oesophageal cell lines representing the non-cancerous oesophagus-Barrett's oesophagus-adenocarcinoma sequence.

High resolution laser scanning confocal microscopy in conjunction with immunofluorescent staining was used to examine subcellular localisation and potential colocalisation patterns of RACK1 and NMHCIIA in oesophageal cell lines representing the normal→Barrett's oesophagus→adenocarcinoma sequence.

3.3.1.1 Analysis of RACK1 and NMHCIIA localisation/colocalisation in resting HET-1A non-cancerous oesophageal cells

The HET-1A cell line was used to represent cells of the non-cancerous oesophageal squamous epithelium(Stoner et al., 1991). In these cells, we observed that both RACK1 and NMHCIIA had a diffuse cytoplasmic distribution, which, in many cells, was particularly concentrated in the perinuclear area. Strong NMHCIIA staining was also observed at the edge of many cells (see yellow arrows in figure 3.1) where it may be localised to peripheral stress fibers. Colocalisation

of RACK1 and NMHCIIA occurred in a significant proportion of cells, particularly in perinuclear areas as indicated by grey arrows in figure 3.1.

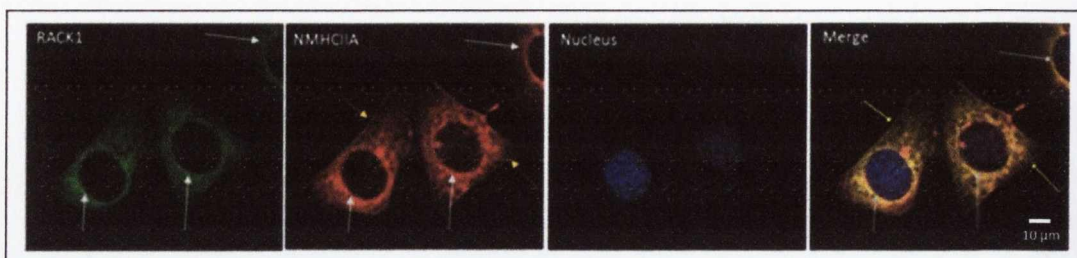


Figure 3.1: Subcellular localisation of RACK1 and NMHCIIA in HET-1A non-cancerous oesophageal cells. Cells were fixed, permeabilised, and stained with hoechst nuclear stain and immunofluorescent dyes conjugated to antibodies specific for RACK1 and NMHCIIA. All images were obtained by confocal microscopy using a 63X objective. Both RACK1 and NMHCIIA exhibited a diffuse cytoplasmic distribution, which was more concentrated in the perinuclear area of many cells. Strong NMHCIIA staining was also observed at the edge of many cells (yellow arrows) where it may be localised to peripheral stress fibers. Colocalisation of RACK1 and NMHCIIA occurred in a significant proportion of cells, particularly in perinuclear areas as indicated by grey arrows.

3.3.1.2 Analysis of RACK1 and NMHCIIA localisation/colocalisation in resting QhTERT Barrett's metaplastic oesophageal cells.

The QhTERT cell line was used to represent metaplastic Barrett's oesophagus (Zhang et al., 2009). RACK1 exhibited a diffuse cytoplasmic expression pattern in this cell line which was most concentrated in the perinuclear region of many, but not all, cells. NMHCIIA also exhibited diffuse cytoplasmic staining with regions of more concentrated staining in perinuclear regions. However more defined NMHCIIA staining was also observed in stress fiber-like structures (see pink arrows in figure 3.2). Strong NMHCIIA staining was also observed at the cell periphery, where it may be localized to peripheral stress fibers (see yellow arrow in figure 3.2). In some cells, a small amount of RACK1-NMHCIIA colocalisation was observed in perinuclear regions, as illustrated by the grey arrow in figure 3.2.

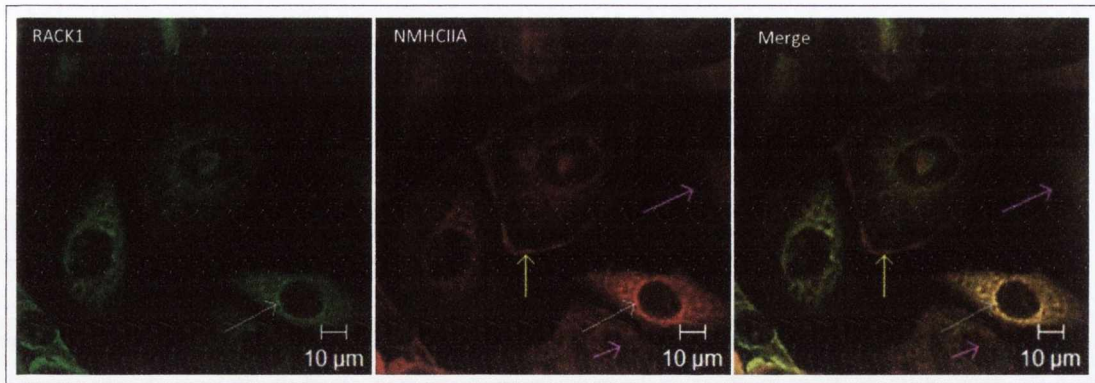


Figure 3.2: Subcellular localisation of RACK1 and NMHCIIA in resting QhTERT Barrett's metaplastic oesophageal cells. Cells were fixed, permeabilised, and stained with immunofluorescent dyes conjugated to antibodies specific for RACK1 and NMHCIIA. All images were obtained by confocal microscopy using a 63X objective. Both RACK1 and NMHCIIA exhibited a cytoplasmic distribution, which was more concentrated in the perinuclear area of many cells. Perinuclear RACK1-NMHCIIA colocalisation occurred in some cells as highlighted by the grey arrow. Strong NMHCIIA staining was also observed at the edge of many cells (yellow arrow) where it may be localised to peripheral stress fibers. NMHCIIA staining was also observed in stress fiber-like structures (pink arrows).

3.3.1.3 Analysis of RACK1 and NMHCIIA localisation/colocalisation in resting GohTERT Barrett's dysplastic oesophageal cells

The GohTERT cell line was used to represent dysplastic Barrett's oesophagus (Huang et al., 2009). Both RACK1 and NMHCIIA exhibited a cytoplasmic distribution in this cell line. In a proportion of cells, particularly strong RACK1 staining was observed in perinuclear areas. In a small proportion of cells, strong NMHCIIA staining was also observed in perinuclear areas, and in such cells, perinuclear RACK1-NMHCIIA colocalisation occurred. Figure 3.3 provides an example of such a cell and this perinuclear colocalisation of RACK1 and NMHCIIA is highlighted by a grey arrow. Strong NMHCIIA staining was observed at the edge of some cells (as illustrated by the yellow arrow in figure 3.3) where it may be localised to peripheral stress fibers. In some cells, NMHCIIA staining was also observed in stress fiber-like structures (see pink arrows figure 3.3). In a significant proportion of cells strong RACK1 staining was observed in punctate structures (highlighted by turquoise arrows in figure 3.3) which resemble staining patterns for focal adhesions.

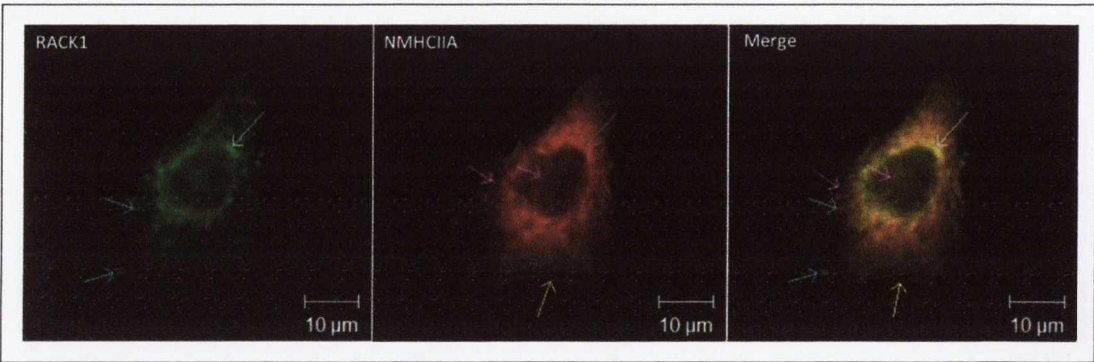


Figure 3.3: Subcellular localisation of RACK1 and NMHCIIA in resting GohTERT Barrett's dysplastic oesophageal cells. Cells were fixed, permeabilised, and stained with immunofluorescent dyes conjugated to antibodies specific for RACK1 and NMHCIIA. All images were obtained by confocal microscopy using a 63X objective. In a small proportion of cells, such as the cell illustrated here, perinuclear RACK1-NMHCIIA colocalisation was observed (grey arrow). Pink arrows highlight NMHCIIA staining in stress fiber-like structures. The yellow arrow highlights strong NMHCIIA staining at the cell periphery where it may be localized to peripheral stress fibers. RACK1 positive punctate structures, which may be focal adhesions, are highlighted by turquoise arrows.

3.3.1.4 Analysis of RACK1 and NMHCIIA localisation/colocalisation in resting SKGT-4 oesophageal adenocarcinoma cells

The SKGT-4 cell line was used to represent Barrett's derived oesophageal adenocarcinoma. Both RACK1 and NMHCIIA exhibited a diffuse cytoplasmic distribution and in many cells staining for both proteins was more concentrated in perinuclear regions. Colocalisation of RACK1 and NMHCIIA occurred in many cells, particularly in perinuclear areas as indicated by grey arrows in figure 3.4.

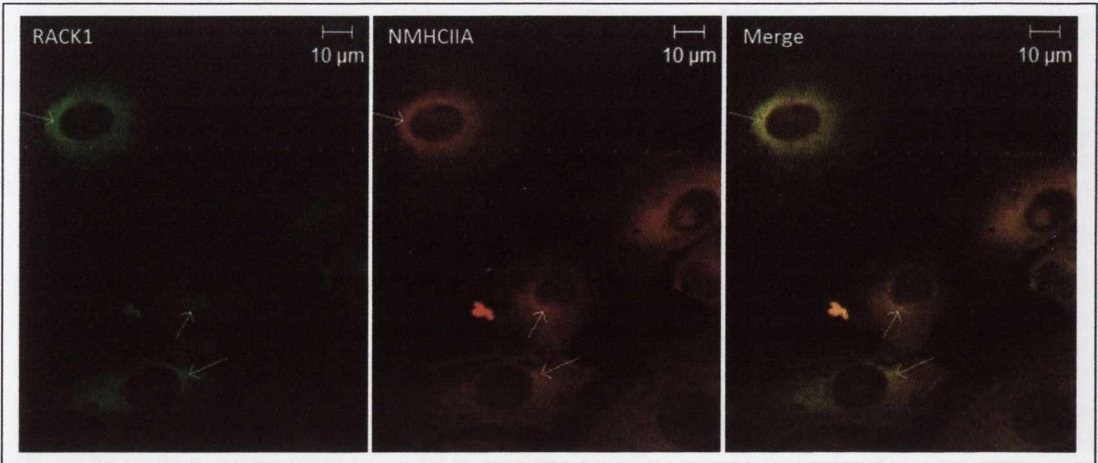


Figure 3.4: Subcellular localisation of RACK1 and NMHCIIA in resting SKGT-4 oesophageal adenocarcinoma cells. Cells were fixed, permeabilised, and stained with immunofluorescent dyes conjugated to antibodies specific for RACK1 and NMHCIIA. All images were obtained by confocal microscopy using a 63X objective. Both RACK1 and NMHCIIA exhibited a cytoplasmic distribution, which was most concentrated in the perinuclear area of most cells. Perinuclear RACK1-NMHCIIA colocalisation occurred in many cells as highlighted by the grey arrow.

The RACK1 and NMHCIIA staining patterns reported above demonstrate that some RACK1-NMHC2A colocalisation occurs in normal, metaplastic, dysplastic and cancerous oesophageal cell lines. Furthermore, in all of the above cell lines this colocalisation was most frequently observed in perinuclear regions. No obvious correlation or pattern linking the extent of colocalisation to cancer staging was observed. The frequency at which colocalisation occurred did vary between the different cells lines but this variation did not correlate with cancer staging; rather colocalisation was most commonly observed in the cell lines which represented each end of the normal → cancer sequence while it was less commonly observed in cells lines representing intermediate stages in the sequence. That is, perinuclear RACK1-NMHCIIA colocalisation was frequently observed in the HET-1A (normal oesophagus) and SKGT-4 (oesophageal adenocarcinoma) cell lines while it was observed less frequently in the QhTERT (Barrett's metaplastic) and GohTERT (Barrett's dysplastic) cell lines. It should be noted that the four cell lines used in this study are not isogenic and some variation between expression and colocalisation patterns may be attributable to this. Nevertheless, we have found that perinuclear RACK1-NMHCIIA colocalisation can occur in normal, dysplastic, metaplastic and cancerous oesophageal cells and is therefore not unique to either healthy or cancerous cells.

3.3.2. In migrating SKGT-4 cells, RACK1 and NMHCIIA exhibit perinuclear colocalisation which is similar to that observed in resting cells.

We next investigated if RACK1 and NMHCIIA colocalise in a manner which is of functional importance to cell migration. Our group has previously shown that, when T-cells are stimulated to migrate, NMHCIIA translocates to the lamellipodial leading edge in association with RACK1. This suggested that RACK1 may be controlling the subcellular localization of NMHCIIA during migration. It was therefore of interest to investigate if RACK1 and NMHCIIA also colocalise at the leading edge of migrating oesophageal adenocarcinoma (SKGT-4) cells. SKGT-4 cells are capable of migrating into a scratch wound made in a layer of confluent cells,

as is demonstrated in chapter 5, figure 5.7. SKGT-4 cells migrating into a scratch wound were therefore imaged and RACK1 and NMHCIIA localization and colocalisation was examined by immunofluorescent staining. However, it was found that, unlike T-cells, SKGT-4 cells, do not migrate as individual cells with the typical polarized morphology of an individually migrating cell and an obvious lamellipodium. Instead, they migrate collectively in cell sheets and do not form individual lamellipodia. Therefore, it was obviously not possible to examine if RACK1 and NMHCIIA colocalise to leading edge lamellipodia in SKGT-4 cells. Nevertheless, RACK1 and NMHCIIA localization and colocalisation were examined in SKGT-4 cells as they migrated into a scratch wound. In these cells, both RACK1 and NMHCIIA exhibited diffuse cytoplasmic localization patterns and staining of both proteins was often strongest in perinuclear regions. Perinuclear RACK1 – NMHCIIA colocalisation was observed in many cells, examples of which are highlighted by the grey arrows in figure 3.5. These localisation and colocalisation patterns were observed both in cells at the wound edge and in cells located further back from the wound in the cell monolayer. They were also similar to the staining patterns observed in resting SKGT-4 cells (see figure 3.4). Therefore, perinuclear RACK1-NMHCIIA colocalisation is observed in migrating SKGT-4 cells but is not specific to migration as it is also observed in resting cells.

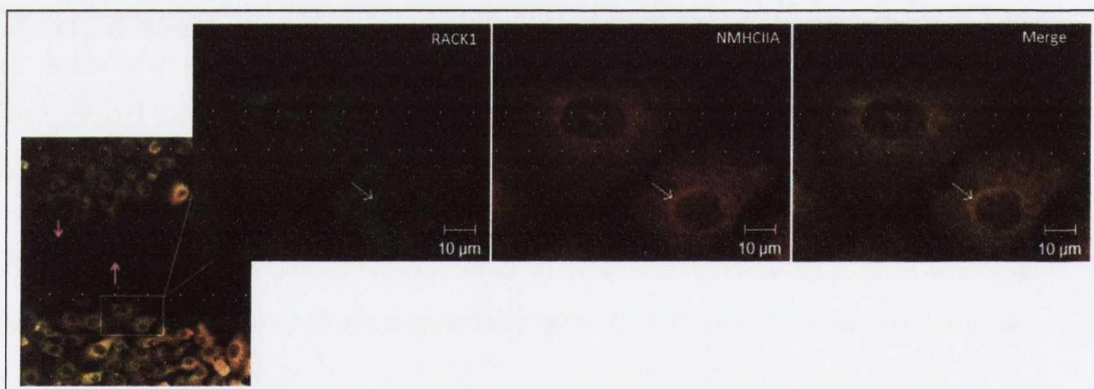


Figure 3.5: Subcellular localisation of RACK1 and NMHCIIA in scratch wounded, migrating SKGT-4 oesophageal adenocarcinoma cells. Cells were grown to confluence and then a scratch wound was made in the cell monolayer (to stimulate migration) using a p200 pipette tip. At 24 hours post wounding, cells were fixed, permeabilised, and stained with immunofluorescent dyes conjugated to antibodies specific for RACK1 and NMHCIIA. The lower image was obtained by confocal microscopy using a 20X objective. The boxed area of this image is magnified in the top panel; images in this top panel were collected by confocal microscopy using a 63X

objective. Pink arrows in the lower image indicate the direction of cell migration into the wound. Both RACK1 and NMHCIIA exhibited a cytoplasmic distribution, which was most concentrated in the perinuclear area of most cells. Perinuclear RACK1-NMHCIIA colocalisation occurred in many cells, an example of which is highlighted by the grey arrow.

3.3.3 RACK1 and NMHCIIA colocalise at the leading edge of migrating MDA-MB-231 epithelial cells. As discussed above, SKGT-4 cells do not undergo single cell migration with an individually polarized morphology and obvious lamellipodium and leading edge. Thus it was not possible to investigate if RACK1 and NMHCIIA colocalise at the lamellipodial leading edge in this cell type. However, some epithelial cancer cell lines do undergo single cell migration; an example of which is the MDA-MB-231 cell line. MDA-MB-231 cells are highly motile and invasive breast cancer cells which have been shown to form tumours and metastasize aggressively in vivo. They exhibit a wide range of morphologies when plated directly on glass, with some cells undergoing spontaneous polarization consistent with single cell migration. However, when MDA-MB-231 cells are plated on collagen IV coated coverslips, the frequency with which they polarize, and display lamellipodia consistent with migration, is increased (Kim and Helfman, 2003). Interestingly, NMHCIIA has been shown to localize at the leading edge of migrating MDA-MB-231 cells (Kim and Helfman, 2003). This cell line therefore represented a suitable model in which to investigate if NMHCIIA and RACK1 colocalise at the leading edge of migrating epithelial cells. MDA-MB-231 cells were thus plated on collagen IV coated coverslips and immunofluorescent staining was then used to examine RACK1 and NMHCIIA colocalisation in cells which had migrated and were exhibiting a polarized morphology. As expected (given previous findings by (Kim and Helfman, 2003) , strong NMHCIIA staining was observed at the leading edge. Furthermore, strong RACK1 staining was also observed at the leading edge and colocalisation of both proteins was observed in this region. This was similar to the RACK1-NMHCIIA colocalisation which had been previously observed at the leading edge of migrating T-cells (Christine Rudolph; unpublished data). Some RACK1-NMHCIIA colocalisation was also observed in the trailing tail of migrating MDA-MB 231 cells; this was again similar to the RACK1-NMHCIIA colocalisation pattern found in migrating T-cells where colocalisation of the two proteins had also been observed in the uropod (Christine Rudolph; unpublished data). These findings indicated that RACK1 and NMHCIIA may undergo an interaction, which is of functional importance to migration, in cells that migrate as polarized, single cells.

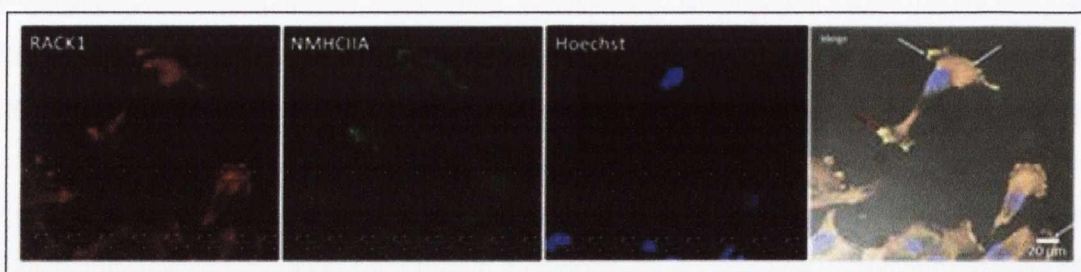


Figure 3.6: RACK1 and NMHCIIA colocalise at the leading edge of migrating MDA-MB-231 cells.

Cells were plated on collagen-IV coated coverslips and allowed to polarize and migrate. They were then fixed, permeabilised, and stained with hoechst nuclear stain and immunofluorescent dyes conjugated to antibodies specific for RACK1 and NMHCIIA. All images were obtained by confocal microscopy using a 63X objective. Strong NMHCIIA staining was observed at the leading edge and also at the cell rear. Strong RACK1 staining was also observed at the leading edge and colocalisation of both proteins is observed in this region, as highlighted by white arrows. Some RACK1-NMHCIIA colocalisation is also observed in the trailing tail of migrating MDA-MB 231 cells, as highlighted by the red arrow.

3.3.4. Identification of an OAC cell line capable of undergoing single cell polarized migration

Immunofluorescent staining of RACK1 and NMHCIIA in migrating MDA-MB 231 cells had indicated that RACK1 and NMHCIIA do localise, and colocalise, at the leading edge of epithelial cells undergoing polarized, single cell migration. We therefore hypothesized that both proteins may also localize (and potentially colocalize) at the leading edge of a migrating OAC cell undergoing single cell, polarized migration. We therefore sought to identify a migrating OAC cell line which was capable of migrating in this way. As reported in Section 3.3.2, SGKT-4 cells do not undergo single cell migration but migrate collectively in cell sheets. We therefore reviewed the literature on all commercially available OAC cell lines in an effort to find an alternative cell line which could serve as an OAC model of single cell, polarized migration. The OACM5.1 C cell line (originally isolated from a lymph node metastasis derived from a primary adenocarcinoma of distal oesophagus with the presence of Barrett's transformed mucosa) was identified for investigation as a potential model. As it was isolated from a metastatic site we hypothesized that it may be highly motile and be capable of single cell, polarized migration similar to that observed in the case of the metastatic MDA-MB 231 cell line.

OACM5.1 C cells were therefore obtained and their migratory potential and mode of migration was examined. However, unlike MDA-MB 231 cells, we found that OACM5.1 C did not undergo any spontaneous polarization consistent with migration. We also tried plating OACM5.1 C cells on different ECM proteins (collagen I, collagen IV, fibronectin, laminin and vitronectin) and on matrigel in an effort to stimulate polarization and single cell migration. However on all of the above ECM proteins, OACM5.1C cells retained a round morphology and remained associated together in clumps. Scratch wounding also failed to stimulate polarized single cell migration. The FLO-1 cell line was next investigated as a potential model of single cell OAC migration. FLO-1 cells are not a metastatic cell line but have been reported to be very motile (Miller et al., 2006). Furthermore, it has been reported that FLO-1 cells form pseudopod structures when scratch wounded and treated with hepatocyte growth factor (HGF) (Watson et al., 2006). FLO-1 cells were therefore obtained and their morphology and mechanism of migration was examined. When seeded at a low density, we found that a small proportion of FLO-1 cells underwent spontaneous polarisation and displayed ruffling lamellipodia. FLO-1 cells were also grown to confluence and scratch wounding was used to stimulate migration. The morphology of cells migrating into the wound at the wound edge was then examined at 12 and 24 hours post migration. Such examination revealed that some cells had individually protruded out into the wound ahead of the other cells at the wound edge and thus appeared to be migrating as single cells.

3.3.5 Immunofluorescent staining indicates that NMHCIIA localises to the leading edge of migrating FLO-1 cells while RACK1 has a diffuse distribution throughout the cytoplasm.

Immunofluorescent staining and confocal microscopy was used to examine RACK1 and NMHCIIA colocalisation in polarised, migrating FLO-1 cells (figure 3.7). NMHCIIA staining was observed at the leading edge (white arrows figure 3.7) while RACK1 had a more diffuse localisation throughout the cytoplasm. Thus, obvious RACK1-NMHCIIA localisation was not observed. Nevertheless, leading edge NMHCIIA staining was directly adjacent to strong RACK1 staining in the lamella which raised the possibility that some transient interactions, between NMHCIIA and a subset of RACK1, might perhaps occur in this region.

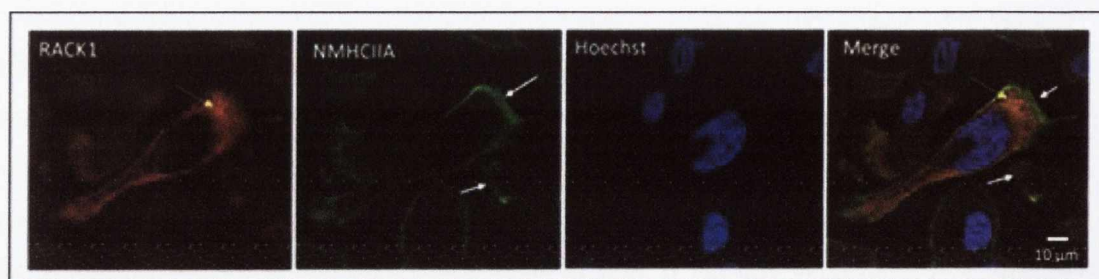


Figure 3.7: Immunofluorescent staining and confocal microscopy was used to examine RACK1 and NMHCIIA colocalisation in polarised, migrating FLO-1 cells. Cells were fixed, permeabilised, and stained with hoechst nuclear stain and immunofluorescent dyes conjugated to antibodies specific for RACK1 and NMHCIIA. All images were obtained by confocal microscopy using a 63X objective. Strong NMHCIIA staining was observed at the leading edge and in lamellipodial ruffles, as highlighted by white arrows. RACK1 staining has a more diffuse localisation pattern throughout the cytoplasm but was particularly strong just behind the leading edge NMHCIIA (yellow arrow).

3.3.6 EGFP tagged RACK1 exhibits a polarized distribution pattern during live cell imaging of migrating FLO-1 cells and is most often concentrated behind the leading edge in and/or close to, the lamella.

A second method of examining RACK1 localisation in FLO-1 cells was employed in order to verify the results obtained in 3.3.5 above. Following transfection optimization, EGFP-RACK1 was successfully expressed in FLO-1 cells. This enabled the localization of EGFP-RACK1 to be examined in live cells thus removing the need for cell fixation, permeabilisation and antibody staining. Furthermore, it enabled RACK1 localisation to be examined over a time period, thus providing more information on RACK1 dynamics during FLO-1 migration.

Localisation of chimeric RACK1-EGFP was compared with localization of a free EGFP control. This helped ensure that RACK1-EGFP localization was being directed by RACK1 rather than by EGFP. As expected, localization of RACK1-EGFP was significantly different to localisation of the free EGFP control; the latter was distributed throughout the cell, exhibiting a predominately non-specific expression pattern although some nuclear accumulation was apparent. In contrast, RACK1-EGFP was absent from the nuclear area and exhibited a more specific, cytosolic expression pattern.

Fluorescent ratiometric microscopy was then employed to estimate subcellular RACK1-EGFP concentration levels in migrating FLO-1 cells. RACK1-EGFP was co-expressed with free mCherry fluorescent protein in FLO-1 cells. The latter served as a marker of cytoplasmic volume and

corrected for variation in optical path length and fluorescent intensity due to variation in cell shape and thickness. Three types of image were collected and used to produce ratiometric images; phase contrast, EGFP and mCherry. Images representing ratios of EGFP to mCherry fluorescence were produced and overlaid onto the phase contrast images. EGFP signal was thus normalized to the distribution of the cytosol, using mCherry. Subcellular regions to which RACK1 localised could thus be identified by identifying regions where the ratio of EGFP to mCherry was high. A small percentage of FLO-1 cells undergo spontaneous polarization and exhibit ruffling and migration. However scratch wounding and treatment with HGF was also employed to help stimulate migration. Cells which exhibited membrane ruffling and a polarized morphology consistent with cell migration were identified for analysis. In such cells, RACK1-EGFP was most concentrated in an area just behind the ruffling lamellipodium; in the lamella or in a region of cell body just behind the lamella (see black arrows figure 3.8). Another region of high RACK1-EGFP concentration was observed at the cell rear (see red arrows figure 3.8). This pattern of RACK1-EGFP localization was observed both in HGF treated cells and in cells undergoing spontaneous or scratch wound stimulated migration.

Time-lapse movies of migrating FLO-1 cells were also compiled by gathering images of cells at 30 second intervals over a ten minute period. In comparison to more intrinsic migratory cell types such as cells of the immune system, FLO-1 cells migrate very slowly. Thus the distance travelled by these cells within a ten minute period was short and not always noticeable. However, a ten minute period did provide time for membrane ruffling to proceed and for some shape change of the lamellipodium and lamella to occur. Movies representing ratios of RACK1-EGFP to mCherry were produced as described above. Analysis of these movies indicated that RACK1-EGFP dynamics were relatively stationary during the ten minute imaging period although some small, minor shifts in peak RACK1-EGFP concentration occurred within and just below the lamella.

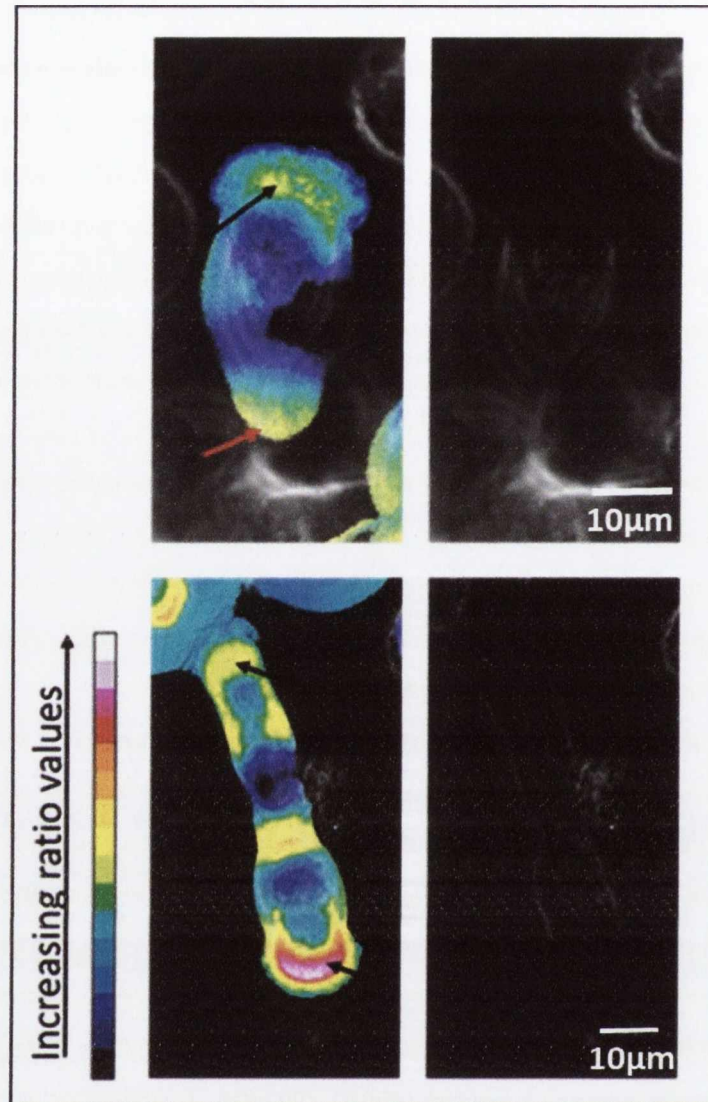


Figure 3.8: EGFP tagged RACK1 exhibits a cytoplasmic distribution pattern, with some accumulation in the lamella and cell rear, during live cell imaging of migrating FLO-1 cells.

Images representing ratios of EGFP-RACK1 to mCherry fluorescence were produced. EGFP-RACK1 signal was thus normalized to the distribution of the cytosol, using mCherry. These ratiometric images were overlaid onto phase contrast images (left hand panels). Subcellular regions to which RACK1 localised could thus be identified by identifying regions where the ratio of EGFP to mCherry was high. Ratio values were particularly high behind the leading edge in the proximity of the lamella (as highlighted by black arrows) but were also high at the cell rear (red arrows). Right hand panels display phase contrast images alone and enable the morphology of the migrating cells, and positioning of lamellipodial ruffles, to be clearly seen.

3.3.7 mCherry tagged NMHCIIA is concentrated in leading edge ruffles during live cell imaging of migrating FLO-1 cells.

Localisation of mCherry tagged NMHCIIA was examined in migrating FLO-1 cells in the same way as localization of EGFP tagged RACK1 was examined in section 3.3.6 above.

Localisation of chimeric NMHCIIA-mCherry was first compared with localization of a free mCherry control. This helped ensure that NMHCIIA-mCherry localization was being directed by NMHCIIA rather than by mCherry. As expected, localization of NMHCIIA-mCherry was significantly different to localisation of the free mCherry control; the latter was distributed throughout the cell, exhibiting a predominately non-specific expression pattern although some nuclear accumulation was apparent. In contrast, NMHCIIA-mCherry exhibited a much more specific, expression pattern with strong fluorescent intensity observed in peripheral cell areas, in stress fiber like structures and in peripheral ruffles.

Fluorescent ratiometric microscopy was then employed to estimate subcellular NMHCIIA-mCherry concentration levels in migrating FLO-1 cells. NMHCIIA-mCherry was co-expressed with free EGFP fluorescent protein in FLO-1 cells. The latter served as a marker of cytoplasmic volume and corrected for variation in optical path length and fluorescent intensity due to variation in cell shape and thickness. Three types of image were collected and used to produce ratiometric images; phase contrast, mCherry and EGFP. Images representing ratios of mCherry to EGFP fluorescence were produced and overlaid onto the phase contrast images. mCherry signal was thus normalized to the distribution of the cytosol, using EGFP. Subcellular regions to which NMHCIIA localised could thus be identified by identifying regions where the ratio of mCherry to EGFP was high. A small percentage of FLO-1 cells undergo spontaneous polarization and exhibit ruffling and migration. However scratch wounding and treatment with HGF was also employed to help stimulate migration. Cells which exhibited membrane ruffling and a polarized morphology consistent with cell migration were identified for analysis. In such cells, NMHCIIA was most concentrated at the leading edge where it was particularly concentrated in membrane ruffles (see figure 3.9, black arrows). This was similar to the NMHCIIA localization pattern observed in immunofluorescently stained FLO-1 cells where strong NMHCIIA staining was observed at the leading edge (see figure 3.7). High NMHCIIA-mCherry concentration was also observed at the cell periphery (see green arrows, figure 3.9) and in stress fibers (see red arrows figure 3.9) which was again consistent with the immunofluorescent NMHCIIA staining patterns shown in figure 3.7. Time-lapse movies of migrating FLO-1 cells were also compiled by gathering images of cells at 30 second intervals

over a ten minute period. In comparison to more intrinsic migratory cell types such as cells of the immune system, FLO-1 cells migrate very slowly. Thus the distance travelled by these cells within a ten minute period was short and not always noticeable. However, a ten minute period did provide time for membrane ruffling to proceed and for some shape change of the lamellipodium and lamella to occur. Movies representing ratios of NMHCIIA-mCherry to EGFP were produced as described above. Analysis of these movies indicated that NMHCIIA-mCherry has quite a dynamic localization, particularly in lamellipodium ruffles where it undergoes constant movement within the ruffles. However the lamellipodium ruffles themselves are dynamic structures and thus it is difficult to say whether NMHCIIA-mCherry was moving of its own accord or if it was simply moving because the structures within which it was localised were moving.

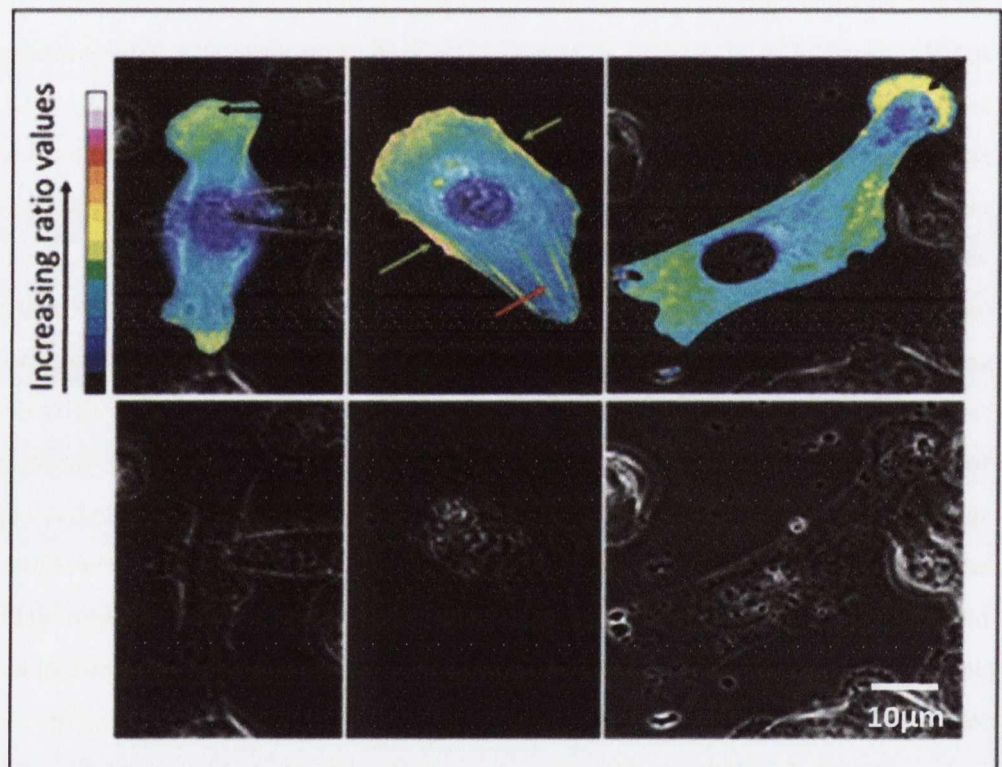


Figure 3.9: mCherry tagged NMHCIIA localises to the leading edge and to ruffling lamellipodia in live, migrating FLO-1 cells.

Images representing ratios of mCherry-NMHCIIA to EGFP fluorescence were produced. mCherry-NMHCIIA fluorescent signal was thus normalized to the distribution of the cytosol, using EGFP. These ratiometric images were overlaid onto phase contrast images (top panels). Subcellular regions to which NMHCIIA localised could thus be identified by identifying regions where the ratio of mCherry to EGFP was high. High ratio values were observed at the leading

edge of migrating cells and in membrane ruffles (black arrows). High ratio values were also observed at the cell periphery (green arrows) of many cells, an example of which is found in the middle panel above. In some cells, such as that in the right hand panel, high ratio values were also found in stress fibres (red arrow). Bottom panels display phase contrast images alone and enable the morphology of the migrating cells, and positioning of lamellipodial ruffles, to be clearly seen.

3.3.8 Analysis of Förster Resonance Energy Transfer (FRET) between RACK1-EGFP and NMHCIIA-mCherry in resting and migrating FLO-1 cells.

The findings described in section 3.3.5, 3.3.6 and 3.3.7 above indicate that RACK1 and NMHCIIA localize to distinct but adjacent subcellular locations in migrating FLO-1 cells. NMHCIIA is most concentrated at the leading edge while RACK1 usually localizes further back in the cell just behind NMHCIIA; it is typically most concentrated in the lamella or in regions of the cell body in proximity to the lamella. Thus the two proteins do not exhibit obvious colocalisation however they do have adjacent sites of localization which may overlap slightly/dynamically. It is therefore plausible that some transient interactions between RACK1 and NMHCIIA may occur in migrating FLO-1 cells.

To investigate if such interactions occurred we employed Förster Resonance Energy Transfer (FRET) Sensitized Emission microscopy. FLO-1 cells were co-transfected with plasmids encoding RACK1-EGFP and NMHCIIA-mCherry. Transfected cells were seeded at high density and allowed to form confluent monolayers. Migration was then stimulated by scratch wounding alone or by scratch wounding and stimulation with 50ng/ml HGF. Cells which had successfully expressed both RACK1-EGFP and NMHCIIA-mCherry were identified for imaging. Phase contrast, EGFP, mCherry and FRET images were collected from live resting and migrating cells and were processed as described in chapter 2. FRET calibration parameters alpha and beta were calculated to be 0.0481 and 0.0515 respectively and the true FRET signal for each image was determined as is also described in chapter 2. This FRET signal was pseudo coloured and overlaid on phase contrast images to facilitate identification of FRET positive subcellular locations. To maximize the chances of detecting transient interactions between RACK and NMHCIIA (and hence transient FRET), both single time frame images and time-lapse videos were analysed. However no significant FRET signal was observed in migrating cells which had been scratch wounded alone (figure 3.10 (b)) or in migrating cells which had been scratch wounded and stimulated with HGF (figure 3.10(c)). Most of the resting cells examined also exhibited negligible FRET signal although some perinuclear FRET signal was observed in a very small proportion of cells, as highlighted by the red arrow in figure 3.10 (a).

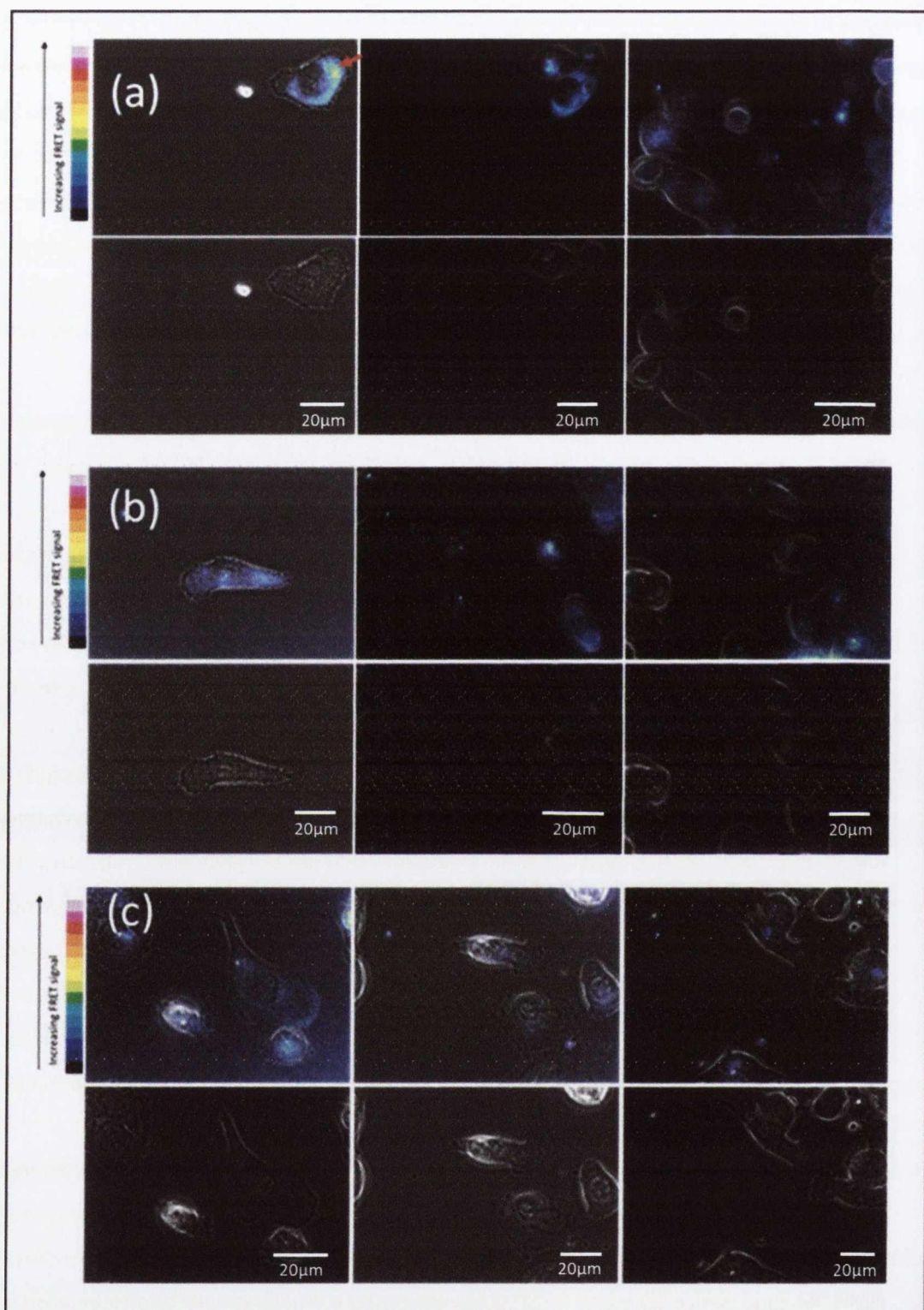


Figure 3.10: No significant FRET signal occurs between RACK1-EGFP and NMHCIIA-mCherry in FLO-1 cells. Signal for FRET between RACK1-EGFP and NMHCIIA-mCherry was pseudo coloured and overlaid on phase contrast images to facilitate identification of FRET positive subcellular locations (top panel in each sub figure). Corresponding phase contrast images (with

no overlaid FRET signal) are included in bottom panels to enable clear visualization of cell morphology and identification of subcellular locations and structures. (a) Most of the resting cells examined exhibited negligible FRET signal although some very weak perinuclear FRET signal was observed in a very small proportion of cells, such as that highlighted by the red arrow. (b) No significant FRET signal was observed in migrating cells which had been scratch wounded to stimulate migration. (c) No significant FRET signal was observed in migrating cells which had been both scratch wounded and stimulated with HGF.

3.4 Discussion

One of the principal aims of this chapter was to determine if RACK1 and NMHCIIA interact in OAC cells and to also determine if this potential interaction is of functional importance to OAC cell migration or to the development or progression of cancer.

Cancer development is a multi-step process whereby a number of changes occur that eventually transform normal cells into carcinoma cells. During this cancer progression process, the subcellular localisation of a protein that is involved in the carcinogenic process may change and the detection of such a change can provide insight into that protein's role in disease progression. Similarly, the co-localisation of two or more proteins involved in disease progression may also change as a cell is transformed. We therefore examined the subcellular localisation and co-localisation of RACK1 and NMHCIIA across a panel of cell lines representing the OAC progression sequence from non-cancerous oesophageal cells through Barrett's metaplasia and dysplasia, to full OAC. We observed co-localisation of RACK1 and NMHCIIA in the perinuclear area of non-cancerous (HET-1A), Barrett's metaplastic (QhTERT), Barrett's dysplastic (GohTERT) and OAC (SKGT-4) cells (figures 3.1 to 3.4). This indicated that RACK1 and NMHCIIA may associate with each other in oesophageal cells but that this perinuclear association or interaction is not specific to any particular disease progression stage or to either healthy or cancerous cells.

When T-cells are stimulated to migrate, NMHCIIA translocates to the lamellipodial leading edge in association with RACK1 which indicates that RACK1 may be controlling NMHCIIA localisation during migration (Christine Rudolph, unpublished data). This finding prompted us to investigate if RACK1 and NMHCIIA interact in a similar way during OAC cell migration. SKGT-4 cells were grown to full confluence and then scratch wounded to stimulate migration. The co-localisation of RACK1 and NMHCIIA was then examined in cells migrating into the wound. This revealed that RACK1 and NMHCIIA do co-localise in migrating SKGT-4 cells but that this co-localisation occurs in the perinuclear area, just as it does in the case of resting SKGT-4 cells.

Therefore, this co-localisation is not specific to migration and thus may not have any functional involvement in the migration process.

Our analysis of migrating SKGT-4 cells revealed that they use a very different mechanism of migration to that employed by T-cells. Unlike T-cells, SKGT-4 cells do not migrate individually as single cells with clearly defined lamellipodia. Therefore it became clear to us that they are a poor model in which to investigate a potential lamellipodial RACK1-NMHCIIA interaction during polarised cell migration. However, some epithelial cells do migrate as single cells with individual lamellipodia. One such cell type is the MDA-MB-231 metastatic breast carcinoma cell line. Interestingly, NMHCIIA has been previously shown to localise to the leading edge of migrating MDA-MB-231 cells (Kim and Helfman, 2003). We therefore investigated if RACK1 and NMHCIIA co-localise at the leading edge of migrating MDA-MB-231 cells; our analysis indicated that such co-localisation does take place (see figure 3.6.). This indicated that RACK1 and NMHCIIA may also co-localise in an OAC cell line capable of undergoing single cell migration. The FLO-1 cell line represents such a cell line. However, upon analysing RACK1 and NMHCIIA localisation in this cell line (using immunofluorescent staining and confocal microscopy) we found that the two proteins do not co-localise at the leading edge as we had observed in MDA-MB-231 cells. Strong NMHCIIA occurred at the leading edge of migrating FLO-1 cells while RACK1 had a more diffuse distribution throughout the cytoplasm with particularly strong staining just behind the leading edge NMHCIIA (see figure 3.7).

Immunofluorescent staining of fixed cells can yield valuable information regarding the expression levels and subcellular localisation of endogenous, unmodified proteins. However, the fixation and permeabilization protocols involved can potentially lead to artifacts which can influence observed localization patterns. Furthermore, some antibodies exhibit non-specific binding due to cross-reactivity with other proteins (Stadler et al., 2013). The anti-RACK1 antibody used in this project is probably the most widely used and best regarded commercially available anti-RACK1 antibody and is widely cited in literature concerning RACK1. However Western Blotting using this antibody sometimes yields a non-specific band at approximately 70kDa. Thus, it was important to consider the possibility that some non-specific staining could occur when conducting immunofluorescent staining with this antibody. Therefore, we also employed a second method of analyzing RACK localisation in FLO-1 cells. Fluorescent ratiometric microscopy was used to examine the relative concentration of endogenous RACK1-EGFP in migrating FLO-1 cells. Results of this agreed with what we had found by immunofluorescent staining in showing that RACK1 was widely distributed throughout the cytoplasm (confirming specificity of the antibody). However they also indicated that RACK1-EGFP was especially concentrated in an area just behind the ruffling lamellipodium; in the

lamella or in close proximity to the lamella (see black arrows figure 3.8). Another region of high RACK1-EGFP concentration was observed at rear of the cell (see red arrows figure 3.8). Fluorescent ratiometric microscopy was also used to examine the relative concentration of endogenous NMHCIIA-mCherry in migrating FLO-1 cells. Results of this reported that NMHCIIA-mCherry was most concentrated at the leading edge where it was particularly concentrated in membrane ruffles (see figure 3.9, black arrows). This was similar to the NMHCIIA localization pattern observed in immunofluorescently stained FLO-1 cells where strong NMHCIIA staining was observed at the leading edge (see figure 3.7). High NMHCIIA-mCherry concentration was also observed at the cell periphery (see green arrows, figure 3.9) and in stress fibers (see red arrows figure 3.9) which was again consistent with the immunofluorescent NMHCIIA staining patterns shown in figure 3.7.

Therefore, both immunofluorescent staining and fluorescent ratiometric imaging have indicated that RACK1 and NMHCIIA have different localisation patterns in migrating FLO-1 cells. However, the two proteins do appear to localize in close proximity to each other in the region where the lamellipodia ends and lamella begins; which raised that possibility that some transient interactions between small subsets of both proteins might possibly occur. As discussed in chapter 1, RACK1 is a scaffolding protein which can temporarily bind to other proteins and shuttle them to subcellular destinations. We therefore hypothesized that RACK1 might transiently interact with NMHCIIA, perhaps in order to deliver it its site of action at the leading edge. FLO-1 cells were therefore co-transfected with both RACK1-EGFP and NMHCIIA-mCherry and Förster Resonance Energy Transfer (FRET) Sensitized Emission microscopy was employed to investigate if any interactions occurred between the two endogenous proteins. However, no evidence of any interaction between RACK1 and NMHCIIA in migrating cells was found using this technique.

Our finding that NMHCIIA localizes to the lamellipodial leading edge of migrating FLO-1 cells suggests that it may play a role in the migration process. Non-muscle myosin II is widely believed to contribute to contraction of the cell rear during migration (Vicente-Manzanares et al., 2009, Ridley et al., 2003). Therefore, our observation of NMHCIIA at the rear of migrating FLO-1 cells is not surprising. However, it has been claimed that non-muscle myosin II does not reside in, or seem to play a role in, the physical organization of the lamellipodium (Vicente-Manzanares et al., 2009). However, some studies in non-cancerous cells are beginning to suggest roles for myosin II in anterior protrusive events related to cell migration (Gupton et al., 2005, Betapudi et al., 2006). Furthermore, our group's previous finding that NMHCIIA (with RACK1) translocates to the lamellipodial leading edge of migrating T-cells has been

complemented by a study in migrating MDA-MB-231 breast cancer cells which also reported NMHCIIA localisation at the leading edge of lamellipodial structures (Kim and Helfman, 2003). Therefore, our finding that NMHCIIA localises to the leading edge lamellipodia of migrating FLO-1 cells is unusual, but not wholly unprecedented. Our observation that NMHCIIA localises to stress fibre like structures also suggests that NMHCIIA may be important for the regulation of some aspect of cytoskeletal dynamics in FLO-1 cells. Therefore, an investigation into the role of NMHCIIA in FLO-1 cell migration and cytoskeletal regulation will be the subject of chapter 4 of this thesis.

3.5 Conclusion

In conclusion we have found no evidence for an interaction between RACK1 and NMHCIIA in oesophageal cells that is of functional significance to cell migration or cancer progression. We have found that NMHCIIA localizes to the lamellipodial leading edge of migrating FLO-1 cells and also localizes to stress-fibre like structures in this cell type. This suggests that NMHCIIA could perhaps be involved in FLO-1 cellular processes such as cell migration and cytoskeletal organization. This will be investigated in the next chapter.

Chapter 4: Non Muscle Myosin Heavy Chain IIA regulates cell morphology, stress fibre structure and cell migration in FLO-1 oesophageal adenocarcinoma cells.

4.1 Introduction

Non-muscle myosin heavy chain IIA (NMHCIIA) is a member of the non-muscle myosin II family of actin-based molecular motors that catalyse the conversion of chemical energy into directed movement and force and thus serve as important regulatory components of the eukaryotic cytoskeleton. Non-muscle myosin II isoforms cyclically interact with adenosine triphosphate and F-actin to promote cytoskeletal force generation in important cellular processes such as cell migration and invasion, morphology changes, cytokinesis, adhesion dynamics and endo- and exo-cytosis (Heissler and Manstein, 2013). As a force-producing protein with actin cross-linking properties, non-muscle myosin II is believed to play important roles in cell body translocation and retraction of the posterior of the cell during migration (Betapudi et al., 2006, Ridley et al., 2003, Vicente-Manzanares et al., 2009).

Non-muscle myosin II functions as a heterohexamer; a non-muscle myosin II molecule is composed of a pair of heavy chains of 230 kDa, two 20 kDa regulatory light chains that regulate non-muscle myosin II activity and a pair of 17 kDa essential light chains that stabilise the heavy chain structure (Vicente-Manzanares et al., 2009). Three different heavy chain isoforms of non-muscle myosin II exist in mammalian cells, namely non-muscle myosin heavy chain IIA (NMHCIIA), non-muscle heavy chain IIB (NMHCIIIB) and non-muscle heavy chain IIC (NMHCIIIC) which are encoded by three different genes, *MYH9*, *MYH10* and *MYH14*, respectively. The heavy chain isoform determines the non-muscle myosin II isoform. For non-muscle myosin II motors to retain their normal, native and active conformations, the light chains must be bound to the heavy chains. Deletion of a specific heavy chain results in the loss of that non-muscle myosin II isoform (Vicente-Manzanares et al., 2009).

Non-muscle myosin II appears to have different functions depending on cell type but it has been found to associate with a wide variety of molecules involved in cell migration including actin microfilaments, microtubules, S100A4 and cadherin and integrin complexes (Li and Bresnick, 2006, Liu et al., 2016, Vicente-Manzanares et al., 2009). It is an important regulator of adhesion, polarity and actin stress fibre organisation; all of which are important processes required for cell migration. Each of the different non-muscle myosin isoforms affects these processes differently (Vicente-Manzanares et al., 2009).

The precise function of NMHCIIA appears to vary with cell type. It is a protein which has been linked to both tumour progression and suppression; again its precise function in relation to cancer progression appears to vary depending on the cancer type or subtype. Similarly, a number of cell migration-associated roles for NMHCIIA have been described but whether this motor protein promotes or suppresses migration appears to depend on the cellular setting and environment in which it is functioning (Ouderkirk and Krendel, 2014).

NMHCIIA appears to promote migration and invasion of breast carcinoma cells. Comparative proteomic analysis of an invasive and non-invasive variant of MCF-7 breast cancer cells revealed that the two variants had a strikingly identical proteomics pattern, except for the finding that NMHCIIA was present in the invasive MCF-7/6 variant, but hardly detectable in the non-invasive MCF-7/AZ variant. Inhibition of NMHCIIA subsequently blocked the invasion of MCF-7/6 cells in three-dimensional invasion substrata such as embryonic chick heart fragments and type I collagen gels (Derycke et al., 2011). In MDA-MB-231 breast carcinoma cells, NMHCIIA is recruited to spreading lamellipodia and NMHCIIA depletion impairs migration but enhances lamellar spreading (Betapudi et al., 2006). Loss of NMHCIIA also blocks the ability of MDA-MB-231 cells to compress a matrix and results in a matrix with decreased stiffness compared to parental controls. This indicates that NMHCIIA is also involved in matrix remodeling and mechanical homeostasis in breast cancer cells, making it a potential therapeutic target not just for blocking metastasis but also for blocking the effects of matrix stiffness on tumor proliferation and progression (Hindman et al., 2015).

NMHCIIA is overexpressed in gastric cancer tissues and a recent study reported that inhibition of NMHCIIA decreased the migratory and invasive abilities of SGC-7901 and MGC-803 gastric cancer cell lines. The role of NMHCIIA in migration and invasion of gastric cancer cells may be mediated, at least in part, via the JNK signalling pathway as NMHCIIA depletion also decreased the levels of p-JNK and p-c-Jun in these cells (Liu et al., 2016).

NMHCIIA promotes migration and invasion of renal cell carcinoma by interacting with the chemokine receptor cysteine (C)-X-C receptor (CXCR4), a G-protein-coupled receptor that exerts a vital role in distant metastasis of this type of cancer. NMHCIIA facilitates the nuclear translocation of CXCR4, an event which is required for its pro-migratory function (Xu et al., 2016).

A study in SK-CO15 colonic adenocarcinoma cells demonstrated multiple functions for NMHCIIA in the regulation of migration and invasion. Such functions included regulation of the F-actin cytoskeleton and cell-matrix adhesions as well as the control of intracellular signalling and protein expression. Interestingly, this study reported that NMHCIIA depletion resulted in

decreased 2-D migration into a scratch wound but increased 3-D invasion. The reduction in 2-D migration was associated with increased cell adhesiveness to collagen and laminin and increased expression of β 1-integrin and paxillin. On a 2-D substrate, NMHCIIA deficient cells lacked focal adhesions and F-actin stress fibres. Meanwhile, the enhanced invasion of NMHCIIA depleted cells was dependent on the activation of Raf-ERK1/2 signalling, enhanced calpain activity, and increased calpain-2 expression (Babbin et al., 2009).

Research conducted in oesophageal SCC has demonstrated that strong NMHCIIA expression is associated with significantly shorter patient survival. In oesophageal SCC tissues, elevated NMHCIIA expression level was significantly correlated with increased lymph node metastases poorer cancer differentiation, and advanced tumour stage. NMHCIIA depletion from KYSE-510 oesophageal squamous cancer cells increased cell matrix adhesion and reduced their migration ability in both scratch wound and transwell migration assays(Xia et al., 2012).

In other carcinoma cells, NMHCIIA depletion has been found to increase 2-D migration in scratch wound assays, for example NMHCIIA depletion from A549 lung carcinoma cells decreased the number of stress fibres and focal adhesions and increased the rate of cell migration in a wound-healing assay (Sandquist et al., 2006). In a study in squamous cell carcinoma NMHCIIA depletion stimulated invasive squamous cell carcinoma formation in tumour-susceptible backgrounds, indicating that NMHCIIA may function as a tumour suppressor and an inhibitor of metastasis in this cancer setting. Interestingly, this study found that NMHCIIA not only exerted its influence via actin-related processes but also regulated posttranscriptional p53 stabilization in squamous cell carcinoma (Schramek et al., 2014). Other studies suggest that NMHCIIA acts as a negative regulator of migration by regulating actomyosin–microtubule crosstalk and microtubule dynamics. Even-Ram and colleagues reported that NMHCIIA depletion gave rise to marked defects in cellular contractility, focal adhesions, actin stress fibre organization and tail retraction. However it also increased cell migration and exaggerated membrane ruffling, which was dependent on Rac1, the Rac1 activator Tiam1 and the microtubule motor kinesin Eg5. NMHCIIA depletion stabilised microtubules and shifted the balance between actomyosin and microtubules with increased microtubules in active membrane ruffles(Even-Ram et al., 2007).

More studies are required to understand and reconcile the divergent findings on the role of NMHCIIA as a promoter of migration versus an inhibitor of migration and as a tumour suppressor versus a metastasis promoter. It may be that NMHCIIA simply functions differently in different cells, in different microenvironments and in different cancer types.

4.2 Objectives

In chapter 3 we found that NMHCIIA localises to the lamellipodial leading edge and retracting rear of migrating FLO-1 cells. We also observed NMHCIIA localisation to stress fibre-like structures in FLO-1 cells. This indicated that NMHCIIA may be involved in the regulation of FLO-1 cell migration. Interestingly, we observed a different NMHCIIA localisation pattern in SKGT-4 oesophageal adenocarcinoma cells; it did not localise to the leading edge of migrating cells but exhibited a perinuclear localisation pattern in both resting and migrating cells. In this chapter we sought to further investigate the role of NMHCIIA in both SKGT-4 and FLO-1 cells and to compare and contrast its function in both cells with a particular focus on cell migration. This study is timely as the role of NMHCIIA has not been previously examined in oesophageal adenocarcinoma.

4.3 Results

4.3.1 Expression of non-muscle myosin heavy chain II isoforms in SKGT-4 and FLO-1 oesophageal adenocarcinoma cells.

Three different isoforms of the non-muscle myosin heavy chain exist, namely non muscle myosin heavy chain IIA (NMHCIIA), non-muscle myosin heavy chain IIB (NMHCIIB) and non-muscle myosin heavy chain IIC (NMHCIIC). Cancer cells of epithelial origin have varying expression and relative abundance of each non muscle myosin II heavy chain isoform (Ma et al., 2010). Each isoform has different biochemical properties and functions, however some redundancy between isoforms does exist and it is possible that poorly expressed isoforms may not be as physiologically relevant as those isoforms which are expressed in abundance (Babbin et al., 2009, Bao et al., 2005, Bao et al., 2007). It was therefore of interest to examine the expression of each NMHCII isoform in oesophageal adenocarcinoma cells prior to further examination of the role of NMHCIIA in oesophageal adenocarcinoma cell migration and other functions. Western blotting was used to examine the expression of NMHCIIA, NMHCIIB and NMHCIIC (figure 4.1). This revealed strong protein expression of NMHCIIA in both SKGT-4 and FLO-1 cells. Strong NMHCIIB expression was also observed in FLO-1 cells while its expression in SKGT-4 cells was barely detectable. NMHCIIC expression was not detected by Western blotting analysis in SKGT-4 cells. In FLO-1 cells, NMHCIIC was not detected in two out of three Western blots and was barely detectable in one blot.

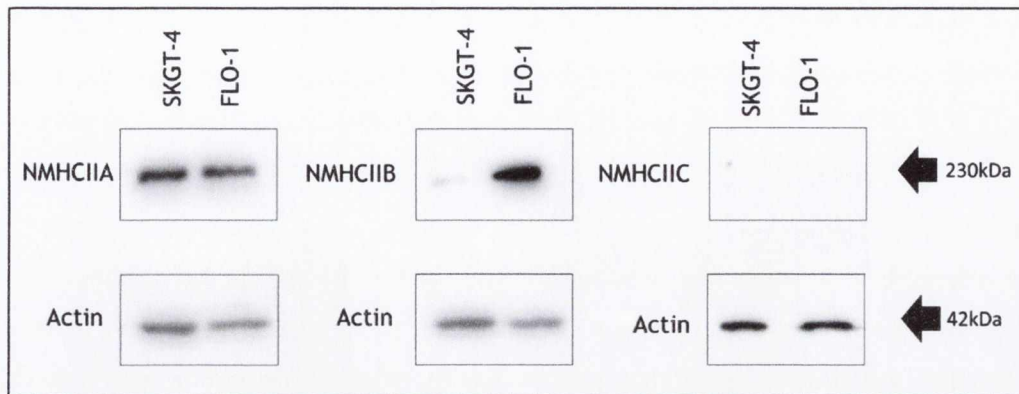


Figure 4.1: Expression of the three NMHCII heavy chain isoforms in FLO-1 and SKGT-4 oesophageal adenocarcinoma cells: NMHCIIA is strongly expressed in both FLO-1 and SKGT-4 cells. NMHCIIIB is strongly expressed in FLO-1 cells but is barely detectable by Western blotting in SKGT-4 cells. NMHCIIIC expression was not detectable by Western blotting in SKGT-4 cells. In FLO-1 cells, NMHCIIIC was barely detectable in one Western blot replicate and was not detected at all in two replicates.

4.3.2 NMHCIIA depletion reduces migration of FLO-1 cells but does not affect migration of SKGT-4 cells in a wound healing assay.

In chapter 3 we demonstrated that NMHCIIA localizes to the lamellipodial leading edge of migrating FLO-1 cells. We also observed NMHCIIA at the rear of migrating cells and in stress fibre like structures (see chapter 3; section 3.3.5 and section 3.3.7). These results indicated that NMHCIIA may play a role in FLO-1 cell migration. In contrast, SKGT-4 cells do not migrate as individual cells with well-defined, visible lamellipodia and we did not observe NMHCIIA localisation at the leading edge of migrating SKGT-4 cells (see chapter 3; section 3.3.2).

To further investigate the role of NMHCIIA in FLO-1 and SKGT-4 cell migration we used an siRNA approach to deplete cells of NMHCIIA and assessed migration using a scratch wound assay. The % NMHCIIA depletion achieved using siRNA was very similar in SKGT-4 and FLO-1 cells; approximately 80% depletion was observed, on average, in each cell line. We observed no reduction in migration in NMHCIIA depleted SKGT-4 cells (figure 4.2a.) However, we observed a significant reduction in migration when FLO-1 cells were depleted of NMHCIIA (figure 4.2b). Thus, NMHCIIA appears to be important for the migration of some, but not all, oesophageal adenocarcinoma cells.

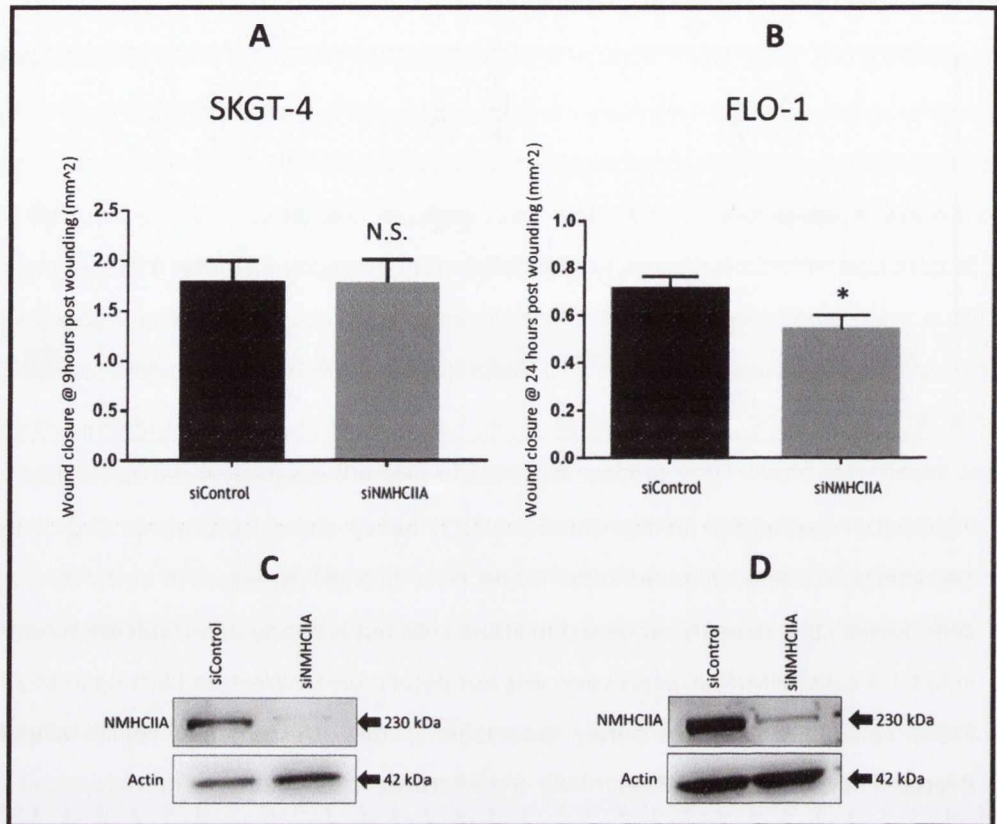


Figure 4.2: NMHCIIA depletion reduces FLO-1 cell migration but does not reduce SKGT-4 cell migration.

NMHCIIA was depleted from SKGT-4 or FLO-1 cells using siRNA. Cells were allowed to form a confluent monolayer which was then wounded using a p200 pipette tip. Migration into the wound was assessed by measuring the area of wound closure after 9 hours (in the case of fast moving SKGT-4 cells) or 24 hours (in the case of slower moving FLO-1 cells). (A) In SKGT-4 cells, there was no significant difference in wound closure between NMHCIIA depleted and control cells. N.S. stands for not significant; i.e. $P > 0.05$, unpaired t test. Data presented are mean $\pm$ SEM. N=3. (B). In FLO-1 cells, the rate of wound closure was significantly reduced in NMHCIIA depleted cells in comparison to controls $*P \leq 0.05$, unpaired t test. Data presented are mean $\pm$ SEM. N=3. (C – D) Representative blots show successful NMHCIIA depletion in SKGT-4 (C) and FLO-1 cells (D).

4.3.3 NMHCIIA colocalises with F-actin at the lamellipodial leading edge of migrating FLO-1 cells but does not colocalise with F-actin at the leading edge of migrating SKGT-4 cells.

We have shown that NMHCIIA expression is required to maintain normal migration rate in cells (FLO-1) in which NMHCIIA localises to the leading edge during migration (as defined by a

wound healing assay). We found no role for NMHCIIA in maintaining normal migration rate in cells (SKGT-4) in which NMHCIIA does not localise to the leading edge during migration. We therefore sought to gain further insight into the potential role of NMHCIIA at the leading edge and to analyse its localisation in relation to leading edge F-actin during migration of both FLO-1 and SKGT-4 cells.

Migrating cells were therefore co-stained with immunofluorescently tagged phalloidin (which selectively binds F-actin) and immunofluorescent dye conjugated to an antibody specific for NMHCIIA. This revealed that NMHCIIA co-localises with F-actin at the lamellipodial leading edge of migrating FLO-1 cells (figure 4.3b). SKGT-4 cells do not display visible lamellipodia but a 'leading edge front' can be identified which faces in the direction of migration; strong F-actin staining was observed at this leading front and was also observed around most of the cell periphery. We observed no NMHCIIA co-localisation with F-actin at the leading edge front of SKGT-4 cells (figure 3.4a). Thus, we have observed that NMHCIIA co-localises with F-actin at the leading edge of FLO-1 cells and this protein is required to maintain the migration rate of FLO-1 cells. In contrast, NMHCIIA does not co-localise with F-actin in SKGT-4 cells, whose rate of migration is not reduced when they are depleted of this protein.

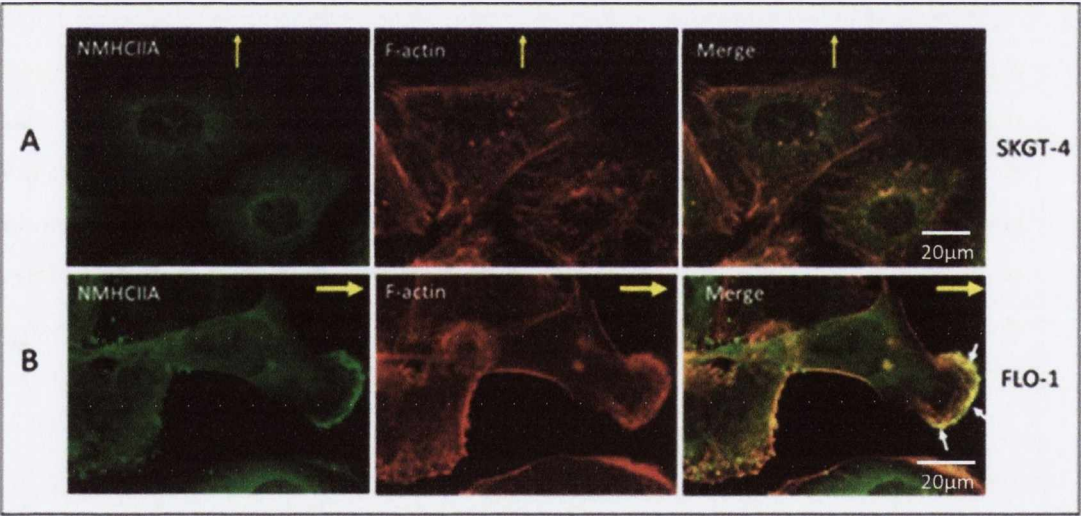


Figure 4.3. NMHCIIA co-localises with F-actin at the lamellipodial leading edge of migrating FLO-1, but not SKGT-4, cells. Cells were grown to confluence and a scratch wound was then made in the cell monolayer (to stimulate migration) using a p200 pipette tip. At 24 hours post wounding, cells were fixed, permeabilised, and stained with phalloidin and immunofluorescent dye conjugated to an antibody specific for NMHCIIA. Images were collected by confocal microscopy using a 63X objective. Yellow arrows indicate direction of migration into scratch wounds. (a) SKGT-4 cells migrate collectively in cell sheets and do not display visible, individual

lamellipodia. NMHCIIA does not localize to the leading edge front of migrating SKGT-4 cells and does not colocalise with F-actin in this cell type. (b) FLO-1 cells do display lamellipodia when migrating and NMHCIIA localises to the lamellipodial leading edge in these cells where it colocalises with F-actin; as is highlighted by white arrows.

4.3.4 NMHCIIA depletion alters FLO-1 cell morphology and F-actin organisation

We observed that NMHCIIA depleted FLO-1 cells had an altered cell morphology and a 'ragged' cell periphery as highlighted by a white arrowhead in figure 4.4a. This observation, in addition to our finding that NMHCIIA co-localises with F-actin in this cell type, led us to hypothesise that NMHCIIA may play a role in regulating or organising F-actin structures in FLO-1 cells. To test this we depleted FLO-1 cells of NMHCIIA using siRNA and then used phalloidin staining to analyse F-actin in control and NMHCIIA depleted cells. This revealed that NMHCIIA depleted cells had an increased number of very thick, F-actin-rich stress fibres; an example of such a stress fibre is highlighted by the white arrows in figure 4.4a. Cells displaying four or more of these thick stress fibres were counted and expressed as a percentage of total cells in the population. This demonstrated that the NMHCIIA depleted population of cells had a statistically significant increase in the percentage of cells with $\geq$ four thick stress fibres; as is illustrated graphically in figure 4.4b.

These results indicate that NMHCIIA is required for the maintenance of normal F-actin dynamics in FLO-1 cells; our findings indicate that it may be required to regulate F-actin bundling, to control the size and integrity of stress fibres, or to promote the efficient turnover of stress fibres. Thus, the reduced rate of migration observed in NMHCIIA depleted FLO-1 cells may be due to altered F-actin dynamics and organisation.

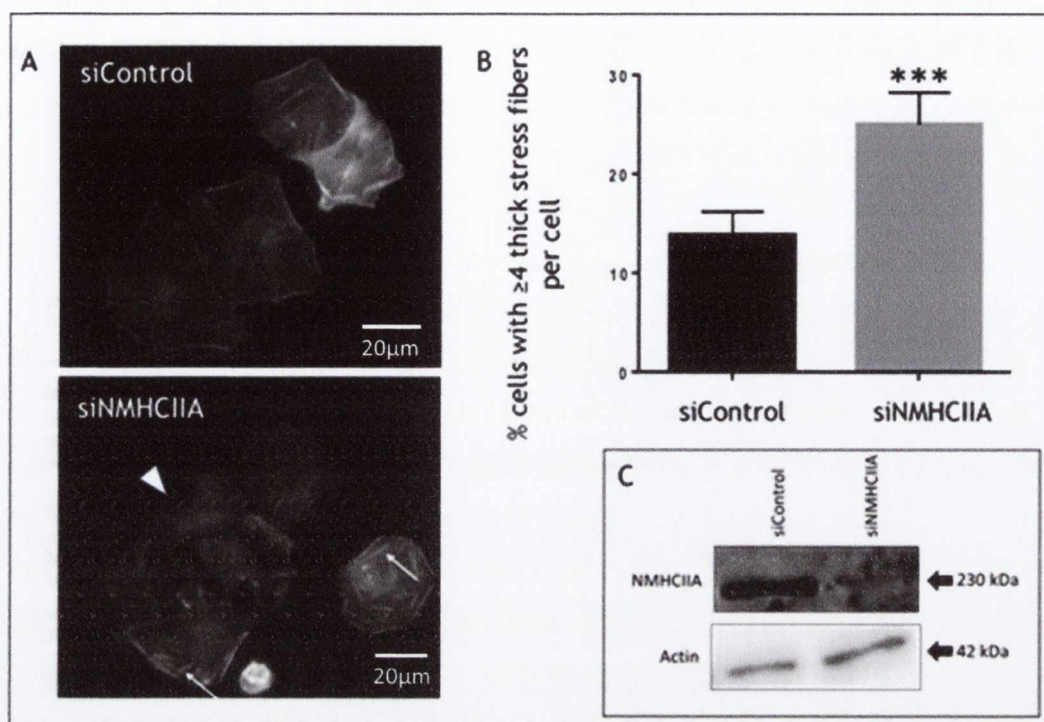


Figure 4.4: NMHCIIA depletion alters FLO-1 cell morphology and F-actin organisation. (A) Phalloidin stained F-actin in control and NMHCIIA depleted FLO-1 cells. NMHCIIA depleted cells have extra thick stress fibres (white arrows) and an altered morphology with ragged cell edges (white arrow head). (B) Percentage cells with ≥ 4 thick stress fibres is significantly increased in NMHCIIA depleted cells. $N=3$ *** $P \leq 0.001$. Data presented are mean $\pm$ SEM analysed using unpaired Student's t-test. (C) Representative Western blot showing successful siRNA-mediated NMHCIIA depletion from FLO-1 cells.

4.3.5 NMHCIIA depletion does not affect FLO-1 cell proliferation as assessed by MTT assay

We also examined the impact of NMHCIIA depletion on FLO-1 cell proliferation (figure 4.5). Using an MTT assay we found no difference in proliferation between NMHCIIA-depleted and control FLO-1 cells. This indicates that the NMHCIIA-depletion induced decrease in FLO-1 cell migration that is reported in this chapter is independent of proliferation-associated processes. However it is important to note the MTT assay does have limited sensitivity; the limitations of the MTT assay are discussed in detail in section 5.4). Therefore the possibility that NMHCIIA depletion does has a subtle impact on FLO-1 cell proliferation (that is too small for an MTT assay to detect) cannot be ruled out.

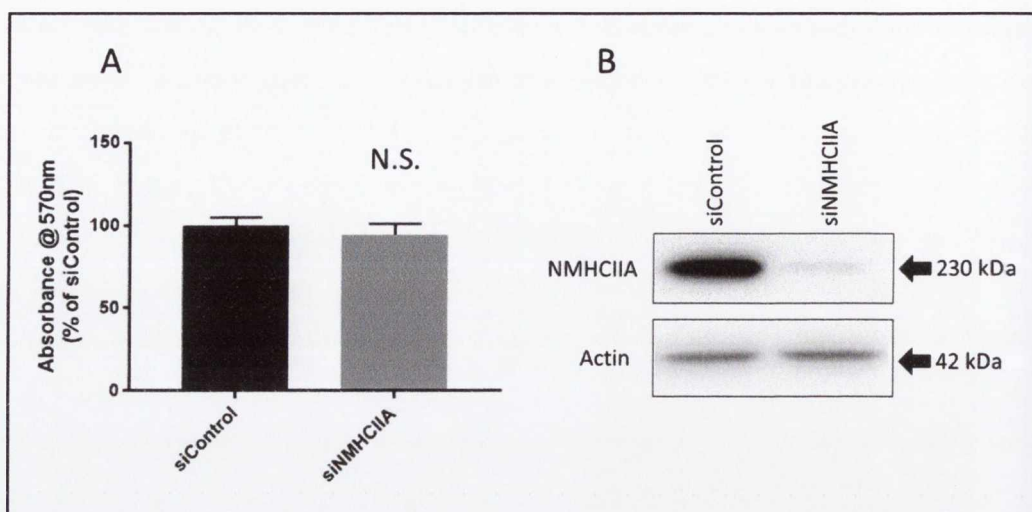


Figure 4.5: NMHCIIA depletion does not affect FLO-1 cell proliferation as assessed by MTT assay.

NMHCIIA was depleted from FLO-1 cells using an siRNA approach. (A) Cell proliferation was assessed by MTT assay. Data presented are mean $\pm$ SEM analysed by unpaired Student's t-test. N=4. N.S. stands for not significant; i.e. $P > 0.05$. (B) Representative Western blot showing successful depletion of NMHCIIA from FLO-1 cells.

4.4 Discussion

In chapter 3 we demonstrated, using both immunofluorescent staining of endogenous NMHCIIA and fluorescent ratiometric imaging of recombinant NMHCIIA-mCherry, that NMHCIIA localises to the lamellipodial leading edge of migrating FLO-1 cells and also to the retracting cell rear. This polarised expression in migrating cells indicated that NMHCIIA may play a role in FLO-1 cell migration. In contrast to FLO-1 cells, we found that migrating SKGT-4 cells do not exhibit NMHCIIA localisation at the leading edge. In this current chapter we further investigated the potential roles of NMHCIIA in oesophageal adenocarcinoma cell migration and also examined its function in cell proliferation.

Three heavy chain isoforms of non-muscle myosin II have been identified in vertebrates, namely NMHCIIA, NMHCIIIB and NMHCIIIC which are encoded by three separate genes; MYH9, MYH10 and MYH14 respectively. Non muscle myosin isoforms exhibit a high degree of sequence similarity at the amino acid level. However each isoform does have different enzymatic and biochemical properties and thus they can have both interchangeable and unique functions(Vicente-Manzanares et al., 2009, Heissler and Manstein, 2013). The relative

expression of each NMHCII isoform varies with different cell and tissue types, with some cells/tissues expressing all three isoforms in varying proportions while other cells/tissues express only one or two of the three isoforms (Ma et al., 2010). When studying the role of NMHCII isoform(s) in a particular cell type it is useful to examine the expression of each isoform, given the fact that some redundancy of isoform function can exist and the most highly expressed isoform may play the most significant physiological role (Babbin et al., 2009, Bao et al., 2005, Bao et al., 2007). Therefore, prior to commencing a more detailed study of the role of NMHCIIA in OAC cells, we examined the expression of all three NMHCII isoforms in both SKGT-4 and FLO-1 cells.

Using Western blotting, we observed strong protein expression of NMHCIIA in both SKGT-4 and FLO-1 cells. We observed strong NMHCIIIB expression FLO-1 cells while its expression in SKGT-4 cells was much lower. NMHCIIIC expression was not detected by Western blotting analysis in SKGT-4 cells and was barely detectable in FLO-1 cells (figure 4.1). It was interesting to observe the difference in NMHCIIIB expression between FLO-1 and SKGT-4 cells. This finding illustrates that NMHCII isoform expression varies not only between cells isolated from different organs but also between cancer cells isolated from the same cancer type. It is possible that this variation in expression might, at least in part, account for the sometimes conflicting reports with regard to the role of NMHCII isoforms in cancer. Having demonstrated that NMHCIIA is abundantly expressed by both SKGT-4 and FLO-1 cells, we considered it worthy of further study in these cells.

An siRNA approach was used to deplete FLO-1 and SKGT-4 cells of NMHCIIA. Scratch wound assays were then used to compare the rate of migration between NMHCIIA-depleted and control cells. NMHCIIA depletion had no impact on the rate of migration in SKGT-4 cells (figure 4.2a). In contrast, NMHCIIA depletion significantly reduced the speed of migration in FLO-1 cells (figure 4.2b). In chapter 3 we found that NMHCIIA localizes to the lamellipodial leading edge of migrating FLO-1, but not SKGT-4 cells. Thus our finding that NMHCIIA depletion reduces migration of FLO-1, but not SKGT-4 cells, indicates that NMHCIIA might promote FLO-1 cell migration through, at least in part, a role at the lamellipodial leading edge. To further investigate NMHCIIA localisation at the leading edge we examined NMHCIIA localisation in relation to F-actin at the leading edge of migrating cells. This revealed that NMHCIIA co-localises with F-actin in lamellipodial ruffles and at the lamellipodial leading edge of migrating FLO-1 cells (figure 4.3b). SKGT-4 cells do not display obvious, visible lamellipodia but a 'leading edge front', which faces in the direction of migration, can be identified in SKGT-4 cells migrating into a wound; strong F-actin staining was observed at this leading front and was also

observed around most of the cell periphery. We observed no NMHCIIA co-localisation with F-actin at the leading edge front of SKGT-4 cells (figure 4.3a).

Our results indicate that NMHCIIA may contribute to FLO-1 cell migration through an association with F-actin at the lamellipodial leading edge. It has been proposed in the literature that myosin II molecules might also contribute to membrane trafficking events related to leading edge functions. Indeed myosin II has been associated with vesicle trafficking in a number of settings which could be relevant to leading edge functions such as the delivery of adhesion molecules or signalling proteins during migration (Betapudi et al., 2006, Togo and Steinhardt, 2004, Durán et al., 2003).

The different modes of migration employed by FLO-1 and SKGT-4 cells might possibly account for the differences we observed between the two cell types with regards to the role of NMHCIIA in their migration. In vivo, cancer cells can use a variety of migration modes to achieve successful spreading and metastasis. These include collective cell migration, which has been observed in many tumours of epithelial origin that still have cadherin-based cell-cell junctions, and single cell migration which is characteristic of leukaemia cells, lymphoma cells and cells that have originated from solid tumours that have undergone EMT and lost cadherin expression (Ouderkirk and Krendel, 2014, Mierke, 2013). In our hands, SKGT-4 cells migrate collectively, in a tight cell sheet in which contacts between cells are retained. In scratch wound assays, FLO-1 cells also exhibit some collective cell migration but we also observed some single cell migration in FLO-1 cells, whereby they separated from their neighbours during migration into a scratch wound. We could identify individual lamellipodia and a clear front-rear polarity in FLO-1 cells but we observed no visually detectable lamellipodia in SKGT-4 cells.

During single cell migration, the principal engine for movement appears to be at the cell front, where dynamic membrane protrusion occurs and the cell adheres to the ECM. As the lamellipodium extends forward, new integrin-based adhesions form which eventually mature into focal adhesions that serve as anchors for longitudinal acto-myosin cables. These mature focal adhesions are retained until they reach the retracting edge of the cell, where they undergo disassembly (Mayor and Etienne-Manneville, 2016). During collective cell migration, multicellular polarity occurs and 'supracellular' organisation of the actin cytoskeleton generates traction and protrusion force for migration (Friedl and Gilmour, 2009). In collectively migrating 2D sheets of cells, the front row of cells form a continuous rim of lamellipodia that bridge multiple cell bodies and drive the leading edge forward (Friedl and Gilmour, 2009).

Both single cell migration and collective cell migration exhibit a large amount of morphological and functional diversity and a number of subtypes of both forms of migration exist. Myosin

activity is undoubtedly important for many types of cell migration. Single cell migration is highly reliant on cell contractility for which myosin activity is likely to be particularly important. Collective cell migration requires dynamic reorganization of cell-cell junctional complexes and associated cytoskeletal structures to allow cells to change their positions and move without losing cell-cell contacts; myosin activity is likely to be important in such reorganisation (Ouderkirk and Krendel, 2014). However, as our observed differences between SKGT-4 and FLO-1 cells illustrate, the importance of particular forms and isoforms of myosin for migration can vary between cells and is likely to depend on the particular mode of migration employed by a cell. Tumour cells can exhibit plasticity in their ability to switch between forms of migration (which makes treatment to inhibit migration/metastasis more difficult) and differential regulation of myosin isoforms may be important for selection of a particular migration mode (Ouderkirk and Krendel, 2014). Research which improves our understanding of the differential roles of myosin isoforms in different modes of migration is important, as it should deepen our insight into how cancer cell migration and metastasis is regulated in different situations.

In addition to reducing migration, we observed that NMHCIIA depletion also alters the morphology of FLO-1 cells and gives the cell a 'ragged' cell periphery (see the white arrowhead in figure 4.4a). Furthermore, we have found that NMHCIIA depletion leads to an increase in thick, F-actin-rich stress fibres, in FLO-1 cells (see figure 4.4a and 4.4b).

This suggests that NMHCIIA is required to maintain normal F-actin dynamics in FLO-1 cells; our findings indicate that it may be required to regulate F-actin bundling, to control the size and integrity of stress fibres, or to promote the efficient turnover of stress fibres. Thus, the reduced rate of migration observed in NMHCIIA depleted FLO-1 cells may be due to disrupted F-actin dynamics and organisation.

A recent study by Hindman and colleagues also examined the effects of NMHCIIA depletion on cell morphology and actin organisation; this time in MDA-MB-231 breast carcinoma cells (Hindman et al., 2015). As we had observed in FLO-1 cells, NMHCIIA depletion disrupted the normal organisation of actin stress fibres in MDA-MB-21 cells. However, while we observed an increase in thick stress fibres *throughout* the cell, Hindman *et al* found that NMHCIIA depletion from MDA-MB-321 cells resulted in an increase in prominent stress fibres at the *periphery of the cell* and a decrease in *centrally located fibres*. Interestingly however, the Hindman study also examined the impact of NMHCIIIB depletion from MDA-MB-231 cells and found that this yielded a similar phenotype to that which we observed in NMHCIIA depleted FLO-1 cells; NMHCIIIB depletion resulted in the formation of thicker, shorter stress fibres in MDA-MB-231 cells which resembled the thickened stress fibres displayed by NMHCIIA-depleted FLO-1 cells.

This again indicates that the precise nature of NMHCII isoform function varies with cell type and that one isoform may play the role of another isoform when the cell setting in which it is expressed changes. To illustrate this point further, a study using lung carcinoma cell lines found that NMHCIIA depletion from A549 cells caused them to become almost devoid of stress fibres although they did occasionally display some bundled F-actin (Sandquist et al., 2006). In these cells, NMHCIIA depletion also gave rise to a more motile phenotype with a quicker rate of migration in scratch wound assays; thus these findings are almost in direct contrast to our findings in FLO-1 cells and may be due to differences between the two cell types. Meanwhile, results from a study in colonic adenocarcinoma cells (SK-CO15) were similar to our findings in FLO-1 cells, in that NMHCIIA reduced cell migration into a scratch wound and gave rise to cells with a ragged cell morphology. However, in contrast to our finding that NMHCIIA depletion gives rise to an increased number of thick stress fibres in FLO-1 cells, NMHCIIA depleted SK-CO15 cells displayed diffuse unstructured F-actin labelling and no prominent stress fibres were visible (Babbin et al., 2009).

4.5 Conclusion

In conclusion we have shown that NMHCIIA depletion reduces migration of FLO-1 cells. We observed no impact of NMHCIIA depletion on FLO-1 cell proliferation. This indicates that NMHCIIA plays a role in mechanisms which specifically contribute to FLO-1 cell migration under the conditions that we studied. NMHCIIA colocalizes with F-actin at the lamellipodial leading edge of migrating FLO-1 cells. Moreover, NMHCIIA depletion alters FLO-1 cell morphology and leads to an increased number of thick, F-actin-rich stress fibres. This suggests that NMHCIIA may be required to maintain normal F-actin dynamics in FLO-1 cells. Thus, the reduced rate of migration observed in NMHCIIA depleted FLO-1 cells may be due to disrupted F-actin dynamics and organisation.

Chapter 5: An investigation into the expression and function of RACK1 in oesophageal cells

5.1 Introduction

As is discussed in chapter 1, RACK1 is a multifunctional scaffolding protein with roles in numerous cellular processes such as cell adhesion and migration, cell proliferation, survival and apoptosis and transcription and protein synthesis (chapter 1; section 1.3). The expression of RACK1 is altered in a wide range of cancers, and depending on the cancer type, it has been shown to promote or inhibit particular processes involved in carcinogenesis including cell migration, invasion and metastasis (chapter 1; section 1.3.2 and 1.3.3).

To our knowledge, the role of RACK1 in oesophageal adenocarcinoma has not been previously examined. However RACK1 has been studied in a number of other cancers of the digestive system. Several studies have demonstrated a tumour suppressive function for RACK1 in colon cancer. Mamidipudi and coworkers have shown that RACK1 can bind to, and inhibit, Src kinase. They report that RACK1 regulates growth of colon cancer cells by suppressing Src activity at G1 and mitotic checkpoints, thereby delaying cell cycle progression (Mamidipudi et al., 2007, Mamidipudi et al., 2004). RACK1 also induces apoptosis in colon cancer cells, where, in part by inhibiting Src, it promotes mitochondrial cell death and blocks Akt-mediated cell survival (Mamidipudi and Cartwright, 2009). Again, in part through inhibition of Src, RACK1 also promotes cell–cell adhesion and reduces the invasive potential of colon cancer cells (Swaminathan and Cartwright, 2012). However not all studies in colon cancer demonstrate roles for RACK1 which are consistent with a tumour suppressive function. A study in matching apoptosis-sensitive and apoptosis-resistant SW480 colon cancer cells reported that RACK1 downregulates levels of the pro-apoptotic protein Fem1b in the apoptosis-resistant cells and suggest that it may therefore promote apoptosis-resistance during progression of colon cancer (Subauste et al., 2009). Furthermore, an in situ hybridisation mediated analysis of genes expressed in primary colon cancers found that RACK1 was universally overexpressed in cells of all 11 colon cancers examined (Saito et al., 2002). Thus further studies are needed to understand and reconcile these apparently contradictory findings regarding the role of RACK1 in colon cancer.

A study by Deng and colleagues demonstrated that RACK1 expression is reduced in gastric tumour samples and that reduced expression of RACK1 is associated with poor tumour differentiation. In gastric cancer cell lines, RACK1 expression levels were also correlated with

differentiation status, with highest RACK1 expression detected in well-differentiated cells and lowest RACK1 expression in poorly differentiated cells. RACK1 depletion from gastric cancer cells accelerated their anchorage-independent proliferation in soft agar, while RACK1 overexpression decreased their tumourigenicity in nude mice. Interestingly, this study found that RACK1 forms a complex with glycogen synthase kinase Gsk3 β and Axin which promotes the interaction between Gsk3 β and β -catenin and thus stabilises the β -catenin destruction complex. Upon Wnt3a stimulation, RACK1 represses Wnt signalling by inhibiting recruitment of Axin by Dishevelled 2. Thus RACK1 appears to act as a tumour suppressor in gastric cancer and as a negative regulator of the Wnt signaling pathway(Deng et al., 2012).

In other cancers of the digestive system, RACK1 appears to contribute to cancer development or progression. Research by Ruan and colleagues reports that RACK1 is highly expressed in normal liver and frequently upregulated in hepatocellular carcinoma. They found that aberrant expression of RACK1 contributed to both *in vitro* chemoresistance and *in vivo* tumour growth of hepatocellular carcinoma. Interestingly, these effects depended on ribosome localisation of RACK1. Ribosomal RACK1 coupled with PKC β II to promote the phosphorylation of eukaryotic initiation factor 4E, which led to preferential translation of potent factors involved in growth and survival (Ruan et al., 2012). Meanwhile, another study in hepatocellular carcinoma demonstrated that RACK1 promotes carcinoma growth by enhancing mitogen-activated protein kinase 7 activity(Guo et al., 2013).

Wang and colleagues observed increased expression of RACK1 in a majority of recurrent cases of oral squamous cell carcinoma and demonstrated that elevated expression of RACK1 is a strong predictor for poor clinical outcome in patients with this malignancy(Wang et al., 2009). A study in two oral squamous cell carcinoma cell lines found that RACK1 silencing inhibited cell proliferation and decreased the invasive, migratory and adhesive capabilities of the cancer cells(Li et al., 2012b). RACK1 has also been associated with poor prognosis in oesophageal squamous cell carcinoma and RACK1 depletion reduces oesophageal squamous cell proliferation, migration and invasion *in vitro* and *in vivo* (Hu et al., 2013, Wang et al., 2015a). Finally a recent study in pancreatic ductal adenocarcinoma reported an association between RACK1 overexpression and poor patient prognosis. Downregulation of RACK1 impaired growth and induced G1/S cell cycle arrest and apoptosis in pancreatic ductal adenocarcinoma cells. RACK1 silencing was associated with decreased bcl-2 expression and increased cleaved caspase3 expression, suggesting that RACK1 may promote carcinogenesis by driving cell cycle progression and suppressing apoptosis(Li et al., 2016).

As was discussed in chapter 1 (section 1.1), the development of oesophageal adenocarcinoma is strongly associated with chronic gastro-oesophageal reflux disease(Pennathur et al., 2013).

Gastro-oesophageal refluxate contains a mixture of acid, bile acids and other harmful chemicals which damage the oesophagus and promote inflammation (Duggan et al., 2010, Jenkins et al., 2006, Jenkins et al., 2004). Chronic exposure to such refluxate can promote the development of Barrett's oesophagus, which, in some cases, progresses further into oesophageal adenocarcinoma (McQuaid et al., 2011). Evidence is accumulating to suggest that the bile acid component of the refluxate plays an important role in initiating and promoting cancer in Barrett's tissues (McQuaid et al., 2011, Jenkins et al., 2004). Deoxycholic acid (DCA) is an unconjugated, bile acid found in the refluxate which has been particularly implicated in carcinogenesis (Duggan et al., 2010). It is a secondary bile acid which is produced from primary bile acids by microbial action in the gut (McQuaid et al., 2011). The precise mechanisms through which DCA promotes oesophageal adenocarcinoma are still poorly understood but it has been shown to modulate the expression and function of a number of genes and proteins in oesophageal cells (Byrne et al., 2012) (Huo et al., 2011, Jenkins et al., 2004). However the influence of DCA on RACK1 expression and function has not yet been examined.

5.2 Objectives

A wide variety of cancer related roles have been ascribed to RACK1. However, no studies of the expression or function of RACK1 in oesophageal adenocarcinoma exist in the scientific literature. The tumour-promoting bile acid, DCA, is a component of refluxate and is implicated in oesophageal carcinogenesis. DCA has been shown to modulate the expression and function of a range of proteins but the influence of this tumour promoting bile acid on RACK1 had not been previously investigated. Therefore in this chapter we aim to examine the expression and function of RACK1 in oesophageal adenocarcinoma cells.

Specifically we aim to:

- Investigate expression levels of RACK1 across a panel of cell lines representing the normal-Barrett's-adenocarcinoma sequence.
- Examine the effects of DCA on RACK1 expression and localisation in oesophageal adenocarcinoma cells.
- Investigate if RACK1 plays a functional role OAC cell biology, with a particular focus on cell migration.

5.3 Results

5.3.1 RACK1 expression levels generally increase with increasing disease progression in a panel of cell lines representing the normal oesophagus - Barrett's metaplasia - Barrett's dysplasia - adenocarcinoma sequence.

Oesophageal adenocarcinoma typically develops by a multi-step process recognised histologically as the normal-Barrett's metaplasia-Barrett's dysplasia-adenocarcinoma sequence (de Almeida et al., 1997). We examined if RACK1 protein expression was altered during this sequence of cancer development by examining its protein levels by Western blot analysis in cell lines derived from each stage of the sequence (figure 5.1). The HEEC and HET-1A cell lines were used to represent noncancerous oesophageal cells, the QhTERT and GohTERT represent Barrett's metaplasia and dysplasia respectively while three cell lines, SKGT-4, OE33 and FLO-1 represent oesophageal adenocarcinoma. RACK1 protein levels between noncancerous HEEC cells and each other cell line were statistically compared as is described in the legend of figure 5.1. Of the seven cell lines examined, the non-cancerous cell line, HEEC, displayed the lowest protein levels of RACK1. The cell line representing Barrett's metaplasia (QhTERT) also had relatively low levels of RACK1. RACK1 expression was significantly increased by over 100% (in comparison to HEEC levels) in the Barrett's dysplastic cell line (GohTERT) and in two (FLO-1 and OE33) of the three adenocarcinoma cell lines examined. Thus, in general, levels of RACK1 increased across the sequence from normal to cancer. However there were some exceptions to this trend. Firstly, the SKGT-4 adenocarcinoma cell line had much lower levels of RACK1 than those observed in the other adenocarcinoma cell lines and, of all the cell lines examined, it actually had the second lowest level of RACK1. Secondly, the HET-1A cell line had much higher levels of RACK1 than those found in the HEECs cell line and actually had higher levels of RACK1 than those found in the cell line representing the next stage of the sequence, namely the QhTERT cell line representing Barrett's metaplasia. Nevertheless, the HET-1A cell line still had considerably lower levels of RACK1 expression than those found in the Barrett's dysplastic cell line (GohTERT) and in the OE33 and FLO-1 OAC adenocarcinoma cell lines. Furthermore, the ability of the HET-1A cell line to represent the 'normal', non-cancerous oesophageal epithelium has been questioned as it has been reported to have dysplastic properties and to lack some typical epithelial cell characteristics (Underwood et al., 2010). Thus, the HEEC cell line may provide a better representation of RACK1 protein levels in the non-cancerous oesophagus.

The cell lines used in this study were derived from different patients and are therefore not isogenic. Furthermore, actin was used as the sole loading control when examining the

expression of RACK1 across this panel of cell lines. It is possible the actin protein levels could vary between the different cell lines that were examined. Therefore the accuracy of this experiment, in investigating if RACK1 levels change across the OAC cancer progression sequence could be improved by probing for a number of different loading control proteins and in this way calculating relative levels of RACK1 in relation to multiple reference proteins. Unfortunately, time constraints did not permit this to be performed. Therefore it is important to note that this study merely gives us an indication that RACK1 expression is upregulated in oesophageal adenocarcinoma but does not give us conclusive proof of same.

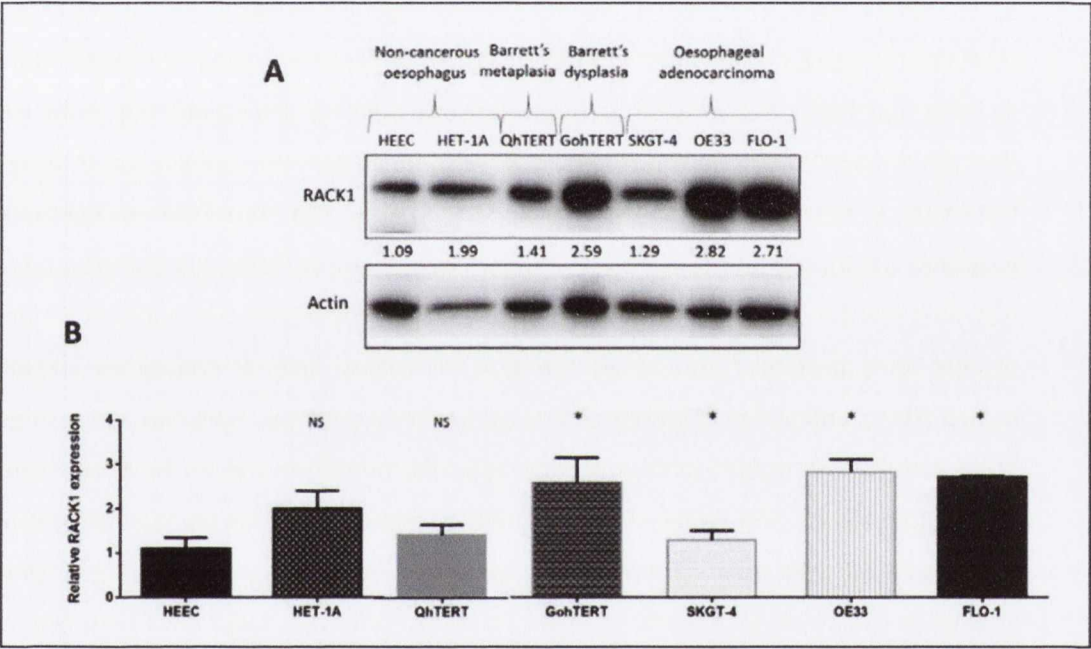


Figure 5.1 RACK1 expression levels generally increase with increasing disease progression in a panel of cell lines representing the normal oesophagus→Barrett's metaplasia→Barrett's dysplasia→ adenocarcinoma sequence. Cells were grown to approximately 70% confluency. Cells were then harvested, protein extracts were prepared and Western blot analysis was performed using an antibody against human RACK1. Blots were also probed with antibody against human actin to control for loading variation. Three independent experiments were performed and one representative blot is shown in (A). Relative RACK1 protein levels were quantified from Western blots using densitometry and normalized against actin. One of the three densitometry values calculated for the non-cancerous HEEC cell line was arbitrarily set to one and all other densitometry values were compared against it. In (A), the values beneath

each RACK1 band represent the mean relative RACK1 protein expression levels for the indicated cell lines calculated from three independent experiments. Values obtained for each cell line were statistically compared to values obtained for the HEEC cell line using Student's t-tests. Results are illustrated graphically in (B). Each bar represents the mean $\pm$ SEM of three independent experiments. Asterisks indicate cell lines whose RACK1 protein levels were significantly higher than those found in the HEEC cell line ($P \leq 0.01$ is represented by **, $P \leq 0.05$ is represented by * and $P > 0.05$ is considered not significant (NS)).

5.3.2 DCA suppresses cellular levels of RACK1 protein in two oesophageal adenocarcinoma cell lines (SKGT-4 and FLO-1).

DCA is present at micromolar concentrations (0-300 μ M) in oesophageal aspirates (Nehra et al., 1999). 300 μ M DCA is capable of inducing NF- κ B expression and activation in oesophageal adenocarcinoma cell lines (Abdel-Latif et al., 2004, Jenkins et al., 2004). NF- κ B can activate transcription of the murine RACK1 gene and may also regulate expression of the human RACK1 gene (Choi et al., 2003). We therefore hypothesized that DCA may upregulate RACK1 protein levels through an NF- κ B mediated increase in RACK1 gene expression. To investigate this we treated SKGT-4 and FLO-1 oesophageal adenocarcinoma cells with 300 μ M DCA or vehicle control (DMSO) for 24 hours. RACK1 protein levels were then examined by Western blot analysis. Blots were also probed with an antibody against actin to control for variations in loading. Three independent experiments were performed for each cell line and results show that treatment with 300 μ M DCA actually reduced RACK1 protein levels in both cell lines (Figure 5.2). RACK1 protein levels were significantly reduced by an average of 31% in DCA treated SKGT-4 cells ($P=0.01$) and by an average of 26% in DCA treated FLO-1 OAC cells ($P=0.03$).

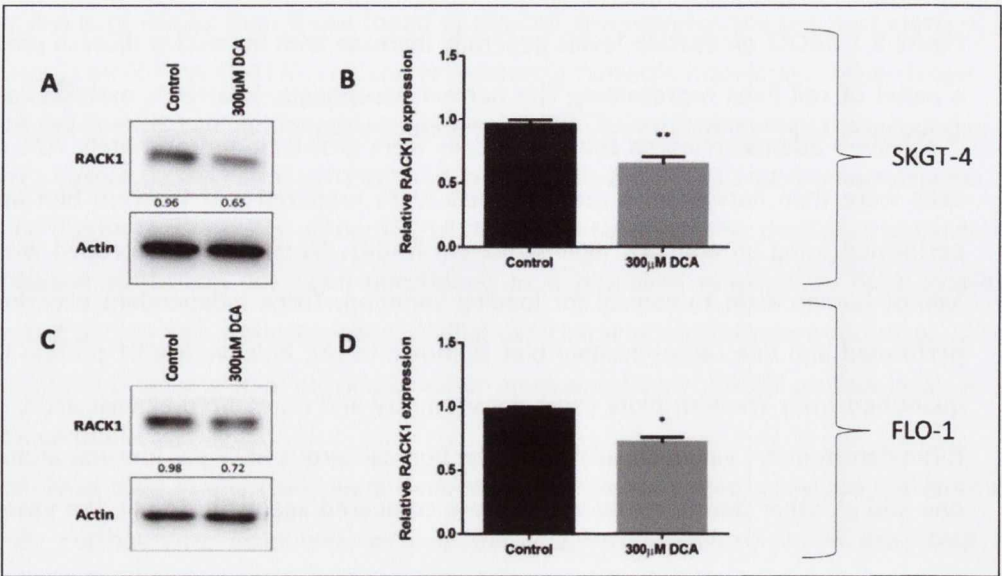


Figure 5.2: DCA suppresses RACK1 protein levels in SKGT-4 and FLO-1 oesophageal adenocarcinoma cells (A and B) SKGT-4 cells were treated with 300 μ M DCA or control vehicle (DMSO) for 24 hours. Cells were harvested, protein extracts were prepared and Western blot analysis was performed using an antibody against human RACK1. Blots were also probed with antibody against human actin to control for loading variation. Three independent experiments were performed and a representative blot is shown in (A). RACK1 protein expression levels were quantified from Western blots using densitometry and normalised against actin. One of the three densitometry values calculated for control cells was arbitrarily set to one and all other densitometry values were compared against it. Values beneath the RACK1 bands in (A) represent mean relative RACK1 protein expression levels calculated from three independent experiments. Results are illustrated graphically in (B). Each bar represents the mean $\pm$ SEM of three independent experiments, analysed using a Student's t-test. ** indicates $P \leq 0.01$. There was a statistically significant difference between the RACK1 protein levels in control and DCA treated cells. In DCA treated cells, RACK1 protein levels were reduced to an average of 69% relative to controls. (C and D) FLO-1 cells were treated in the same way as that described for SKGT-4 cells above and data obtained were also analysed and displayed identically. A representative blot is shown in (C) and results are illustrated graphically in (D). There was a statistically significant difference between RACK1 protein levels in control and DCA treated cells. In DCA treated cells, RACK1 protein levels were reduced to an average of 74% relative to controls. * indicates $P \leq 0.05$.

5.3.3 DCA does not alter the subcellular localization of RACK1 in SKGT-4 and FLO-1 adenocarcinoma cells.

RACK1 plays an important role in shuttling its binding partners around the cell in response to certain stimuli and in targeting or anchoring its partners at particular cellular locations (Adams et al., 2011). DCA can alter the subcellular localization of several RACK1 binding partners including IGF-IR, Src and a number of PKC isoforms (Adams et al., 2011). Thus, it is possible that the subcellular localization of RACK1 might also be altered by DCA. To investigate this, we treated SKGT-4 and FLO-1 cells with 300 μ M DCA for 90 minutes or 24 hours. RACK1 localization was then examined in control and DCA treated cells using immunofluorescence. However, in both cell lines, neither 90 minute nor 24 hour treatment with DCA altered RACK1 subcellular localization which remained predominately cytoplasmic (with particularly strong staining in the perinuclear area) in control and treated cells (figure 5.3a and 5.3b). SKGT-4 and FLO-1 cells were also treated with lower concentrations of DCA (100 μ M) for 90 minutes and 24 hours and this also had no effect on RACK1 subcellular localization (data not shown).

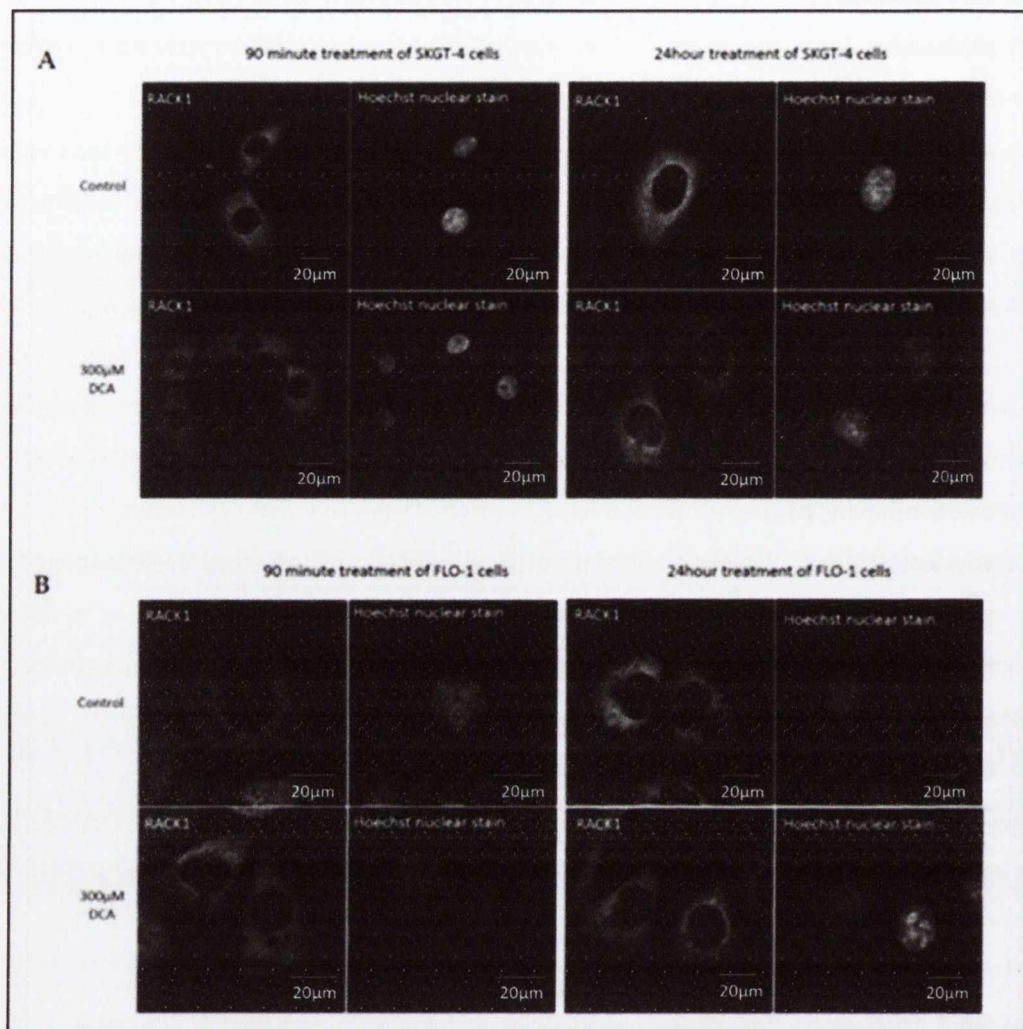


Figure 5.3. RACK1 has a cytoplasmic subcellular localization in SKGT-4 and FLO-1 cells which is not altered by DCA. The subcellular localization of RACK1 was examined using immunofluorescent staining and confocal microscopy. SKGT-4 (A) and FLO-1 cells (B) in the above images were treated with control vehicle or 300 µM DCA for 90 minutes or 24 hours prior to fixation, staining with an anti-RACK1 antibody and imaging by confocal microscopy. RACK1 subcellular localization is similar in both control and DCA treated cells and is predominately cytoplasmic, relatively diffuse in nature and is most concentrated in perinuclear areas.

5.3.4 RACK1 depletion does not affect SKGT-4 oesophageal adenocarcinoma cell proliferation as assessed by MTT assay.

RACK1 was depleted from SKGT-4 cells using an siRNA approach. An MTT assay was then used to assess cell proliferation in control and RACK1 depleted cells. Results of this demonstrated no difference in proliferation between RACK1 depleted cells and control cells treated with non-targeting siRNA. This is illustrated graphically in figure 5.4.a.

5.3.5 RACK1 depletion leads to a trend towards decreased SKGT-4 cell proliferation, when assessed by nuclear count and cell confluency assays, but this is not statistically significant.

In order to investigate the possibility that RACK1 depletion results in a small decrease in SKGT-4 cell proliferation, which may not be detectable by MTT assay, we employed an alternative method of assessing cellular proliferation in SKGT-4 cells. A nuclear count assay was used to examine change in cell number over time in RACK1 depleted and control cells. We observed a trend towards a smaller fold increase in cell number in RACK1-depleted SKGT-4 cells, however this was not statistically significant (figure 5.4b). We also assessed the impact which RACK1-depletion had on the ability of cells to grow, spread out and cover the surface area of wells into which they were plated; i.e. to become confluent. This revealed a trend towards a small decrease in % confluency in RACK1-depleted cell populations in comparison to controls; however this was not statistically significant (figure 5.4c).

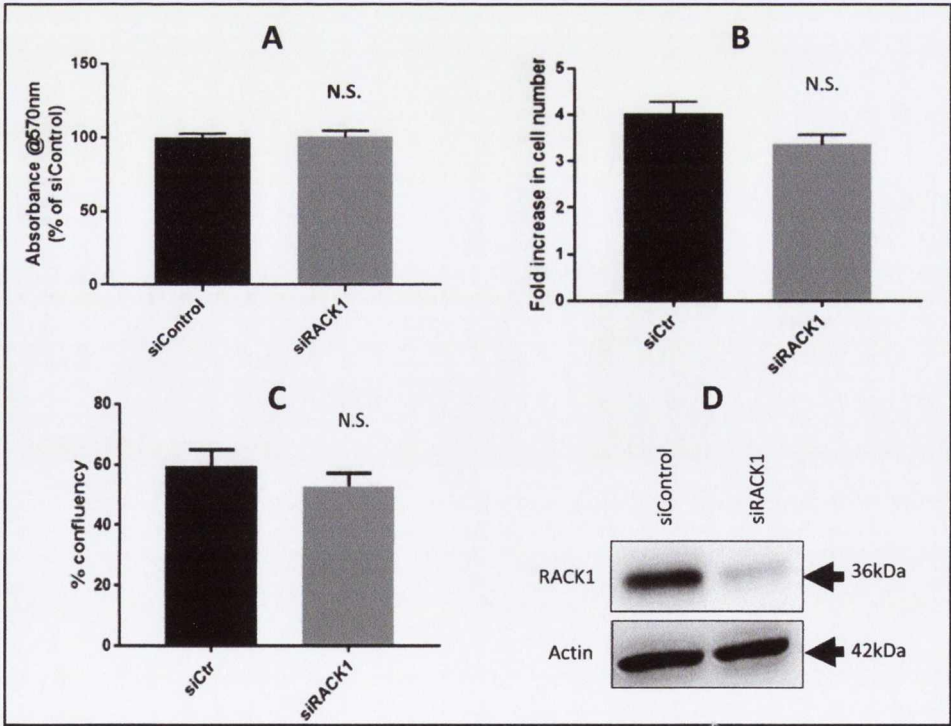


Figure 5.4: Impact of RACK1-depletion on SKGT-4 oesophageal adenocarcinoma cell proliferation. In the case of A, B and C, data presented are mean $\pm$ SEM analysed by Student's

t-test. N=3 in each case. N.S. stands for not significant; i.e. $P > 0.05$. (A) SKGT-4 cells were transfected with anti-RACK1 siRNA or non-targeting control siRNA. Following successful RACK1 depletion, an MTT assay was performed which analysed cell metabolic activity. (B) SKGT-4 cells were transfected with anti-RACK1 siRNA or non-targeting control siRNA. Fold change in cell number from 42 hours post transfection to 96 hours post transfection was measured with the aid of a nuclear count assay to evaluate cell number. (C) SKGT-4 cells were transfected with anti-RACK1 siRNA or non-targeting control siRNA. At 72 hours post transfection % cell confluency was measured by measuring the % surface area of tissue culture plates that was covered by the cells. (D) In the case of A, B and C above, RACK1 depletion was verified for each experiment by Western blotting. Shown is a representative blot.

5.3.6 RACK1 depletion reduces FLO-1 oesophageal adenocarcinoma cell proliferation as assessed by MTT assay. RACK1 was depleted from FLO-1 cells using an siRNA approach. An MTT assay then used to assess cell metabolic activity (as an indicator of cell proliferation) in control and RACK1 depleted cells. Results of this indicated that RACK1-depleted FLO-1 cells have a reduced proliferative ability in comparison to control cells treated with non-targeting siRNA. This is illustrated graphically in figure 5.5.

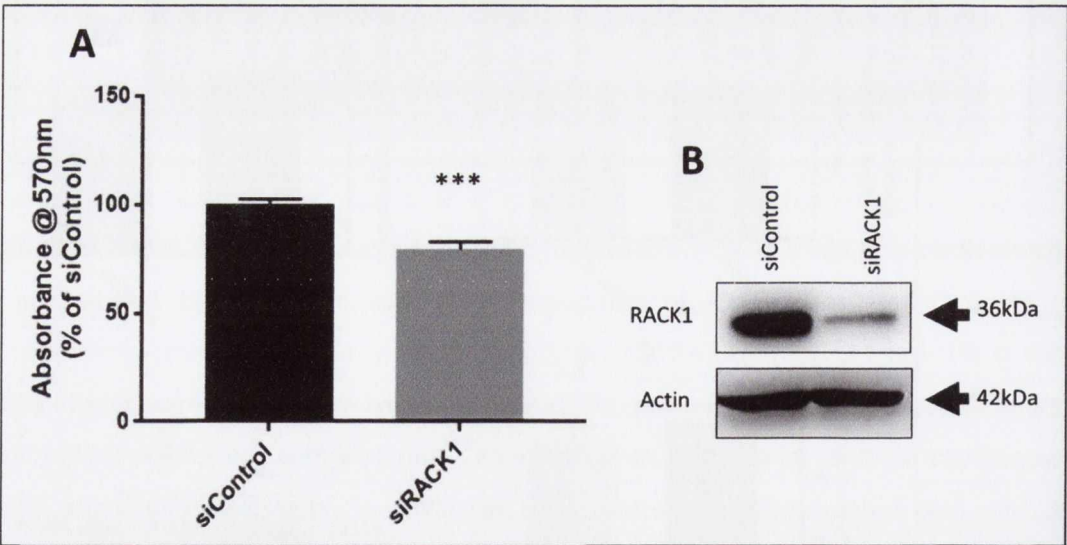


Figure 5.5. RACK1 depletion reduces FLO-1 oesophageal adenocarcinoma cell proliferation as assessed by MTT assay. FLO-1 cells were transfected with anti-RACK1 siRNA or non-targeting control siRNA. Following successful RACK1 depletion, an MTT assay was performed which analysed cell metabolic activity, as an indicator of proliferative ability. RACK1-depleted FLO-1

cells exhibited a reduction in proliferation in comparison to control cells. Data represents mean $\pm$ SEM analysed using Student's t-test. *** $P \leq 0.001$ (N=5). (B) Representative Western blot showing successful siRNA-mediated RACK1 depletion from FLO-1 cell.

5.3.7 RACK1 depletion reduces FLO-1 cell proliferation as assessed on an X-CELLigence label-free real-time cell analysis platform.

FLO-1 cell proliferation in control and RACK1 depleted cells was also analysed in real time over a 40 hour period using an X-CELLigence label-free real-time cell analysis platform. (See more details on this system in chapter 2). Results of this were consistent with our MTT assay findings described above, as they also demonstrated a significant proliferation reduction in RACK1 depleted FLO-1 cells in comparison to cells treated with control siRNA. Results are illustrated graphically in figure 5.6.

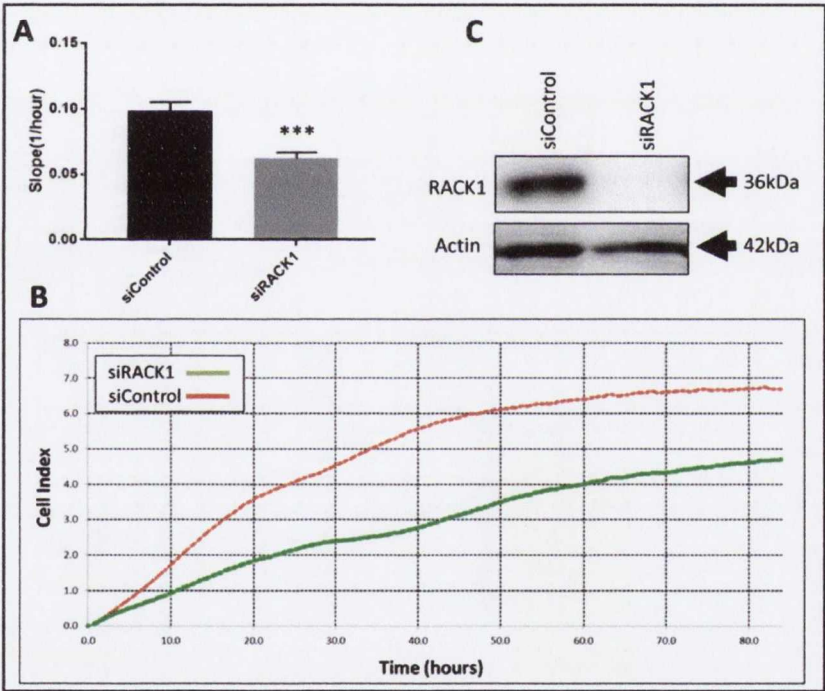


Figure 5.6 RACK1 depletion reduces FLO-1 cell proliferation as assessed on an X-CELLigence label-free real-time cell analysis platform.

Cell proliferation was assessed in real time using the xCELLigence RTCA platform. FLO-1 cells were transfected with anti-RACK1 siRNA or non-targeting control siRNA. At 48 hours post transfection cells were seeded into xCELLigence E plates and proliferation was measured over 80 hours and recorded in terms of cell index. (A) The rate of proliferation as determined by

analysing the slope of the line between the 8 and 48 hour interval. *** $P \leq 0.001$, unpaired t test. $N=3$. Data presented are mean $\pm$ SEM (B) A representative graph of cell index values for control and RACK1 depleted cells recorded in real time over a period of 80 hours. (C) Representative Western blot showing RACK1 depletion from FLO-1 cells using siRNA.

5.3.8 RACK1 depletion reduces 2D cell migration into a scratch wound assay in both SKGT-4 and FLO-1 cells.

RACK1 was depleted from FLO-1 and SKGT-4 cells using an siRNA approach and 2D cell migration was assessed using scratch wound assays. This revealed that both FLO-1 and SKGT-4 cell migration is reduced in RACK1 depleted cells in comparison to cells that had been treated with control siRNA. This is illustrated graphically in figure 5.7

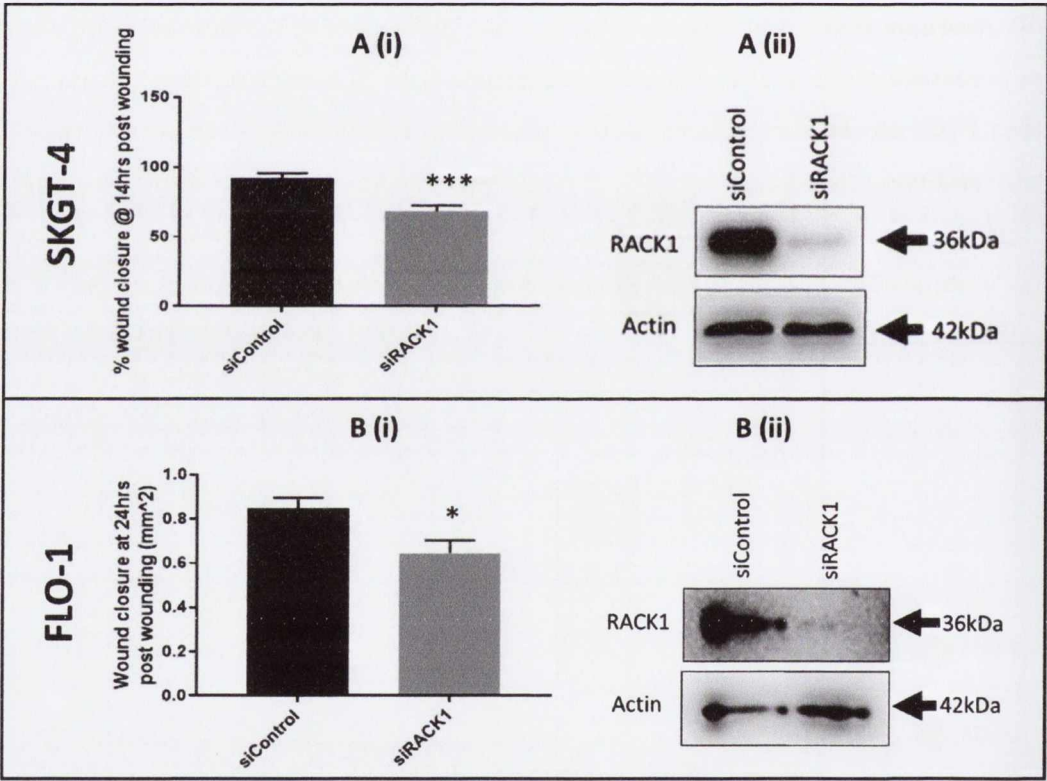


Figure 5.7. RACK1 depletion reduces 2D migration of both SKGT-4 and FLO-1 oesophageal adenocarcinoma cells. RACK1 was depleted from SKGT-4 or FLO-1 cells using siRNA. Cells were allowed to form a confluent monolayer which was then wounded using a p200 pipette tip. Migration into the wound was assessed by measuring the area of wound closure after 14 hours (in the case of fast moving SKGT-4 cells or 24 hours (in the case of slower moving FLO-1 cells). (A)(i) RACK1 depleted SKGT-4 cells exhibited decreased migration in comparison to control

cells treated with non-targeting siRNA. Data presented are mean $\pm$ SEM analysed using unpaired Student's t-test. *** indicates $P \leq 0.001$ (N=3). (A)(ii) Representative blot showing successful depletion of RACK1 from SKGT-4 cells. (B)(i) RACK1 depleted FLO-1 cells also exhibited decreased migration in comparison to control cells treated with non-targeting siRNA. Data presented are mean $\pm$ SEM analysed using unpaired Student's t-test. * indicates $P \leq 0.05$ (N=3). (A)(ii) Representative blot showing successful depletion of RACK1 from FLO-1 cells.

5.3.9 RACK1 depletion reduces 3D FLO-1 cell migration as analysed in real time using the xCELLigence label free real time cell analysis platform.

Having found that RACK1 depletion reduces both proliferation and 2D migration of FLO-1 cells we next investigated if RACK1 is required for the 3D migration of these cells. RACK1 was again depleted from FLO-1 cells using siRNA. 3D cell migration in control and RACK1 depleted FLO-1 cells was then assessed in real time using an xCELLigence cell analysis platform. Serum starved cells were placed into an upper, fibronectin-coated chamber CIM chamber and cell migration down into a lower, chamber containing cell culture medium with 10% serum, was monitored in real time. This revealed that RACK1 depletion significantly reduces the 3D migration of FLO-1 cells (figure 5.8). Thus our findings now indicate that RACK1 is required to maintain a normal rate of migration during both 2D and 3D migration of FLO-1 cells.

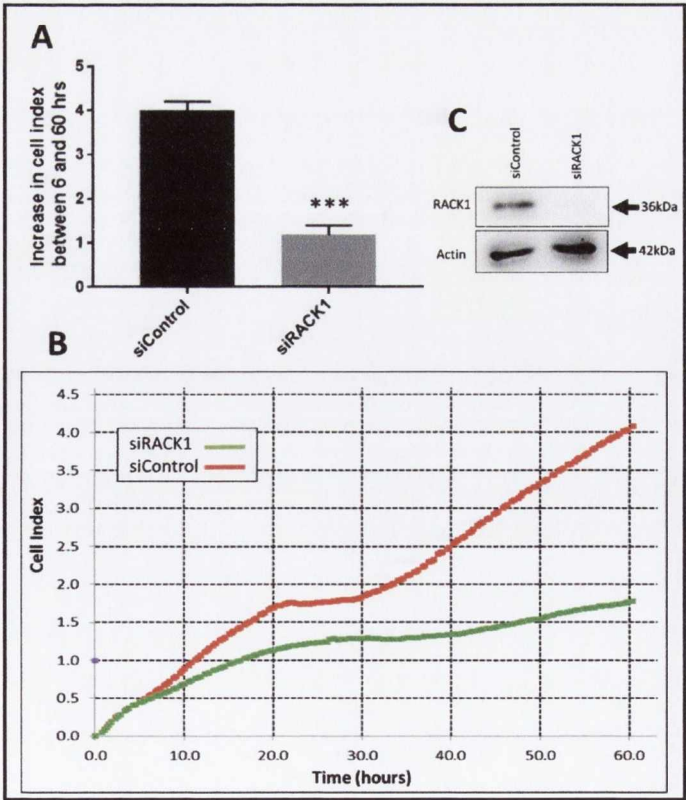


Figure 5.8 RACK1 depletion reduces 3D migration in FLO-1 cells as assessed by X-CELLigence label free real time cell analysis platform. RACK1 was depleted from FLO-1 cells using siRNA. The xCELLigence RTCA platform was used to assess 3D cell migration. Serum starved cells were placed into an upper, fibronectin-coated chamber CIM chamber and cell migration down into a lower, chamber containing cell culture medium with 10% serum, was monitored in real time and recorded in the form of cell index values. (A) The change in cell index between 6 and 60 hours. *** $P \leq 0.001$, $N=3$, unpaired t test. Data presented are mean $\pm$ SEM. (B) A representative graph of cell index values for control and RACK1 depleted cells recorded in real time over a period of 60 hours.(C) Representative Western blot showing RACK1 depletion from FLO-1 cells using siRNA.

5.4 Discussion

Oesophageal adenocarcinoma develops from the normal oesophageal mucosal lining through a multi-step sequence. Initially a metaplastic change takes place whereby the normal squamous epithelium of the oesophagus changes to a specialised or intestinal-type columnar epithelium called Barrett's oesophagus. Barrett's metaplasia can give rise to Barrett's dysplasia and finally, in some cases, to fully malignant adenocarcinoma (de Almeida et al., 1997, Shaheen and Ransohoff, 2002). Bile acids, such as the secondary bile acid, DCA, are believed to play an important role in promoting progression through this sequence and have been shown to modulate the expression of a variety of genes in oesophageal cells (Duggan et al., 2010, Looby et al., 2009, Byrne et al., 2012). However, our understanding of the precise mechanisms through which bile acids promote tumorigenesis is still poor. Furthermore, while Barrett's oesophagus is a significant risk factor for oesophageal adenocarcinoma, only a small percent of patients with Barrett's oesophagus go on to develop cancer (Pennathur et al., 2013). At present, our ability to predict prognosis and to differentiate between Barrett's oesophagus patients who will and will not progress to oesophageal adenocarcinoma is poor (Pennathur et al., 2013). Therefore, it is important that we further our understanding of the mechanisms and protein expression alterations which drive oesophageal adenocarcinoma as this should help us to develop novel strategies to predict, diagnose and treat this serious malignancy. A number of studies have reported that RACK1 expression is altered (usually, but not always, upregulated) in a variety of cancers including oesophageal squamous cell carcinoma, glioma and cancers of the lung and liver (Li and Xie, 2014, Guo et al., 2013, Hu et al., 2013, Deng et al., 2012, Peng et al., 2013). Furthermore, correlations between RACK1 expression and tumour stage and prognosis have also been observed in a number of studies; for example, a study in non-small-

cell lung cancer reported that upregulation of RACK1 was inversely correlated with tumour differentiation and positively correlated with both tumour stage and metastasis (Shi et al., 2012). However, to our knowledge, no studies on the expression and function of RACK1 in oesophageal adenocarcinoma have been published in the scientific literature. Furthermore, the influence of DCA on RACK1 expression and function has not been investigated. Therefore, in this chapter we examined RACK1 expression and function in oesophageal adenocarcinoma cells and also examined the influence which DCA has on RACK1 in these cells.

We examined the expression of RACK1 in a panel of seven oesophageal cell lines representing each stage of the oesophageal adenocarcinoma progression sequence from normal oesophagus to Barrett's metaplasia to Barrett's dysplasia to oesophageal adenocarcinoma. In general, we found that RACK1 protein levels increased as the sequence progressed from normal oesophagus to oesophageal adenocarcinoma. The lowest level of RACK1 expression was found in a cell line (HEEC) derived from non-cancerous oesophageal tissue while the first, second and third highest levels of RACK1 expression were found in the OE33 adenocarcinoma, FLO-1 adenocarcinoma and GohTERT Barrett's dysplastic cell lines respectively (figure 5.1). However two of the cell lines examined did make exceptions to this general trend. Firstly, the SKGT-4 adenocarcinoma cell line displayed low levels of RACK1 which were closer to those observed in non-cancerous or Barrett's metaplastic cells than to those found in the other adenocarcinoma cell lines. Thus, our findings indicate that RACK1 may only be upregulated in some, but not all, adenocarcinoma tumours. This is not all together surprising as cancer is a heterogeneous disease with many different subtypes occurring within the one cancer type which vary in their genetic alterations and their mechanisms of carcinogenesis. Secondly, the HET-1A cell line, which is a non-cancerous oesophageal epithelial cell line, had much higher levels of RACK1 than those found in the other non-cancerous oesophageal cell line examined (HEEC) and also had higher levels of RACK1 than those found in the QhTERT Barrett's metaplastic cell line. The HET-1A cell line was derived from normal oesophageal tissue, has remained non-tumourigenic in athymic, nude mice for over 12 months and is widely used as a model of normal oesophageal epithelium cells (Stoner et al., 1991). However, it has been reported that this cell line appears dysplastic when grown in organotypic culture, does not display evidence of squamous differentiation and is hyper proliferative in comparison to primary oesophageal epithelial cells (Underwood et al., 2010). Furthermore, HET-1A cells do not express the epithelial marker E-cadherin while they do express the mesenchymal marker vimentin (Underwood et al., 2010). A recent study in oesophageal squamous cell carcinoma has reported a positive correlation between RACK1 expression and vimentin, and an inverse correlation between RACK1 expression and E-cadherin (Wang et al., 2015a). If such findings

are considered, it is plausible to suggest that HET-1A cells might have higher RACK1 expression levels than those normally found in non-cancerous oesophageal epithelial cells. Thus, the HEEC cell line probably provides a better representation of RACK1 protein levels in the non-cancerous oesophagus.

Our study of RACK1 expression levels in this panel of oesophageal cell lines provides an insight into the potential modulation of RACK1 expression levels which may occur as oesophageal adenocarcinoma develops. However, the cell lines used in this study were derived from different patients and are therefore not isogenic. Therefore, some of the differences in RACK1 protein levels which we observed between cells may be due to the non-isogenic nature of these cells, rather than being due purely to the cells' particular stage in the cancer progression sequence. Furthermore, actin was used as the sole loading control when examining the expression of RACK1 across this panel of cell lines. It is possible the actin protein levels could vary between the different cell lines that were examined. Therefore the accuracy of this experiment, in investigating if RACK1 levels change across the OAC cancer progression sequence could be improved by probing for a number of different loading control proteins and in this way calculating relative levels of RACK1 in relation to multiple reference proteins. Unfortunately, time constraints did not permit this to be performed. Therefore it should be noted that this study merely gives us an indication that RACK1 expression is upregulated in oesophageal adenocarcinoma but does not give us conclusive proof of same. Nevertheless, by providing an indication that RACK1 may be upregulated in oesophageal adenocarcinoma, we established that further investigation into the role of RACK1 in this malignancy was worthwhile.

DCA has been identified as an important contributor to OAC progression and physiologically relevant concentrations of DCA can alter (both upregulate and downregulate) the expression of a variety of genes in OAC cells (Jenkins et al., 2004, Looby et al., 2009). However, to our knowledge, the effect of DCA on RACK1 expression has not been previously investigated.

300 μ M DCA induces both expression and activation of NF- κ B expression in oesophageal adenocarcinoma cell lines (SKGT-4, OE33 and OE21) (Jenkins et al., 2004, Abdel-Latif et al., 2004). Interestingly, NF- κ B binds to the murine RACK1 promoter and promotes RACK1 gene expression (Choi et al., 2003). Bioinformatic analysis indicates that the human RACK1 promoter also contains binding sites for the NF- κ B transcription factor (Del Vecchio et al., 2009). Therefore, we hypothesized that DCA may induce an increase in RACK1 protein expression through an NF- κ B mediated induction of RACK1 gene expression. However, our results found that 300 μ M DCA actually decreased RACK1 protein levels in SKGT-4 and FLO-1 OAC cells

(figure 5.2). Use of both SKGT-4 and FLO-1 cell lines in this study enabled us to examine the effect of DCA on OAC cell lines with relatively low (SKGT-4) and high (FLO-1) basal levels of RACK1. Western blotting was used in this study in preference to RT-PCR as it enabled us to examine the effects of DCA on actual RACK1 protein levels as opposed to its effects on mere transcription of the RACK1 gene. This is particularly important in the context of DCA as published literature indicates that DCA may sometimes have opposing effects on gene expression and protein levels. For example, various studies in oesophageal cells have indicated that DCA can increase EGFR *gene* expression but decrease EGFR *protein* expression (Jenkins et al., 2004, Byrne et al., 2012). Similarly, in this present study we also report that DCA significantly reduces RACK1 protein levels in oesophageal adenocarcinoma cells, in spite of the literature discussed above which suggested that DCA could potentially increase RACK1 gene expression through its upregulation of NF- κ B. DCA is a well-known inducer of oxidative stress which in turn has negative effects on protein translation and can also influence protein turnover (Duggan et al., 2010, Huo et al., 2011, Vogel et al., 2011). Thus it is possible that DCA does increase RACK1 gene expression through the NF- κ B dependent mechanism proposed above but that this increase in expression is not seen at the protein level due to an oxidative stress mediated reduction in protein translation. If time has permitted it would have been insightful to also assess the impact of DCA on RACK1 gene expression in our SKGT-4 and FLO-1 cell models using RT-PCR. It would also have been useful to assess if the DCA treatments performed in this project had an impact on cell viability/toxicity by performing cell viability/toxicity assays on the DCA treated and control cells.

The DCA mediated decrease in RACK1 protein levels which we have observed may have serious implications for the cell. RACK1 expression is believed to be tightly regulated in healthy cells and changes in its expression are likely to have dramatic consequences for the stability, activity and localisation of its multiple binding partners and could thus impact on a wide range of signalling pathways and cellular processes (Adams et al., 2011). A decrease in RACK1 expression is likely to result in increased competition for RACK1 among its interaction partners. The consequences of decreased RACK1 expression may depend on the outcome of such competition and on the particular proteins which retain or lose their interactions with RACK1 in this situation. For example, RACK1 interacts with and inhibits Src in colon cancer cells which inhibits cell cycle progression and thus reduces proliferation (Mamidipudi and Cartwright, 2009). In contrast, the interaction of RACK1 with other interaction partners can promote cell cycle progression; for example, The interaction of RACK1 with the DNA replication licensing factor, MCM7, promotes its activity and promotes cell cycle progression (Li and Xie, 2014).

Thus, alterations in the RACK1 associated proteome as a result of a DCA mediated reduction in RACK1 expression could potentially have pro- or anti-tumourigenic effects. However, our observation that RACK1 expression levels generally increase across the sequence from non-cancerous cells to adenocarcinoma indicates that RACK1 may promote the development of oesophageal adenocarcinoma.

It might possibly be that DCA-mediated down regulation of RACK1 protein levels may actually select for cells with increased RACK1 expression. DCA simultaneously induces apoptosis and activates survival signalling in oesophageal cells. Interestingly, a study in Barrett's oesophageal cells has shown that DCA induces apoptotic resistance through NF- κ B activation(Huo et al., 2011). Thus it is plausible that DCA may select for cells with higher levels of NF- κ B activation and it is possible that such cells may also have greater levels of RACK1 gene expression. It has been proposed that DCA mediated selection of apoptotic resistant cells plays an important role in neoplastic transformation(Duggan et al., 2010). RACK1 can protect against apoptosis in a variety of cellular settings, for example it can integrate survival signalling or can promote the ubiquitination-mediated degradation of pro-apoptotic proteins such as Fem1b and BimEL (Li and Xie, 2014). Thus, it is possible that the apoptotic resistant cells which DCA selects for may also be cells which have retained greater levels of RACK1 protein than their out-selected counterparts.

We next examined if DCA influences the subcellular localization of RACK1 in SKGT-4 and FLO-1 oesophageal adenocarcinoma cells. RACK1 plays an important role in shuttling its binding partners around the cell and in targeting or anchoring its partners at particular cellular locations(Adams et al., 2011). DCA can alter the localization of a number of known RACK1 binding partners including IGF-IR, Src and a number of PKC isoforms(Morgan and Meuillet, 2009, Looby et al., 2005). Thus, it is possible that RACK1 localization might also be altered by DCA. Furthermore, it is possible that RACK1 may play a role in DCA mediated re-localization of RACK1 binding partners. However, we observed no obvious alterations in RACK1 subcellular localization following treatment of SKGT-4 and FLO-1 cells with 300 μ M DCA for 90 minutes or 24 hours (figure 5.3). Both DCA-treated cells and cells treated with a control vehicle exhibited a predominately cytoplasmic distribution of RACK1 which was relatively diffuse and slightly more concentrated in perinuclear areas.

Thus DCA does not appear to have any obvious effects on RACK1 localization in SKGT-4 or FLO-1 cells. However, it is possible that DCA may modulate the localization of particular subsets of RACK1 (e.g. a subset of RACK1 interacting with a particular binding partner) but that such effects are 'masked' by an opposing effect which DCA has on a different subset of RACK1, or simply by the diffuse distribution of RACK1 throughout the cytoplasm of both cell types. For

example, DCA has been shown to induce endocytosis of the IGF-IR receptor and thus mediate its removal from the cell membrane. RACK1 interacts with the IGF-IR in a variety of cells and plays an important role in IGF-IR mediated signalling (Kiely et al., 2008). Thus it is plausible to suggest that DCA mediated endocytosis of the IGF-IR could alter the localization of an IGF-IR-associated subset of RACK1 and possibly stimulate its translocation away from the membrane. In contrast, DCA could potentially induce the translocation of a PKC-associated subset of RACK1 to the membrane. A report in colorectal cancer cells has shown that DCA can stimulate translocation of PKC β 1 and PKC ϵ from the cytosol to the plasma membrane (Looby et al., 2005). RACK1 is a well-known regulator of PKC function and it is known to interact with a number of PKC isoforms and to shuttle them to sites of action. In glioma, upon PMA mediated PKC activation, RACK1 translocates from the cytosol to the membrane and targets activated PKC ϵ to integrin β chains (Besson et al., 2002). Thus, it is possible that a subset of PKC-associated RACK1 may translocate to the membrane in response to DCA. However, if translocation of another subset of RACK1 (for example IGF-IR associated RACK1) away from the membrane also occurs, both translocations could 'mask' each other and preclude visual, immunofluorescence-mediated detection of such RACK1 translocations. Future studies which investigate the effects of RACK1 depletion on the localization of RACK1 binding partners might be more successful in determining if RACK1 is involved in any DCA mediated translocation of such binding partners. Such studies could focus on examining the localization of RACK1 binding partners by immunofluorescence and examining if DCA mediated translocation of such partners is altered when RACK1 expression is suppressed.

As discussed above, RACK1 has been shown to both promote and suppress cell proliferation and whether it does the former or the latter seems to depend on cell type and/or the particular proteins with which it interacts in a particular cellular setting. To gain an insight into the impact which RACK1 may have on the proliferation of oesophageal adenocarcinoma cells, we examined the impact of siRNA-mediated RACK1 depletion on SKGT-4 and FLO-1 cell proliferation using a variety of assays. The % of RACK1 depletion achieved using anti-RACK1 siRNA was approximately 90% in SKGT-4 cells and approximately 80% in FLO-1 cells (in comparison to RACK1 levels in cells treated with non-targeting control siRNA).

Using an MTT assay, which uses metabolic activity as an indicator of cell viability and proliferative ability, we observed no change in SKGT-4 proliferation following RACK1 depletion. Rather than measuring cell proliferation directly, the MTT assay measures mitochondrial dehydrogenase activity and hence cellular metabolic activity. The MTT assay is an absorbance-based assay. Therefore it is not very sensitive at detecting minor changes in cell proliferation. To investigate the possibility that RACK1 depletion results in a small decrease in SKGT-4 cell

proliferation, which may not be detectable by MTT assay, we employed an alternative method of assessing cellular proliferation in SKGT-4 cells. A nuclear count assay was used to examine the increase in cell number over time for RACK1-depleted and control cells. We observed a trend towards a smaller fold increase in cell number in RACK1-depleted SKGT-4 cells, however this was not statistically significant (figure 5.4b). We also assessed the impact which RACK1-depletion had on the ability of SKGT-4 cells to grow, spread out and cover the surface area of wells into which they were plated; i.e. to become confluent. This revealed a trend towards a small decrease in % confluency in RACK1-depleted SKGT-4 cell populations in comparison to controls; however this was not statistically significant (figure 5.4c). It is important to note that these MTT, nuclear count and cell confluency assays that were employed to assess the impact of RACK1 depletion on SKGT-4 cell proliferation, viability and confluency were performed over a limited time period using transient transfection of cells with anti-RACK1 siRNA. Therefore they lack the sensitivity to detect an impact which RACK1 depletion could potentially have on SKGT-4 cells over a longer period of time. The impact of RACK1 depletion over a longer time period could be investigated by stably transfecting cells with a plasmid that codes for a RACK1 targeting shRNA. The use of alternative cell proliferation assays, such as the xCELLigence real time cell proliferation assay which measures proliferation over a period of time, or the BrdU assay, which measures BrdU incorporation during DNA synthesis, could also provide more insight into the role of RACK1 in SKGT-4 cell proliferation.

In contrast to what was observed in the case of SKGT-4 cells, RACK1 depletion did result in a significant decrease in FLO-1 cell proliferation, as assessed by both MTT assay and by an xCELLigence real time cell proliferation assay.

This difference (regarding the influence which RACK1-depletion has on proliferation) between the two cell lines is interesting given our earlier finding that FLO-1 cells have much higher relative levels of RACK1 protein than those found in SKGT-4 cells (figure 5.1). Indeed, we found that SKGT-4 adenocarcinoma cells had a particularly low level of RACK1 protein, which was closer to the levels of RACK1 found in the non-cancerous (HEEC and HET-1A) and metaplastic (QhTERT) oesophageal cell lines than it was to the levels found in dysplastic (GohTERT) cells or in the other two adenocarcinoma cell lines (OE33 and FLO-1) that we examined (figure 5.1). Given the cell type specific functions of RACK1 and the heterogeneity which is observed in cancer (even in cancers which have originated from the same anatomical site) it is possible that SKGT-4 cells represent a form of oesophageal adenocarcinoma in which RACK1 does not play a particularly important role in regulating proliferation but that FLO-1 cells, in contrast,

represent a subtype of oesophageal adenocarcinoma for which RACK1 is functionally important.

As is discussed at length in chapter 1 (section 1.3.3), RACK1 plays an important role in regulating many different processes which contribute to cell migration. As is the case with other cellular functions of RACK1, the specific contribution which RACK1 makes to cancer cell migration depends on cancer type and it has been associated with both pro- and anti-migratory activity. We assessed the impact of siRNA mediated RACK1 depletion on the migratory ability of SKGT-4 and FLO-1 cells. Using scratch wound assays we demonstrated that the 2D migration of both cell types was slower in RACK1-depleted versus control cells.

We had now demonstrated that FLO-1 cells require RACK1 for both proliferation and 2D cell migration. We had also shown that FLO-1 cells express high levels of RACK1 in comparison to SKGT-4 cells or non-cancerous oesophageal cells. FLO-1 cells were therefore chosen as a model for a more detailed study of the role of RACK1 in oesophageal adenocarcinoma (FLO-1 cells are used exclusively in the studies conducted in subsequent chapters of this thesis). We next investigated if RACK1 is also required for the 3D migration of FLO-1 cells. Using the xCELLigence label free cell analysis platform we found that 3D FLO-1 cell migration was significantly reduced in RACK1 depleted cells in comparison to controls. Thus we have shown that RACK1 is required for both 2D and 3D FLO-1 cell migration. To appreciate the potential *in vivo* implications of these results it is worth noting that wound healing is an important response to the impacts of chronic gastro-oesophageal acid reflux, where the stratified squamous epithelium is sloughed off exposing the basement membrane epithelial cells. These epithelial cells must migrate to enable wound closure (Byrne et al., 2015). Thus RACK1 may contribute to this type of wound healing in the oesophagus. As was discussed in chapter 1, the ability of a cancer cell to migrate can promote metastasis as it helps the cell to change position within tissues, break free from a primary tumour and enter lymphatic and blood vessels for dissemination into the circulation (Polacheck et al., 2013, Yamaguchi and Condeelis, 2007). Thus RACK1 may also be involved in facilitating metastasis of oesophageal adenocarcinoma cells. Like our finding in FLO-1 cells, RACK1 has been shown to facilitate 3D migration of oesophageal squamous cell carcinoma cell lines and RACK1 expression is positively associated with lymph node metastasis in tissue samples from oesophageal squamous cell carcinoma patients (Hu et al., 2013, Wang et al., 2015a). It is important to note that oesophageal adenocarcinoma and oesophageal squamous cell carcinoma are two very different cancer subtypes with differing etiologies, epidemiology, genetic alteration patterns and pathogenic pathways (Agrawal et al., 2012, Pennathur et al., 2013, Chow et al., 1998). Nevertheless, our

findings indicate that RACK1 plays a functional role in oesophageal adenocarcinoma cell migration, as it has been previously shown to do in oesophageal squamous cell carcinoma.

5.5 Conclusion

In conclusion we have found that the RACK1 protein is strongly expressed in a number of OAC cell lines. Our findings indicate that RACK1 plays a significant functional role in FLO-1 OAC cells; RACK1 depletion from this cell type reduced cell proliferation and viability and also reduced both two- and three-dimensional cell migration. Thus further examination of the RACK1 scaffolding protein in this cellular model is warranted.

Chapter 6: A mass spectrometry based proteomics screen of RACK1 associated proteins in oesophageal adenocarcinoma cells.

6.1 Introduction

Protein-protein interactions are essential for virtually all cellular activities and processes. Proteins do not act in isolation but instead function as part of an interconnected network composed of multiple other proteins with which they interact to fulfil their functions. Indeed, most processes within the cell are conducted and regulated by proteins acting in macromolecular complexes. Thus we can learn a huge amount about any one particular protein by identifying and studying the proteins with which it interacts (Link et al., 1999, Huang and Kim, 2013).

Furthermore, disruption of protein-protein interactions may alter protein function and cellular and physiological processes, thus leading to the development of pathological states or disease. Therefore, the identification of interacting proteins is necessary to further our understanding of both normal biological processes and pathogenesis, and to develop new therapeutic strategies (Oti et al., 2006, Higuero et al., 2013, Gandhi et al., 2006). In the last twenty plus years great advances have also been made in the development of small molecule inhibitors which target specific protein-protein interactions as a therapeutic strategy (Arkin et al., 2014). Such therapeutics arise from an in-depth understanding of protein-protein interactions and their function in health and disease.

All of the above is particularly relevant to RACK1, which interacts with a plethora of different interaction partners and which, as a scaffolding protein, also facilitates interactions between other proteins. The binding partners of RACK1 may associate with it constitutively or they may bind in a transient, stimulus-dependent manner, often via interactions between the WD repeats of RACK1 and modular structural domains of its binding partners (Gandin et al., 2013b). The role of RACK1 in multi-protein complexes varies. For example, RACK1 may facilitate cross-talk between specific signalling pathways, recruit other signalling proteins into protein

complexes, modulate the activity of kinases to which it is bound or shuttle proteins to their subcellular sites of action (Gandin et al., 2013b). As has been discussed in previous chapters, the function of RACK1 can vary greatly between different cellular/tissue/disease settings and much of this variation may be due to the different binding partners with which RACK1 interacts in different cellular contexts. Thus it is insightful to examine the RACK1 interactome in specific cellular contexts in order to gain an insight into the functions of RACK1 in those contexts.

The application of mass spectrometry to cell and molecular biology has provided huge benefits to the field of proteomics (Turriziani et al., 2014). The ability to identify numerous potential interaction partners of a selected protein simultaneously has enabled us to gain a more thorough, information-rich insight into the overall function of the selected protein than that which could be gained from using lower throughput techniques such as Western blotting or microscopy which require antibodies or epitope-tagged versions of each potential interaction partner. Mass spectrometry based proteomics have also made it easier and quicker to identify novel interaction partners of a selected protein.

Mass spectrometry based proteomic interaction protocols have typically involved the isolation of the protein complex to be analysed and the elution of the bait and its interacting proteins. The eluted sample is then separated by one or two dimensional SDS PAGE, stained and individual bands or spots are excised. The proteins then undergo in-gel digestion and the peptides are analysed by mass spectrometry. The processing, washing and digesting steps involved in sample preparation using such a protocol can introduce significant contamination from keratin and other substances. Furthermore, the multiple steps involved in in-gel protocols can lead to the loss of material, in particular for the lower abundant proteins which may lead to their detection failure. In order to lessen the risk of such contamination and increase our chances of detecting less abundant RACK1 interaction partners (and thus increase the chance of identifying novel RACK1 interaction partners which had not been detected in previous screens) we used an on-bead digestion method instead of the traditional in-gel digestion approach when preparing our samples for mass spectrometry. This on-bead digestion strategy has been utilised by Turriziani and others and has been proven to be a fast, reliable and sensitive tool for interactome analysis (Turriziani et al., 2014).

6.2 Objectives

The function of RACK1 as a scaffolding protein is centred on its ability to interact with other proteins, modulate or facilitate their function, and in this way, exert its influence on a range of cellular processes. A plethora of different binding proteins/partners for RACK1 have been identified. The particular proteins with which RACK1 interacts are likely to vary depending on cell or tissue context and this may be responsible, at least in part, for the differences in RACK1 function that have been observed in different cellular settings. Therefore, the identification and characterization of interaction partners of RACK1 in a particular cell/tissue/disease setting can provide much insight into its function in that setting. Therefore, in this chapter we aim to examine the RACK1-associated proteome in FLO-1 oesophageal adenocarcinoma cells.

We aim to:

- Immunoprecipitate RACK1 from FLO-1 cells and to use a mass spectrometry based proteomics screen to examine the protein composition of the RACK-1 co-immunoprecipitated complex.
- Carry out *in silico* analysis of the functional role of proteins detected in the RACK1-associated complex.
- Characterise the proteins which we detect in this complex and to identify proteins of particular interest for validation and further analysis.

In addition to gaining an insight into the RACK1 interactome in oesophageal adenocarcinoma cells, this approach should identify novel RACK1 interaction partners not previously detected in any cellular setting.

6.3 Results

6.3.1 Isolation and mass spectrometry mediated identification of RACK1 immunoprecipitates (IPs).

In the previous chapter, we demonstrated that RACK1 is of functional importance to FLO-1 cells. To gain further insight into the function of RACK1 in FLO-1 cells, we sought to identify proteins which interact with RACK1 in these cells. RACK1 was immunoprecipitated from FLO-1 cell lysates using a RACK1-specific IgM antibody and protein G sepharose (agarose) beads. Control immunoprecipitates (IPs) were also prepared where a non-specific IgM antibody (isotype control) was used in place of the RACK1 specific antibody. Following on bead digestion, the composition of the IPs was analysed by mass spectrometry. In the case of the

RACK1 IPs, 95% of the RACK1 protein sequence was covered by identified peptides (i.e. 95% coverage was obtained).

39% coverage for RACK1 was observed in the control IP group so it was not completely RACK1-free. Nevertheless, the large difference between the quality of RACK1 detection in control versus RACK1 IPs (as is indicated in figure 6.1b) indicates that the RACK1 IPs were very much enriched for RACK1 and for RACK1 associated proteins in comparison to the control IPs. A protein heatmap generated from the mass spectrometry data, using PEAKS software, also makes it clear that the RACK1 IPs are very much enriched for RACK1 in comparison to controls (figure 6.1a).

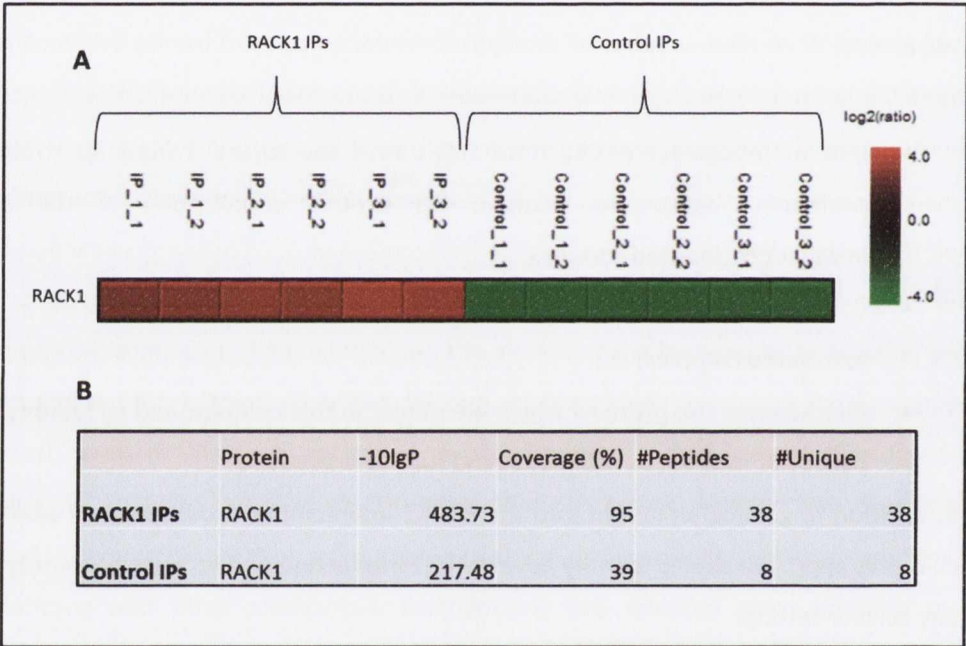


Figure 6.1: Detection of RACK1 in control versus RACK1-specific IPs by mass spectrometry. Protein complexes were immunoprecipitated from FLO-1 lysates using either RACK1 specific IgM antibody (RACK1 IP) or non-specific control IgM antibody (control IP). This was performed three times in three independent experiments, giving three biological replicates (N=3). Each biological replicate was split into two to provide two technical replicates of each sample for analysis on the mass spectrometer. Thus six RACK IP and six control IP samples can be seen in the protein heatmap in (A). Cell colour represents, for RACK1, the log2(ratio) to the average area across different samples. Much greater RACK1 detection is observed in the six RACK1 IP samples in comparison to control IPs, indicating that the IP experiments were successful in isolating RACK1 protein complexes from FLO-1 cells. The table in (B) also shows that RACK1

detection by mass spectrometry was much stronger for the RACK IPs than for the control IPs; 95% of the RACK1 protein sequence was covered by peptides identified by the mass spectrometer (i.e. 95% coverage achieved) in the case of RACK1 IPs but only 39% of RACK1 coverage was detected in control IPs.

6.3.2 Identification of 293 proteins unique to the RACK1 IPs.

Lists of proteins detected by the mass spectrometer in the control and RACK1 IPs were generated using PEAKS software. Lists of proteins present in (i) the RACK1 IP only, (ii) present in both control IP and RACK1 IP and (iii) present in control IP only were created. These lists can be found, in full, in appendix 3. As is illustrated in the Venn diagram in figure 6.2, 293 proteins unique to the RACK1 IP were detected, 250 proteins were detected in both RACK1 and control IPs while 64 proteins were detected in control IP only. A virtual protein-protein interaction network which included RACK1 and the 293 proteins unique to the RACK1 IP was generated using the Cytoscape bioinformatics platform. The protein–protein interactions for this network were retrieved from the STRING protein interaction database. As can be seen in figure 6.3, this analysis revealed that a number of previously identified direct RACK1 interaction partners were present in the 293 proteins, in addition to proteins for which no interaction with RACK1 has been previously identified.

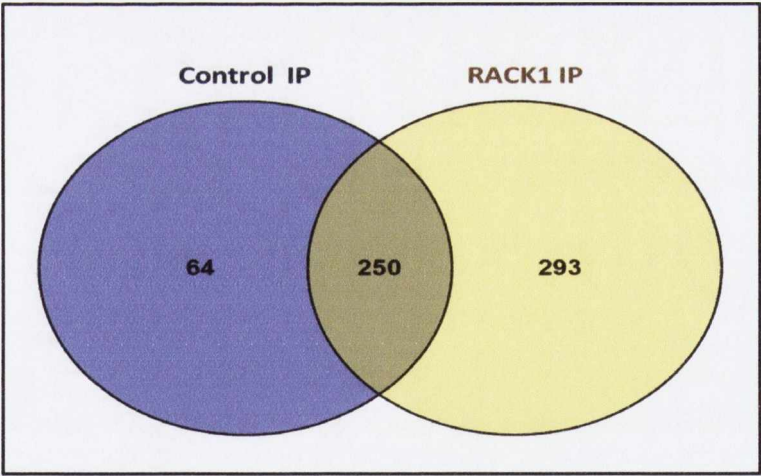


Figure 6.2: Venn diagram of control IP and RACK1 IP proteomic lists. A total of 293 proteins were unique to the RACK1 IP list detected by mass spectrometry and identified using PEAKS.

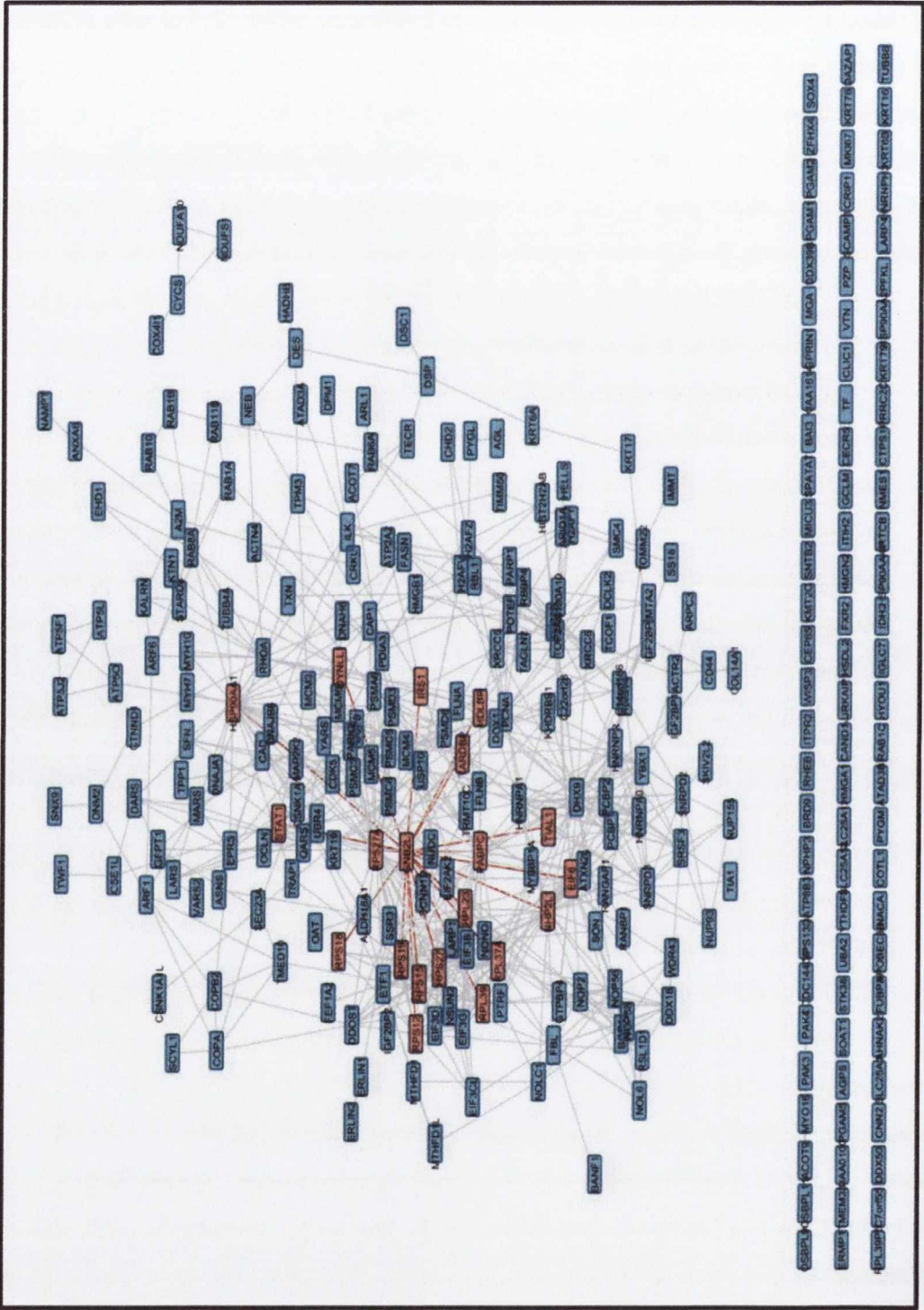


Figure 6.3: Protein-protein interaction network of RACK1 co-immunoprecipitating proteins. A protein-protein interaction network was generated using the Cytoscape bioinformatics software platform. Protein-protein interactions were retrieved from STRING. RACK1 (GNB2L1)

and its previously identified direct interactors are highlighted in red. Unconnected nodes are included at the bottom of the image.

6.3.3 RACK1 co-immunoprecipitating proteins are involved in ribosomal related activities, RNA & DNA regulation, cell cycle, metabolism, trafficking, integrin signalling, inflammation and cytoskeletal regulation.

Enrichment analysis was performed on the list of 293 proteins which had been identified as unique to the RACK1 IP. Enrichment analysis assesses the statistical enrichment of biological processes or pathways in a given data set on the basis of information in Gene Ontology (GO) or other databases. It therefore enabled us to identify which biological process terms were over or under-represented in our dataset of proteins unique to the RACK1 IP.

During enrichment analysis a 'biological process' refers to a biological objective to which a gene or gene product contributes. Examples of broad biological process terms include 'signal transduction' or 'cell growth and maintenance'. Examples of more specific terms are 'cAMP biosynthesis' or 'translation' (Ashburner et al., 2000). GO is an ontology and consortium which aims to use a hierarchy of biological terms to unify all knowledge about biological processes, cellular components and molecular functions. It provides a gold-standard reference of gene functions against which any data set can be assessed (Dutkowski et al., 2013). We therefore analysed our dataset of 293 RACK1 IP unique proteins for GO biological process term enrichment (see figures 6.4 and 6.5).

REACTOME is an expert-authored, peer-reviewed database of reactions, pathways and biological processes which can also be used to conduct valuable enrichment analysis (Croft et al., 2014). We therefore also examined our RACK1 IP unique database for REACTOME term enrichment (see figures 6.6 and 6.7).

As can be seen in figures 6.4 to 6.7, these enrichment analysis studies have demonstrated that the list of 293 proteins unique to the RACK1 IP is enriched with proteins involved in translation and other ribosomal related processes, RNA and DNA regulation, cell cycle, metabolism, cytoplasmic/intracellular protein transport and actin regulation.

The proteins unique to the RACK1 IP were also analysed using PANTHER DB which found that this group of proteins is enriched for molecules that contribute to pathways involved in integrin signalling, inflammation and cytoskeletal regulation (see figure 6.8). Excel sheets with complete, detailed results of GO_BP, Reactome and PANTHER DB analysis are included in Appendix 3.

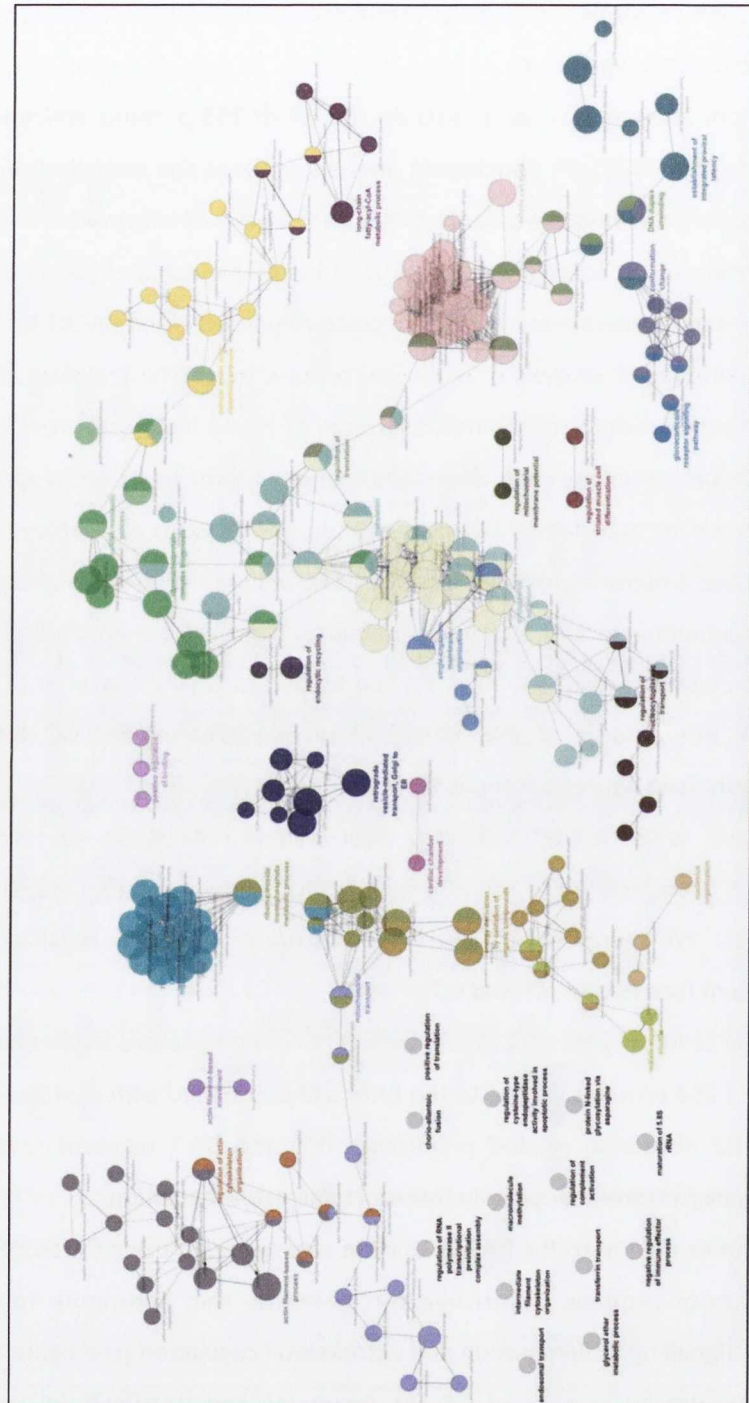


Figure 6.4: Gene Ontology (Biological Processes) analysis of RACK1 co-immunoprecipitating proteins. The 293 proteins unique to the RACK1 IP were analysed for GO_BP term enrichment using ClueGO within Cytoscape (version 3.2.1). Node cluster colour reflects the colour of the most significant term associated with that cluster. Key clusters of GO terms centre around

actin organisation, cell cycle control, translation, metabolism and trafficking pathways. Larger node size indicates more significant P values.

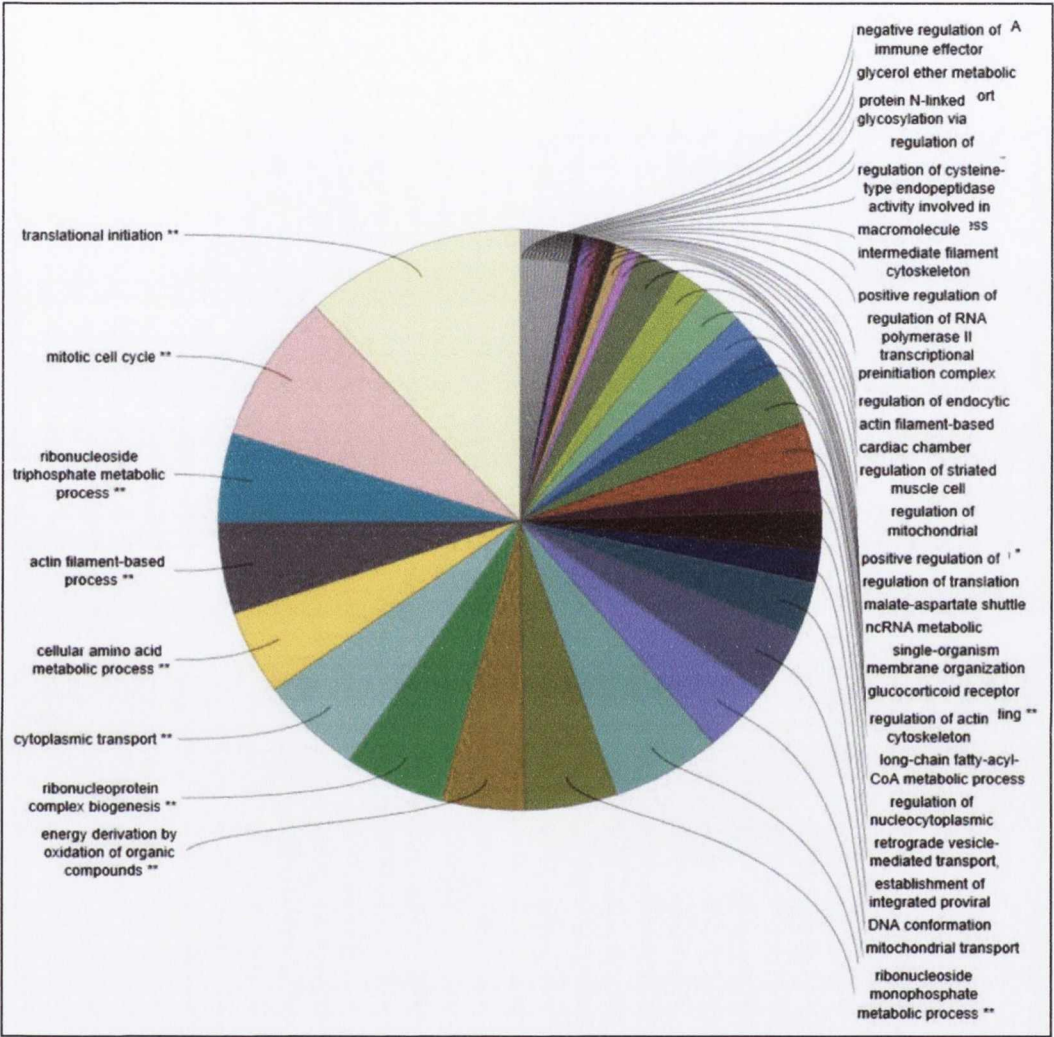


Figure 6.5: Results of Gene Ontology (Biological Processes) analysis of RACK1 co-immunoprecipitating proteins in pie chart format. The 293 proteins unique to the RACK1 IP were analysed for GO_BP term enrichment using ClueGo within Cytoscape (version 3.2.1).

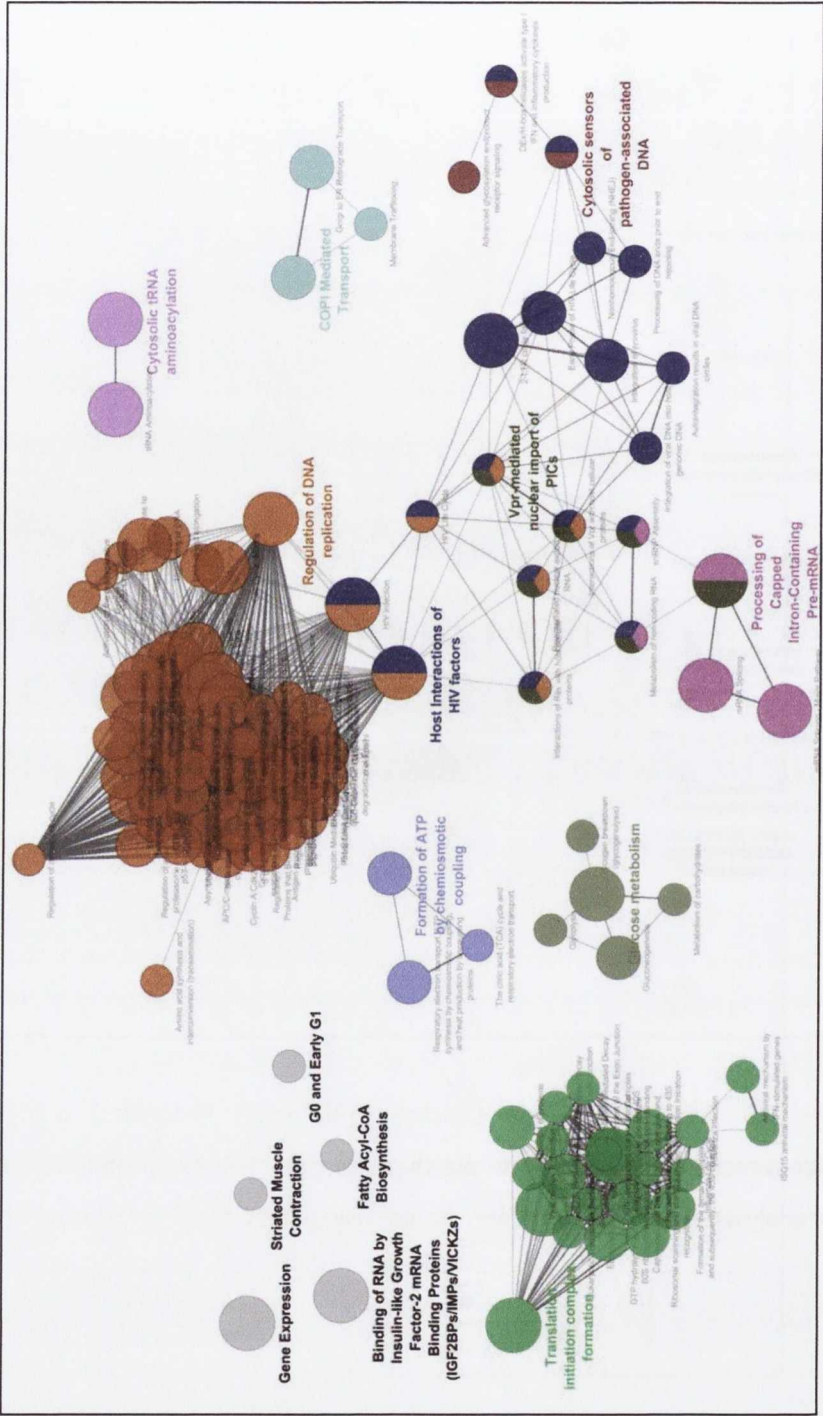


Figure 6.6: Reactome analysis of RACK1 co-immunoprecipitating proteins. The 293 proteins unique to the RACK1 IP were analysed for REACTOME term enrichment using ClueGO within Cytoscape (version 3.2.1). Node cluster colouring reflects the colour of the most significant

(Benjamini and Hochberg corrected P value) term associated with that cluster. Larger node size indicates more significant P values.

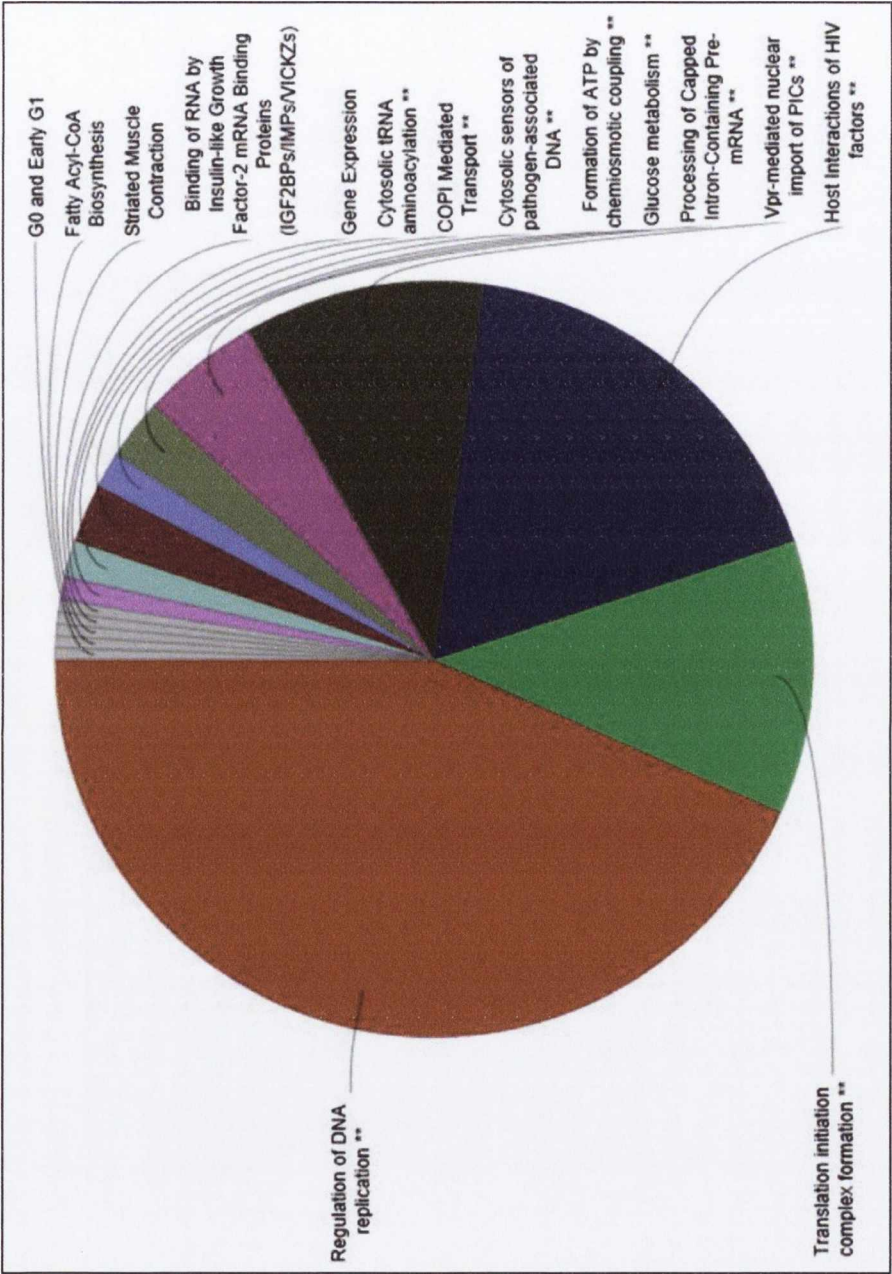


Figure 6.7: Results of Reactome analysis of RACK1 co-immunoprecipitating proteins in pie chart format. The 293 proteins unique to the RACK1 IP were analysed for REACTOME term enrichment using ClueGO within Cytoscape (version 3.2.1).

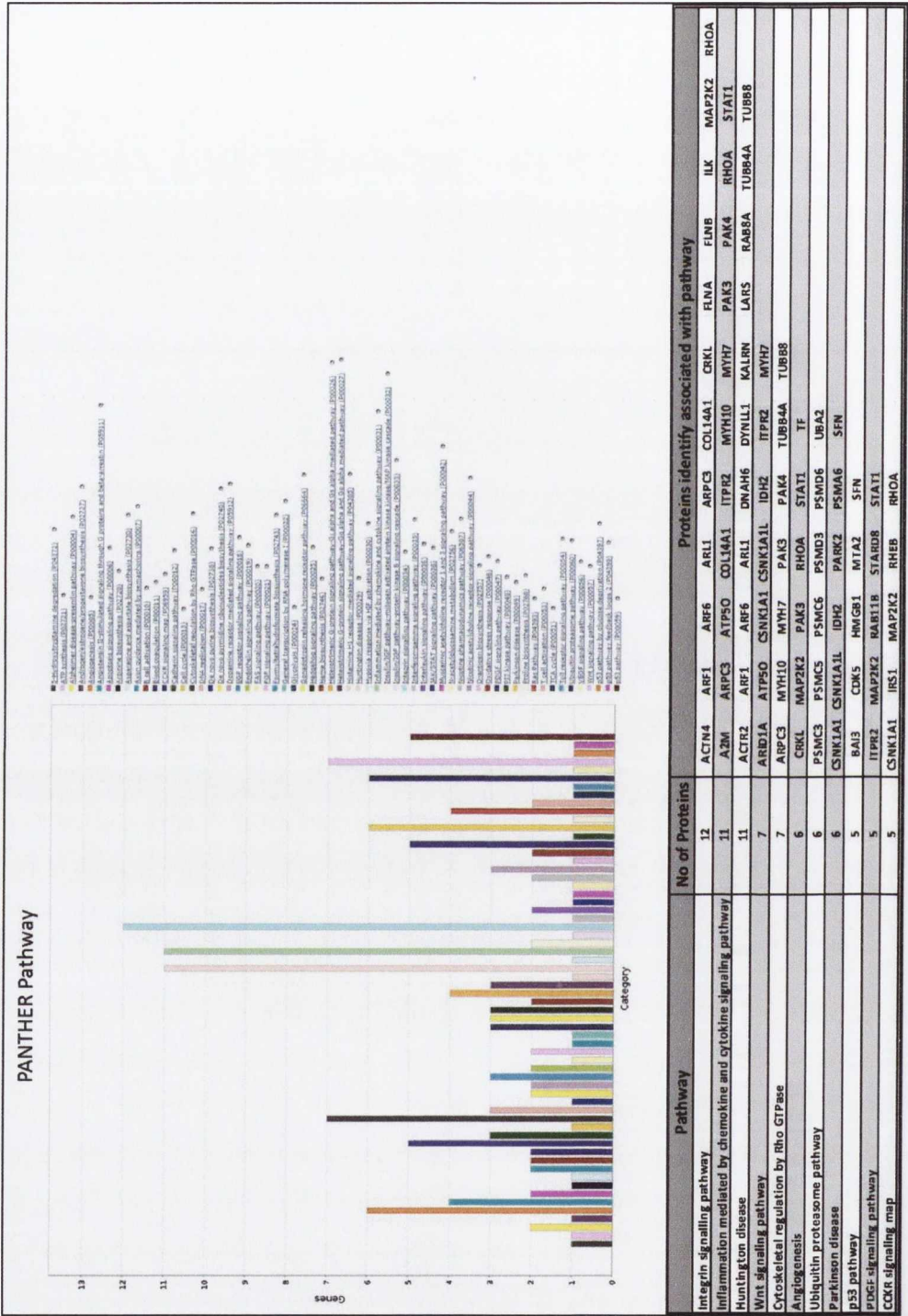


Figure 6.8 PANTHER DB pathway analysis of RACK1 co-immunoprecipitating proteins. Pathway analysis of RACK1 interacting proteins highlights enrichment of proteins which function in pathways involved in integrin signalling, inflammation and cytoskeletal regulation. The table highlights pathways with 5 or more proteins mapping to them.

6.3.4 MaxQuant high stringency analysis of mass spectrometry data generated from analysis of control and RACK1 IPs indicates that the RACK1 interactome in FLO-1 cells is enriched for nucleic acid binding proteins.

The data generated by mass spectrometry-mediated analysis of control and RACK1 IPs were also analysed using MaxQuant. MaxQuant is an quantitative proteomics software package designed for analysing and interpreting large-scale proteomic data sets based on tandem mass spectrometry (Zhou et al., 2016). It is composed of a set of algorithms, which includes peak detection and scoring of peptides. MaxQuant performs mass calibration and database searches for protein identification and it quantifies identified proteins. One of the major strengths of MaxQuant is that, through the application of advanced algorithms, it substantially improves mass accuracy (Cox and Mann, 2009).

Using MaxQuant, our raw mass spectrometry data was searched against the Homo sapiens subset of the Uniprot Swissprot database, for peptides cleaved with trypsin. Label-free quantification (LFQ) values were extracted from the MaxQuant analysis for each of our technical replicates across the 3 biological replicates. Analysis of LFQ values between control and RACK1 IPs was performed using Perseus. Perseus is a companion software of MaxQuant and a bioinformatics platform which can perform statistical analyses of MaxQuant output.

Identification of enrichment of protein class was performed using the PANTHER classification system database. This found that proteins with a strong probability of being RACK1 interactors were greatly enriched for nucleic acid binding proteins which was mainly due to the presence of high numbers of ribosomal proteins.

Statistical analysis of the LFQ values in Perseus was performed at 2 stringency levels; $P=0.05$ and $P=0.01$. At $P=0.01$, 66 proteins were deemed to co-immunoprecipitate with RACK1 (figure 6.9). At $P=0.05$, 116 protein were deemed to co-immunoprecipitate with RACK1 (see figure 6.10). At both stringencies, a large grouping of proteins related to the ribosome and RNA processing, was identified. RACK1 is well documented to interact with ribosomal proteins (Sengupta et al., 2004) (Gandin et al., 2013b) which lends weight to our analysis indicating that the 66 or 116 proteins identified represent the potential RACK1 interactome in FLO-1 cells.

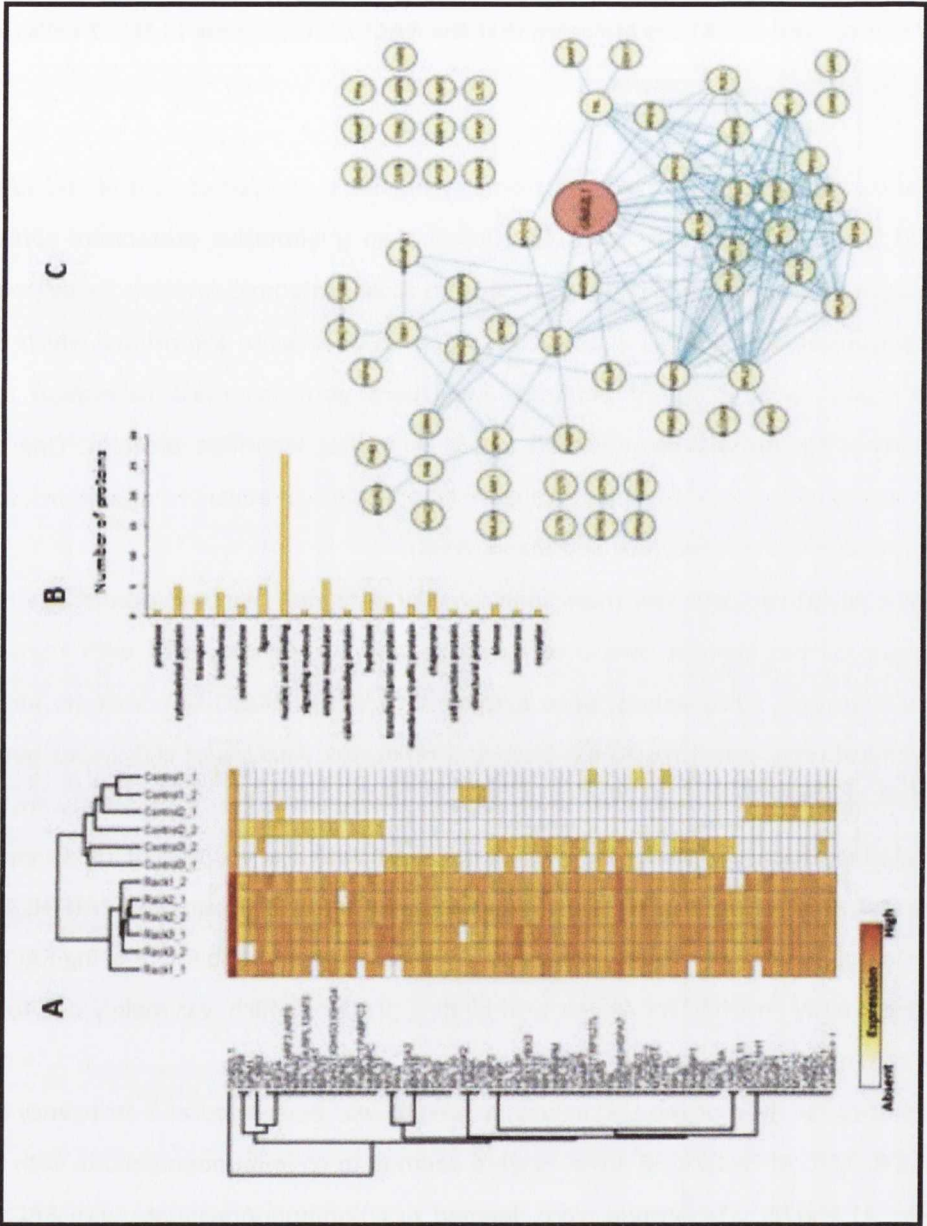


Figure 6.9: Proteomics approach to identifying the GNB2L1 (RACK1) interactome. Statistical analysis of the LFQ values in Perseus was performed at a stringency of $p=0.01$ which identified 66 proteins deemed to co-immunoprecipitate with GNB2L1. A) Hierarchically clustered heat map of the 66 proteins which were significantly enriched as putative interactors of GNB2L1. B) Break down of different protein classes represented in the list of 66 co-immunoprecipitating proteins, highlighting 27 of the 66 proteins are involved in nucleic acid binding. C) Protein-protein interaction network of 66 GNB2L1 co-immunoprecipitating proteins. Interactions between proteins represent previously experimentally validated interactions (STRINGDB version10). This highlights the interaction between GNB2L1 and a family of ribosomal proteins.

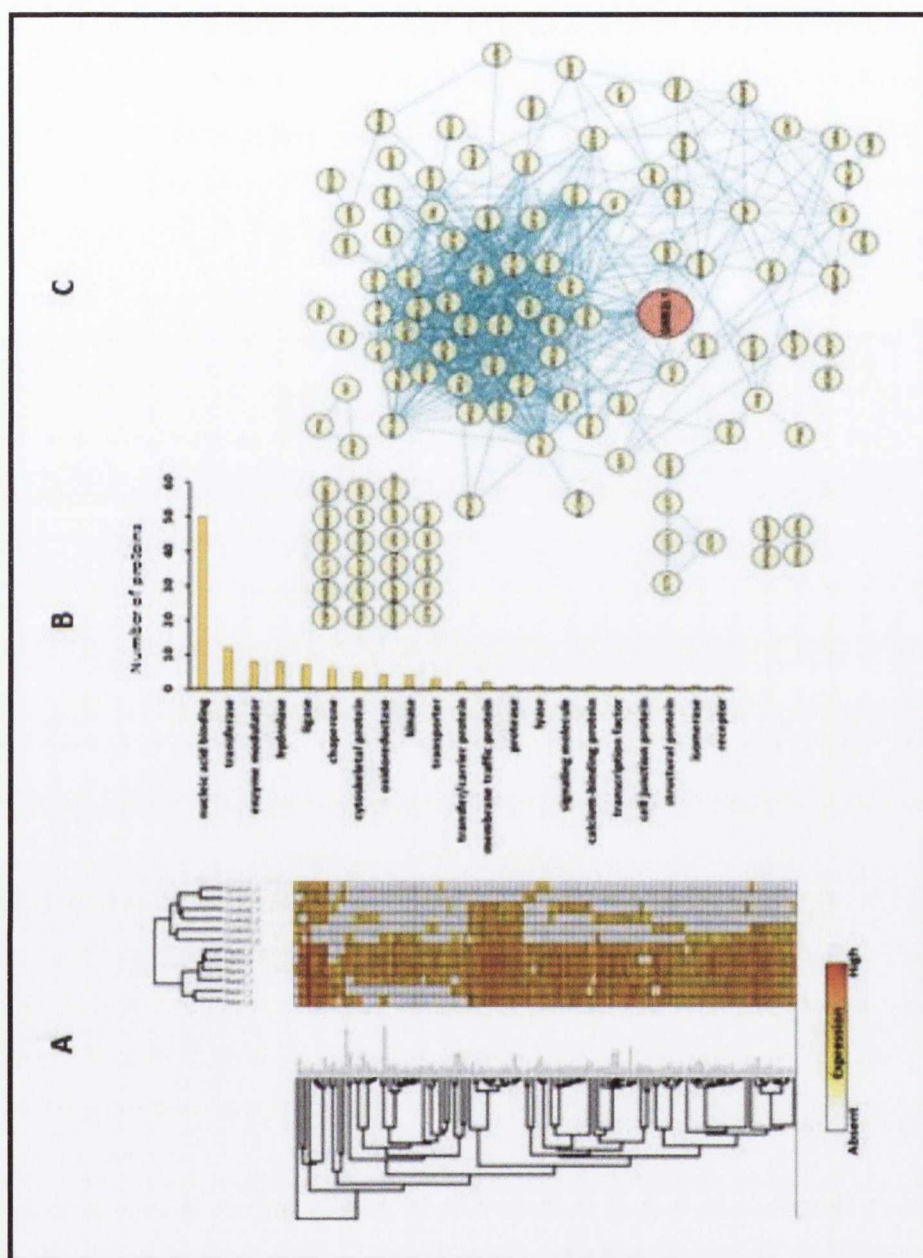


Figure 6.10: Proteomics approach to identifying the GNB2L1 (RACK1) interactome. Statistical analysis of the LFQ values in Perseus was performed at a stringency of $p=0.05$ which identified 116 proteins deemed to co-immunoprecipitate with GNB2L1. A) Hierarchically clustered heat map of the 116 proteins which were significantly enriched as putative interactors of GNB2L1. B) Break down of different protein classes represented in the list of 116 co-immunoprecipitating proteins, highlighting 50 of the 116 proteins are involved in nucleic acid binding. C) Protein-protein interaction network of 116 GNB2L1 co-immunoprecipitating proteins. Interactions between proteins represent previously experimentally validated interactions (STRINGDB version10). This highlights the interaction between GNB2L1 and a family of ribosomal proteins.

6.3.5 Analysis of potential interaction between RACK1 and vigilin (HDLBP) in FLO-1 cells

As outlined in the previous section, our most stringent analysis of the mass spectrometry data generated from analysing control and RACK1 IPs resulted in the generation of a list of 66 proteins deemed to be putative RACK1 interaction partners (see figure 6.9). One of the proteins included in this list was vigilin, also known as high-density lipoprotein-binding protein (HDLBP).

There is strong evidence to indicate that the yeast homologue of vigilin, Scp160p, interacts with the yeast functional homologue of RACK1, Ascp1 (Sonja et al., 2004, Coyle et al., 2009). However, to our knowledge, no previous evidence of an interaction between mammalian RACK1 and vigilin has been published. Vigilin represents one of a number of ribosomal-binding proteins which were identified in our screen. Vigilin/Scp160p has been associated with a number of biological processes including translational control and RNA transport and metabolism (Hirschmann et al., 2014, Mendelsohn et al., 2003, Kruse et al., 2000, Gelin-Licht et al., 2012, Weber et al., 1997, Sonja et al., 2004, Frey et al., 2001). Interestingly, vigilin has also been associated with carcinogenesis (Yang et al., 2014). For these reasons, we chose to further investigate the potential interaction between RACK1 and vigilin.

6.3.5.1 Efforts to verify that RACK1 and vigilin co-immunoprecipitate from FLO-1 lysates

In order to verify that RACK1 and vigilin co-immunoprecipitate from FLO-1 cells, RACK1 was immunoprecipitated from FLO-1 cell lysates using the same IP protocol which had been used to prepare samples for the mass spectrometry screen. The co-immunoprecipitated proteins were then separated by SDS PAGE and Western blotting was used to investigate if vigilin was present in the RACK1 IP. As can be seen in figure 6.11a, our first replicate of this experiment identified a faint band corresponding to vigilin in the RACK1 IP which was absent in the control IP. However, when this experiment was repeated a second and third time, we could not detect vigilin co-immunoprecipitation with RACK1 and thus our results are inconclusive. In an effort to obtain a more conclusive result, a second IP technique was employed whereby the protein G sepharose beads used in the original IP protocol were replaced with magnetic 'dynabeads' (See chapter 2, section 2.21 for full details of both protocols). Using this IP protocol, we found no evidence of vigilin co-immunoprecipitating with RACK1 (see figure 6.11b).

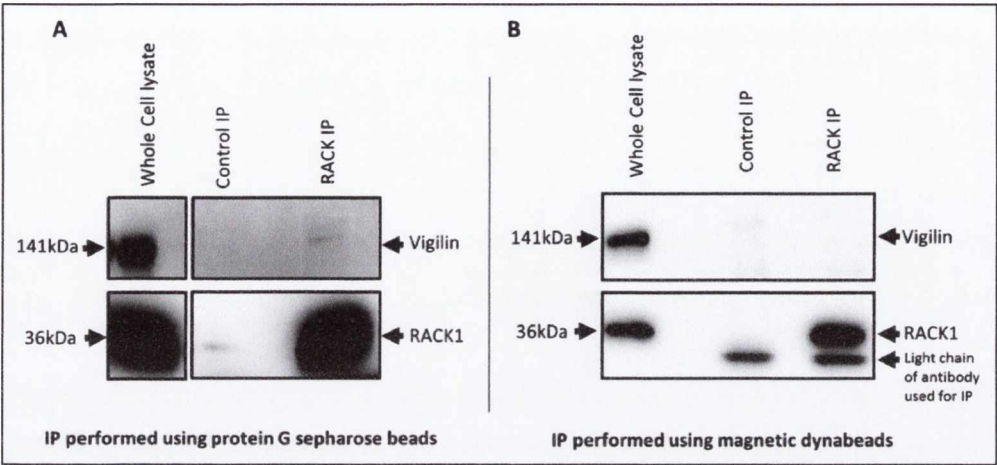


Figure 6.11. Investigating if vigilin co-immunoprecipitates with RACK1 in FLO-1 cell lysates. RACK1 was immunoprecipitated from FLO-1 cell lysates using an anti-RACK1 IgM antibody. In control IPs, a non-specific IgM was used in place of the anti-RACK1 antibody. Proteins in the IP complexes were separated by SDS PAGE and Western blotting was used to investigate if vigilin was present in them. Two different immunoprecipitation protocols were employed. The co-immunoprecipitation protocol used in (A) was the same protocol as that used to prepare samples for the mass spectrometry based proteomics screen described above. (B) An alternative co-immunoprecipitation protocol was also employed which utilised magnetic dynabeads in place of protein G sepharose beads. The blot in (B) is representative of three independent experiments.

6.3.5.2 Analysis of RACK1 and vigilin localization and potential colocalization in FLO-1 cells

We next investigated if RACK1 and vigilin colocalize in FLO-1 cells using immunofluorescent staining and confocal microscopy. This revealed that the two proteins have distinct localization patterns in FLO-1 cells but also revealed that some partial RACK1-vigilin colocalization does occur, as is highlighted by white arrows in figure 6.12. Thus we cannot rule out the possibility that vigilin and RACK1 interact or associate in FLO-1 cells, but as of yet, we have been unable to conclusively show that they do.

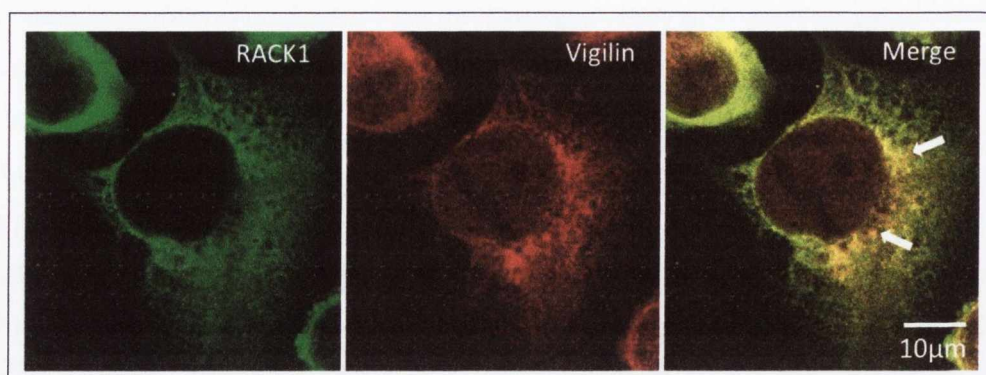


Figure 6.12: RACK1 and vigilin in FLO-1 cells. Cells were fixed, permeabilised, and stained with immunofluorescent dyes conjugated to antibodies specific for RACK1 and vigilin. Images were obtained by confocal microscopy using a 63X objective. RACK1 and vigilin exhibit distinct colocalization patterns but some partial colocalization is observed and is highlighted by white arrows.

6.3.6: RACK1 and nicotinamide phosphoribosyltransferase (NAMPT) co-immunoprecipitate from FLO-1 cells

Nicotinamide phosphoribosyltransferase (NAMPT) was another protein present in the list of 66 proteins deemed to be putative RACK1 interaction partners (see figure 6.9). NAMPT is a nicotinamide adenine dinucleotide (NAD) biosynthetic enzyme and also has cytokine-like activity (Garten et al., 2015). To our knowledge, no previous interaction between RACK1 and NAMPT has been identified, making it a novel potential RACK1 interaction partner. NAMPT is also interesting in that it has been previously associated with obesity, inflammation, oxidative stress and cancer and is thus of interest in the context of oesophageal adenocarcinoma (Garten et al., 2015).

In order to verify our mass spectrometry result, which indicated that RACK1 and NAMPT co-immunoprecipitate in FLO-1 cells, RACK1 was immunoprecipitated from FLO-1 cell lysates using the same IP protocol which had been used to prepare samples for the mass spectrometry screen. The co-immunoprecipitated proteins were then separated by SDS PAGE and Western blotting was used to investigate if NAMPT was present in the RACK1 IP. As can be seen in figure 6.13, NAMPT was detected in the RACK1 IP but was absent in the control IP, verifying our mass spectrometry finding that NAMPT co-immunoprecipitates with RACK1 in FLO-1 cells. As a result, the interaction between NAMPT and RACK1 will be investigated in more detail in chapter 7.

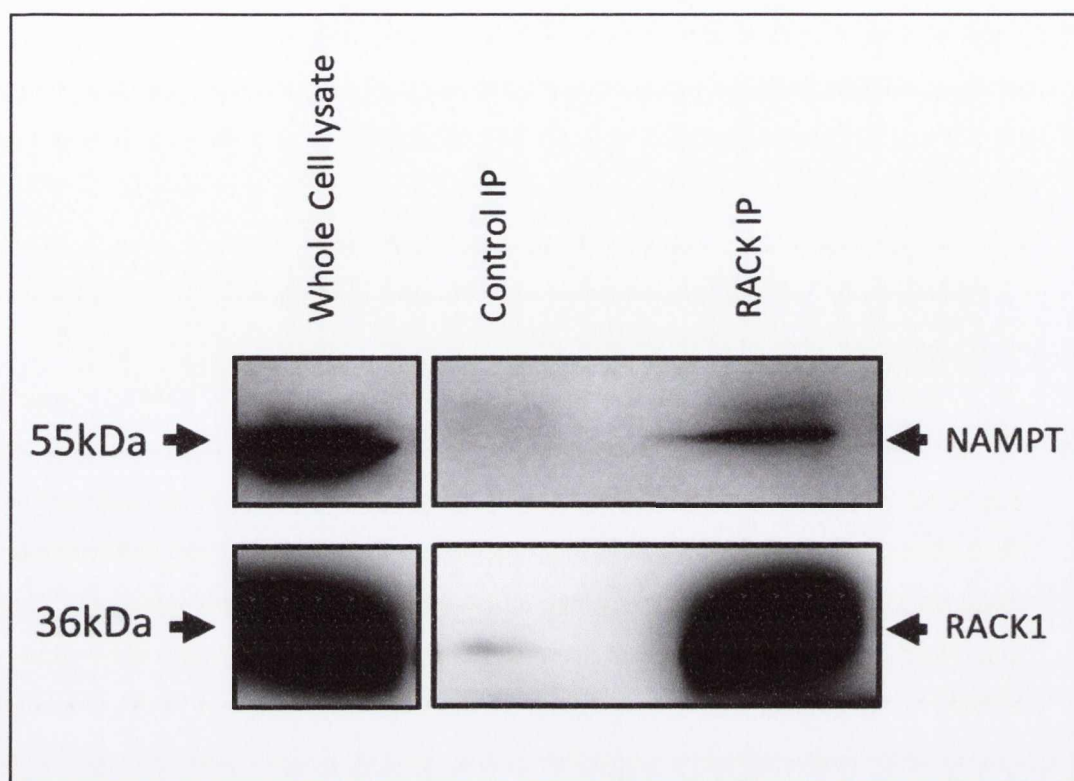


Figure 6.13: NAMPT co-immunoprecipitates with RACK1 from FLO-1 cells: RACK1 was immunoprecipitated from FLO-1 cell lysates using the same protocol that was used to prepare samples for the mass spectrometry screen. A RACK1-specific IgM antibody was used to immunoprecipitate RACK1 from FLO-1 cells. A non-specific IgM antibody was used, in place of the anti-RACK1 antibody, in the control IP. Proteins in the IP complexes were separated by SDS PAGE and Western blotting was used to investigate if NAMPT was present in them. NAMPT was detected in the RACK1 IP and was absent in the control IP, indicating that RACK1 and NAMPT complex in FLO-1 cells. The blot shown is representative of two independent experiments.

6.4 Discussion

As has been discussed in previous chapters, RACK1 contributes to a diverse range of cellular functions which vary greatly with cell and cancer type. Some of the variation in the function of RACK1 is likely to be due to the different binding partners and associated proteins with which RACK1 interacts in any particular cell type. Thus an examination of the RACK1 associated proteome in a particular cell or disease setting has the potential to further our understanding of the functions of RACK1 in that setting and to also further our understanding of the

molecular mechanisms underlying these functions. Therefore, in an effort to improve our understanding of the functions of RACK1 in oesophageal adenocarcinoma, we conducted proteomic profiling of RACK1 associated proteins in FLO-1 oesophageal adenocarcinoma cells. RACK1 was immunoprecipitated from FLO-1 cell lysates using an anti-RACK1 IgM antibody. Control IPs were prepared using a non-specific IgM control antibody in place of the anti-RACK1 antibody. Proteins present in the RACK1 and control IPs were detected by mass spectrometry and the resultant data was analysed using a range of software programmes to identify proteins with a high probability of being genuine RACK1-associated proteins.

As is detailed in section 6.3 above, a list of 293 proteins unique to the RACK1 IP samples was initially identified by PEAKS analysis of the mass spectrometry data. This list included numerous ribosomal and RNA and DNA regulatory proteins as well as multiple proteins involved in the cell cycle, trafficking processes, integrin signalling, inflammation and cytoskeletal regulation. More stringent analysis of the mass spectrometry generated data using MaxQuant in conjunction with Perseus produced a list of 66 proteins deemed to have a higher probability of co-immunoprecipitating with RACK1. This list was heavily enriched with nucleic acid binding proteins, which was mainly due to the presence of large number of ribosomal proteins in the list. This finding adds to the credibility of our mass spectrometry results as RACK1 is well known to be a ribosome associated protein with important roles in translation. Studies with ribosomes from yeast, fungi, unicellular algae, plants, protozoa and mammals have identified RACK1 as a key component of the ribosomal machinery and several structural studies have shown that RACK1 interacts with the 40S subunit of the eukaryotic ribosome(Adams et al., 2011). X-ray crystallography studies have identified RACK1 association with a number of ribosomal proteins, for example, an X-ray crystal structure obtained for the 40S eukaryotic ribosomal subunit (from *T. thermophile*) has demonstrated RACK1 bound to this ribosomal subunit and interacting with the co-associated rpS3e, rpS16e and rpS17e proteins(Adams et al., 2011).

It is likely that the ability of RACK1 to act as a scaffolding protein is responsible for much of its ribosomal related functions. For example, RACK1 can recruit various factors to the ribosome and may also recruit ribosomes themselves to particular subcellular locations(Nielsen et al., 2017). RACK1 is an important recruiter of activated PKC β II and PKC-mediated translational control has been shown to be RACK1-dependent. PKC β II-mediated stimulation of translation has been proposed to occur, as least in part, through its phosphorylation of eukaryotic initiation factor 6 (eIF6) which regulates ribosomal subunit association. This phosphorylation of eIF6 by PKC β II is thought to occur on the 60S subunit as a result of an interaction with eIF6 and PKC β II

bound to a nearby 40S subunit via RACK1(Ceci et al., 2003, Sengupta et al., 2004). RACK1 has also been shown to bind to eIF6 directly(Ceci et al., 2003).

In our mass spectrometry screen of RACK1 immunoprecipitates from FLO-1 cells, eIF6 was among our initial list of 293 proteins that were present in RACK1 IPs but absent in control IPs. However eIF6 did not make it into the lists of 116 or 66 proteins deemed to have a high probability of co-immunoprecipitating with RACK1 which were created using more stringent analysis of the mass spectrometry generated data using MaxQuant and Perseus. A number of other eukaryotic translation initiation factors (eIFs) were present in these lists however. The list of 116 potential RACK1 co-immunoprecipitating proteins (generated using a stringency of $P=0.05$) included eIF3A, eIF3D, eIF4G1 and eIF2S3. Meanwhile, the list of 66 proteins deemed most probable to co-immunoprecipitate with RACK1 (generated using a higher stringency of $P=0.01$) included eIF3A (see figures 6.9 and 6.10).

While studies such as those just described demonstrate a role for RACK1 in promoting translation, other studies have suggested a gene repression function for RACK1 at the ribosome. RACK1 can interact with constituents of the microRNA-induced gene silencing complex and facilitate in the recruitment of this complex to translation sites. This indicates that RACK1 may support a post-initiation mode of miRNA-mediated gene repression (Gerbasí et al., 2004, Jannot et al., 2011). In addition, research by Kuroha and colleagues has shown that RACK1 bound to the 40S subunit facilitates nascent peptide-dependent translation arrest(Kuroha et al., 2010).

RACK1 may act as a signalling hub in the quality control of newly synthesized peptides under stress conditions. PMA induced stimulation of PKC signalling results in phosphorylation and recruitment of stress-induced c-Jun N-terminal kinase (JNK) to ribosomes via RACK1. This results in phosphorylation of the eukaryotic translation elongation factor 1A isoform 2 (eEF1A2) which promotes proteasome-mediated degradation of newly synthesized proteins (Gandin et al., 2013a). In our mass spectrometry screen of RACK1 immunoprecipitates from FLO-1 cells, eEF1A2 was among our initial list of 293 proteins that were present in RACK1 IPs but absent in control IPs. Indeed, reasonable sequence coverage (30% coverage) was detected for eEF1A2 in the screen although it did not make it into the lists of 116 or 66 proteins deemed to have a high probability of co-immunoprecipitating with RACK1 which was created using more stringent analysis of the mass spectrometry generated data using MaxQuant and Perseus.

It has been proposed that RACK1 may also play a role in recruiting ribosomes to specific subcellular locations(Nielsen et al., 2017). For example, the interaction between RACK1 and β -integrin has been suggested to facilitate the recruitment of ribosomes to focal adhesions

(Sengupta et al., 2004). Adhesion of fibroblasts to fibronectin triggers translation in a β -integrin dependent manner and there is plenty of research to show that ribosomes, mRNA, mRNA binding proteins and translation initiation factors are present in nascent focal adhesions (Nielsen et al., 2017, de Hoog et al., 2004, Willett et al., 2010, Chicurel et al., 1998). Similarly, it is also well established that RACK1 can localize to nascent focal adhesions and RACK1 overexpression can result in an increased number of focal adhesions and actin stress fibres (de Hoog et al., 2004, Cox et al., 2003). These studies support the idea that RACK1 may recruit the translational machinery to focal adhesions via simultaneous interaction with ribosomes and integrins although solid evidence to prove the existence of such a complex does not yet exist (Nielsen et al., 2017). As is discussed in chapter 1, RACK1 recruits FAK and PDE4D5 to newly formed integrin adhesions and to the leading edge of polarized cells. This FAK-RACK1-PDE4D5 complex is required for squamous cell carcinoma cells to sense direction and to migrate towards chemotactic stimuli. It is also required for the construction of actin-based protrusive structures at the edges of polarized or spreading cells (Serrels et al., 2010, Serrels et al., 2011). Whether RACK1 also recruits the ribosome when scaffolding such a complex, and whether local translation at such adhesion sites is involved in this type of directional cell migration, is not yet known. However, it is plausible to suggest that local translation may be required for the reorganization of the actin cytoskeleton and for the focal adhesion turnover that is required for cell migration (Nielsen et al., 2017).

Considering the involvement of RACK1 in cytoskeletal dynamics and focal adhesion related processes, it is interesting to note that our proteomics screen of RACK1 immunoprecipitates from FLO-1 cells identified some members of the Arf family of small GTPases. Arf1 and Arf6 were included in our initial PEAKS-generated list of 293 proteins unique to the RACK1 IP while Arf1 also made it into the MaxQuant/Perseus generated list of 66 proteins deemed most probable to genuinely co-immunoprecipitate with RACK1. A recent mass spectrometry screen of RACK1 co-immunoprecipitated proteins from rat hippocampus also identified Arf1 as a RACK1 interacting protein (Dwane et al., 2014). The Arf family of proteins play important roles in regulating actin cytoskeleton dynamics and they function in a number of actin-based processes, including cell adhesion and migration (Lewis-Saravalli et al., 2013, Myers and Casanova, 2008). Interestingly Arf1 has been shown to mediate paxillin recruitment to focal adhesions and potentiate rho-stimulated stress fibre formation in Swiss 3T3 cells (Norman et al., 1998). Arf1 is also upregulated in highly invasive breast cancer and regulates migration of breast cancer cell lines by controlling Rac 1 signalling (Lewis-Saravalli et al., 2013). Our finding that Arf1 may interact with RACK1 in FLO-1 cells is interesting, given that RACK1 depletion reduces both two and three dimensional FLO-1 cell migration, as we have shown in chapter 5.

In yeast, the mRNA binding protein Scp160p has been shown to associate with the ribosome at a site close to ribosomal Asc1p, the functional homologue of RACK1(Sonja et al., 2004). A direct interaction between Scp160p and Asc1p has been suggested by research showing that Scp160p dissociates from polysomes when Asc1p is mutated in such a way that its binding with ribosomes is impaired (Sonja et al., 2004, Coyle et al., 2009). Scp160p associates with specific mRNAs and it has thus been suggested that recruitment of Scp160p to Asc1p may influence the translation of specific mRNAs(Sonja et al., 2004). Thus it is possible that, by recruiting proteins that regulate translation of specific mRNAs in different ways, Asc1p/RACK1 may have varying effects on gene expression and may regulate the translation of a subset of specific mRNAs rather than regulating global translation(Sonja et al., 2004, Gandin et al., 2013b).

Interestingly, our mass spectrometry screen of RACK1 immunoprecipitates from FLO-1 oesophageal adenocarcinoma cells identified the mammalian homologue of Scp160p, namely vigilin (also known as HDLBP) as a RACK1 co-immunoprecipitating protein; 25% sequence coverage for vigilin was detected in the RACK1 IPs and it was also included in our list of 66 proteins deemed to co-immunoprecipitate with RACK1 following our most stringent analysis of the mass spectrometry data. To our knowledge, this is the first study to report a potential interaction between mammalian RACK1 and vigilin.

Vigilin copurifies in a complex with elongation factor 1A and transfer RNA and has been implicated in a range of processes including translational control, heterochromatin formation, nuclear export of transfer RNA, cytoplasmic transport of RNA, metabolism of specific mRNAs and sterol metabolism(Kruse et al., 1998, Hirschmann et al., 2014, Mendelsohn et al., 2003, Wang et al., 2005, Wintersberger et al., 1995, Huertas et al., 2004, Kruse et al., 2000, Gelin-Licht et al., 2012, Sonja et al., 2004, Frey et al., 2001, Weber et al., 1997, Dodson and Shapiro, 1997, Chen et al., 2003). A recent study by Mobin and coworkers indicated a role for vigilin at the site of protein synthesis and identified it as a positive regulator of translation for mRNAs coding for a subset of proteins of the secretory pathway(Mobin et al., 2016).

Vigilin has also been implicated in carcinogenesis, which was of interest to us in light of the fact that we have identified a possible RACK1-vigilin interaction in oesophageal adenocarcinoma cells. Vigilin upregulation has been reported in hepatocellular carcinoma human specimens and cell lines. Yang and colleagues examined protein levels of vigilin in normal human liver, liver cirrhosis, adjacent non-tumor liver and hepatocellular carcinoma tumour tissues and found that vigilin expression increased progressively from the liver cirrhosis tissue to adjacent non-tumour liver tissue and then to hepatocellular carcinoma tumour cells. They also found that vigilin depletion from BEL7402 hepatocellular carcinoma cells significantly inhibited their proliferation, colony formation and cell migration while

enhancing cisplatin treatment-induced growth inhibition of these cells in culture (Yang et al., 2014). In contrast, a study in breast cancer cell lines found that vigilin suppressed cellular motility and invasion and acted like a tumour suppressor by binding to the 3' untranslated mRNA region of the c-fms proto-oncogene and hence destabilizing this mRNA and inhibiting its translation. Thus vigilin appears to be capable of inhibiting or promoting carcinogenesis depending on cell or cancer type or the particular tissue context.

Considering the studies that indicate that Ascp1 and Scp160p interact, and our identification of vigilin among our mass spectrometry hits of potential RACK1 interaction partners, it is likely that RACK1 and vigilin interact in human cells and in cancer cells. If this is the case, and if RACK1's interaction with vigilin is required for its role in a particular tumour promoting or tumour suppressing function (such as controlling the translation of particular mRNAs) then it may be possible to target particular RACK1-vigilin interactions as a therapeutic strategy.

Therefore, we sought to further investigate the potential interaction between vigilin and RACK1 in oesophageal adenocarcinoma cells which we had identified by mass spectrometry. To verify that RACK1 and vigilin co-immunoprecipitate from FLO-1 cells, RACK1 was immunoprecipitated from FLO-1 cell lysates using the same IP protocol which had been used to prepare samples for the mass spectrometry screen. The co-immunoprecipitated proteins were then separated by SDS PAGE and Western blotting was used to investigate if vigilin was present in the RACK1 IP. The first replicate of this experiment indicated that vigilin did co-immunoprecipitate with RACK1 (figure 6.11a). However, when this experiment was repeated a second and third time, vigilin did not co-immunoprecipitate with RACK1. In an effort to obtain a more conclusive result, an alternative IP technique was employed but this failed to detect any evidence of vigilin co-immunoprecipitating with RACK1 (see figure 6.10b). Thus we cannot conclusively say that RACK1 and vigilin co-immunoprecipitate in FLO-1 cells, despite the fact that our mass spectrometry results indicated that they do.

It is important to note that the failure to detect co-immunoprecipitation of two proteins using IP experiments by no means proves that they do not interact in the cell; several phenomena which occur post cell lysis and during the IP procedure can disrupt or break the interactions between two proteins which were associated with each other prior to cell lysis. In particular, loss of interacting partners can occur during the IP wash process. Obtaining the optimal balance between a wash that is sufficiently vigorous to maximally remove non-specifically bound proteins, while at the same time retaining co-precipitated interacting proteins can be challenging (Huang and Kim, 2013). Variability between antibody batches may also influence whether two proteins can be successfully co-immunoprecipitated or not.

We also examined the subcellular localization of RACK1 and vigilin in FLO-1 cells by immunofluorescence analysis and confocal microscopy. This revealed that the two proteins have distinct localization patterns but also revealed colocalization between them, indicating that small subsets of both proteins may associate with each other. Thus, our results regarding whether or not RACK1 and vigilin associate or interact are inconclusive. For this reason, we chose to investigate other hits from our mass spectrometry screen for further study. However, we have certainly gained a strong indication that RACK1 and vigilin may interact and it may be interesting to investigate this further in the future.

NAMPT was another protein included in our list (generated by our most stringent MaxQuant-aided analysis of our mass spectrometry screen data) of 66 proteins deemed to co-immunoprecipitate with RACK1. To our knowledge, no previous interaction between RACK1 and NAMPT has been identified, making it a novel potential RACK1 interaction partner. Furthermore, in contrast to our difficulty in validating that RACK1 and vigilin co-immunoprecipitate, we successfully detected NAMPT co-immunoprecipitation with RACK1 using IP experiments followed by SDS PAGE and Western blotting to detect NAMPT in RACK1 IPs (figure 6.13).

NAMPT is a multifunctional protein which appears to act as both a cytokine and an NAD biosynthetic enzyme. It is of interest in the context of oesophageal adenocarcinoma, a cancer that is associated with obesity and inflammation, as NAMPT has been associated with modulating processes involved in the pathogenesis of obesity and related diseases through its influence on the oxidative stress response, inflammation, lipid and glucose metabolism, insulin resistance and cell growth and apoptosis (Garten et al., 2015). Furthermore, increased NAMPT expression has been associated with the development of several cancers including breast, prostate, endometrial, ovarian, stomach and colorectal cancer as well myeloma, astrocytoma and melanoma (Garten et al., 2015). A recent study in osteosarcoma found that NAMPT could significantly promote the in vitro migration and invasion of osteosarcoma cells while a number of other studies have indicated roles for NAMPT in cancer migration and invasion through induction of the epithelial mesenchymal transition or upregulation of pro-migratory proteins (Adya et al., 2008a, Soncini et al., 2014, Wang et al., 2016). Indeed a variety of mechanisms for the role of NAMPT in cancer progression have been proposed and these are discussed in chapter 1 (section 1.5) and in chapter 7 (section 7.1).

Therefore, NAMPT, as a protein associated with obesity, inflammation, cancer and cancer cell migration and invasion, is an interesting potential RACK1 interaction partner to study in the context of oesophageal adenocarcinoma. For these reasons, we chose to investigate further

whether RACK1 and NAMPT interact directly and such investigations are the subject of the next chapter.

6.5 Conclusion

In conclusion, using a mass spectrometry based proteomics screen, we have gained an insight into the RACK1-associated proteome in FLO-1 oesophageal adenocarcinoma cells. We have identified a large number of ribosomal proteins associated with RACK1 in this cell type, which is consistent with previous reports describing RACK1 as a ribosome associated protein with key roles in the translation machinery(Adams et al., 2011). Thus, at least a proportion of the role of RACK1 in oesophageal adenocarcinoma cells is likely to involve its function in ribosome associated processes. We also identified a novel potential RACK1 interaction partner in the form of NAMPT, a protein which functions as both an NAD biosynthetic enzyme and cytokine/adipokine and which has been previously associated with obesity, inflammation and cancer(Garten et al., 2015). We have used co-IP experiments in association with SDS-PAGE and Western blotting to validate our mass spectrometry finding which found that NAMPT co-immunoprecipitates with RACK1. Further studies, in the next chapter, will investigate the nature of the interaction between RACK1 and NAMPT and the potential functional consequences of this interaction.

Chapter 7: RACK1 interacts with Nicotinamide Phosphoribosyltransferase (NAMPT) in FLO-1 Oesophageal Adenocarcinoma Cells

7.1 Introduction

As discussed in chapter 1, RACK1 is an adaptor protein which contributes to multiple signal transduction pathways and cellular processes through its interaction with various binding partners. In chapter 6, we used a mass spectrometry based proteomics screen of RACK1 immunoprecipitates, prepared from FLO-1 cells, to identify potential RACK1 binding partners within the setting of oesophageal adenocarcinoma. One of the proteins identified in this screen, whose association with RACK1 was subsequently verified by co-immunoprecipitation and Western blotting experiments, was nicotinamide phosphoribosyltransferase (NAMPT). NAMPT is also known as visfatin or pre-B cell colony enhancing factor 1 (PBEF)(Dahl et al., 2012).

NAMPT is a multifunctional protein which functions both as a pro-inflammatory cytokine and as an enzyme(Dahl et al., 2012). It can activate human leukocytes and induce cytokine production (Moschen et al., 2007). NAMPT induces IL-1 β , TNF- α , IL-6 production in CD14+ monocytes and also increases surface expression of costimulatory molecules CD54, CD40, and CD80 (Moschen et al., 2007). Plasma levels of NAMPT are elevated in patients with inflammatory bowel disease and its mRNA expression levels are increased in the colonic tissue of Crohn's and ulcerative colitis patients compared with healthy controls (Moschen et al., 2007). A number of studies have indicated that NAMPT plays a role in the pathophysiology of metabolic disorders associated with obesity and inflammation (Dahl et al., 2012). Interestingly, it has been shown, by comprehensive meta-analysis, that, of all cancers, oesophageal adenocarcinoma has the strongest association with BMI (Renehan et al., 2008). In particular, abdominal or visceral adiposity significantly increases the risk of oesophageal adenocarcinoma(Singh et al., 2013). Visceral adipose tissue is a metabolically active organ and is believed to contribute to carcinogenesis, at least in part, by promoting inflammation in viscerally obese patients (Lysaght et al., 2011). Therefore, NAMPT represents a particularly interesting potential RACK1 interaction partner to study in the context of oesophageal adenocarcinoma.

Moreover, NAMPT augments macrophage-induced inflammation and can upregulate extracellular matrix metalloproteinase inducer expression and matrix metalloproteinase-9 and matrix metalloproteinase-2 production as a result of increased nuclear factor kappa B levels (Dahl et al., 2012, Belo et al., 2015, Adya et al., 2008b). This is again interesting in relation to oesophageal adenocarcinoma where matrix metalloproteinase-9 expression is upregulated with visceral obesity and is associated with poor tumour differentiation (Allott et al., 2013). Furthermore, nuclear factor kappa B expression levels increase across the sequence from normal oesophagus to Barrett's oesophagus to adenocarcinoma and its activation is also increased in Barrett's oesophagus and oesophageal adenocarcinoma (O'Riordan et al., 2005).

NAMPT is the rate-limiting enzyme in the mammalian nicotinamide adenine dinucleotide (NAD) salvage pathway (Sampath et al., 2015). NAD is an important metabolite that serves as a substrate for a number of different enzymes and regulatory proteins (Sampath et al., 2015). It is a cofactor and second messenger for a variety of cellular processes that are essential for survival, such as metabolism and the maintenance of genomic stability (Sampath et al., 2015). NAD can be synthesised *de novo* from tryptophan or recycled from nicotinamide through the NAMPT-dependent salvage pathway. Alternatively, nicotinic acid can be converted to NAD through a nicotinic acid phosphoribosyltransferase domain containing 1 (NAPRT-1) dependent pathway (Sampath et al., 2015).

Cancer cells turnover NAD rapidly in order to meet their high energy demands. NAD-dependent enzymes, such as poly (ADP-ribose) polymerases (PARPs), mono-ADP ribosyltransferases (MARTs) and sirtuins (SIRTs), which are required for DNA repair, genome stability and proliferation, are also highly active in cancer cells (Schuster et al., 2015, Sampath et al., 2015). However, cancer cells do not efficiently utilize the *de novo* synthesis pathway, frequently do not express NAPRT-1 (O'Brien et al., 2013) and have low levels of nicotinic acid (Kirkland, 2009). Therefore, many cancer cells are highly reliant on the NAMPT dependent salvage pathway for NAD production and thus NAMPT is an attractive therapeutic target for cancer (Sampath et al., 2015).

NAMPT overexpression has been shown to increase the activity of NAD-dependent SIRTs and protect cells from death due to Poly (ADP-ribose) polymerase (PARP) overexpression. SIRT1 plays a role in a number of biological processes including carcinogenesis (Bi and Che, 2010). This function of NAMPT may be of particular interest in the setting of oesophageal adenocarcinoma, where cellular stress, due to chronic exposure of the oesophagus to gastric refluxate, plays an important role in pathogenesis (Dvorak et al., 2007).

The primary amino acid sequence of NAMPT does not include a cytokine signal sequence. Despite this, NAMPT is released extracellularly by a variety of cells (Dahl et al., 2012). It had

been previously speculated that this release of extracellular NAMPT was simply due to cell lysis or death but a more recent study has indicated that NAMPT is positively secreted through a non-classical secretory pathway(Revollo et al., 2007).

Thus NAMPT exists in two forms which are commonly described as intracellular NAMPT (iNAMPT) and extracellular NAMPT (eNAMPT). iNAMPT is commonly observed in the cytoplasm and, depending on the cell type, may also be found in the nucleus. Some studies have reported that iNAMPT is also present in the mitochondria although this has been disputed(Bi and Che, 2010) (Stein and Imai, 2012, Garten et al., 2015). iNAMPT is widely expressed and has been well characterised as an NAD biosynthetic enzyme as discussed above. The precise function of eNAMPT is less clear, although potential pro-inflammatory (Kieswich et al., 2016, Moschen et al., 2007), NAD biosynthetic (Yoon et al., 2015) and insulin-mimetic functions have been ascribed to it(Dahl et al., 2012). However these putative functions are controversial and have been disputed(Hara et al., 2011, Garten et al., 2009). Some of this uncertainty regarding the function of eNAMPT may be due to the occurrence of structurally and functionally distinct monomeric and dimeric forms of the protein (Kieswich et al., 2016) Dimerisation is believed to be essential for the biosynthetic functions of NAMPT while the eNAMPT monomer appears to have NAD-independent pro-inflammatory functions (Kieswich et al., 2016). There is further uncertainty regarding the question of whether NAMPT occurs in the same form or configuration within the cell as it does in the extracellular space(Garten et al., 2015).

NAMPT over expression has been observed in a number of cancers including colorectal, breast, thyroid, gastric, ovarian and pancreatic cancers (Dalamaga, 2012, Bi and Che, 2010, Sawicka-Gutaj et al., 2015, Shackelford et al., 2010).

Genetic polymorphisms of NAMPT have been correlated with susceptibility to oesophageal squamous cell carcinoma (Zhang et al., 2015). Just recently, a study in oesophogastric junction adenocarcinoma has found iNampt expression to be an independent prognostic indicator for patients with this malignancy(Li et al., 2017). Interestingly, this study also identified an association between iNAMPT, eNAMPT and visceral obesity. However, to our knowledge, the function or expression of NAMPT has not been previously studied in the context of oesophageal adenocarcinoma.

7.2 Objectives

In this chapter we sought to investigate the nature, and functional significance, of the interaction between RACK1 and NAMPT in FLO-1 oesophageal adenocarcinoma cells. Specifically we aimed to;

- (i) Determine if RACK1 and NAMPT interact directly and, if they do, to map the site(s) on RACK1 which mediate their interaction.
- (ii) Investigate if NAMPT is of functional importance to FLO-1 cell biology.
- (iii) Search for functional implications of the RACK1-NAMPT interaction in FLO-1 cells.

7.3 Results

7.3.1 NAMPT interacts directly with RACK1.

As shown in chapter 6, NAMPT co-immunoprecipitates with RACK1 in lysates prepared from FLO-1 cells. This indicates that RACK1 and NAMPT exist in a complex in this cell type. We next investigated if RACK1 interacts directly with NAMPT using peptide array technology. This investigation was conducted in collaboration with Dr Patrick Kiely (University of Limerick, Ireland).

Peptides arrays spanning the entire RACK1 protein were first generated on nitrocellulose membranes. Scanning libraries of overlapping 18-mer peptides covering the entire sequence of RACK1 were produced by automatic SPOT synthesis and synthesized on continuous cellulose membrane supports on Whatman 50 cellulose using Fmoc (9-fluorenylmethyloxycarbonyl) chemistry with the AutoSpot-Rosbot ASS 222 (Intavis Bioanalytical Instruments). (See chapter 2 for more details on the production of RACK1 peptide arrays).

The interaction of NAMPT with the RACK1 array was then investigated by overlaying the cellulose membranes with 3.3 µg/ml of recombinant NAMPT. Bound protein was detected with specific rabbit antisera for NAMPT and a secondary anti-rabbit antibody. Results of this indicated that NAMPT directly interacts with RACK1. As illustrated in figure 7.1, the region of RACK1 with which NAMPT interacted was mapped to the WD1 and WD2 region of RACK1.

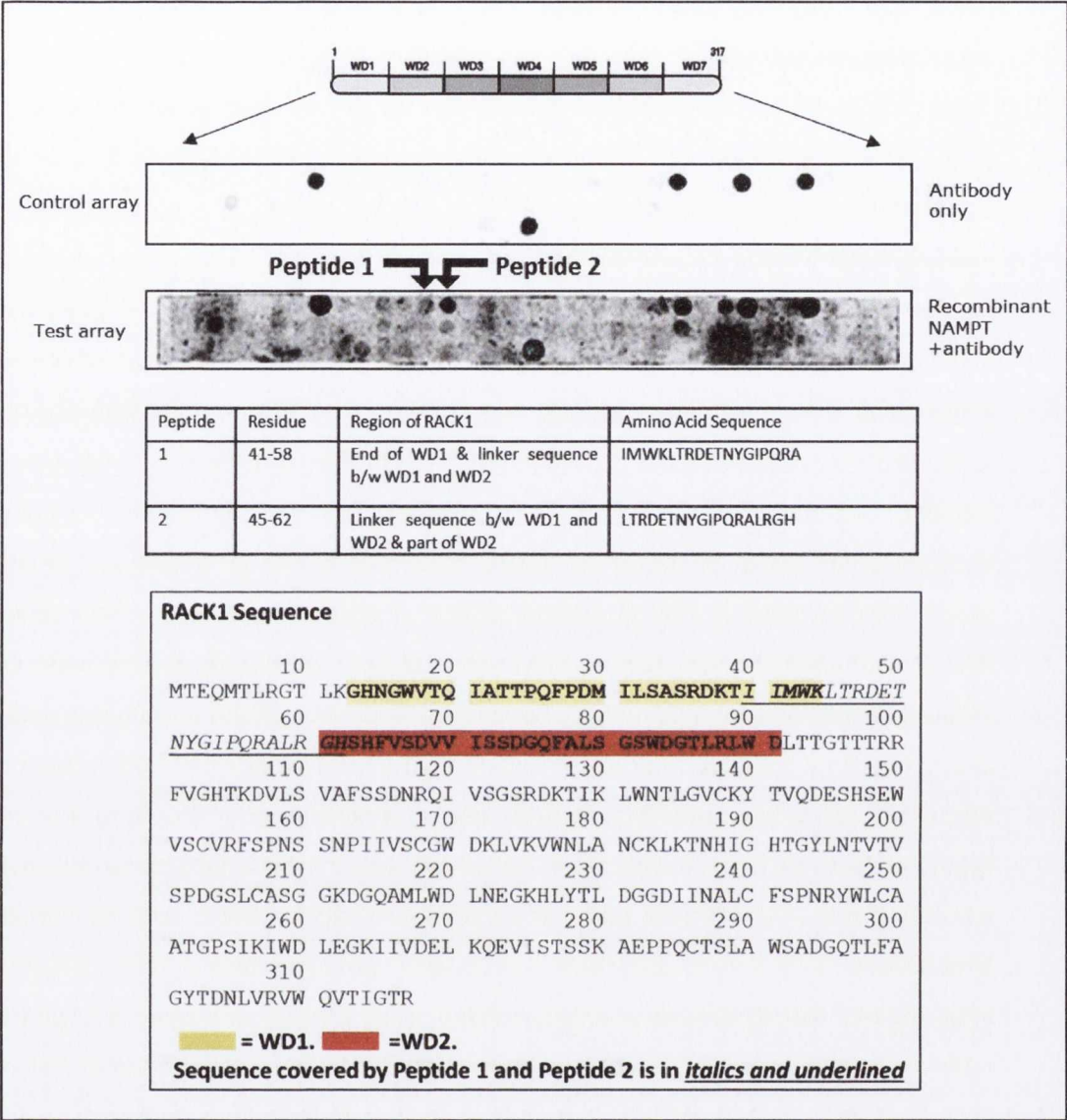


Figure 7.1: Identification of NAMPT as a direct RACK1 interaction partner. Peptide arrays encompassing the entire RACK1 sequence were generated and probed with recombinant NAMPT. Dark spots are indicative of peptide binding. Two binding peptides (numbered 1 and 2) unique to the array probed with NAMPT were identified. The table displays the amino acid identity of Peptides 1 and 2 and their location within the entire RACK1 sequence is illustrated below the table.

7.3.2 NAMPT interacts with arginine 47 in the linker region between WD1 and WD2 of RACK1 and is also very likely to interact with lysine 44 in WD1 and arginine 60 in the linker region between WD1 and WD2.

Having shown that NAMPT interacts directly with RACK1, we next sought to identify specific amino acid residues on RACK1 which mediate this interaction. Specific alanine scanning substitution arrays were generated for selected RACK1 peptides of interest. These peptides correspond to 'Peptide 1' and 'Peptide 2' described in figure 7.1 above. They were generated using the same technique as was described for the RACK1 peptide arrays in section 7.3.1 above. Peptide 1 corresponds to the amino acid sequence from residues 41 to 58 of the RACK1 sequence and maps to the end of WD1 and to most of the linker region between WD1 and WD2. Peptide 2 corresponds to the amino acid sequence from residue 45 to 62 of the RACK1 sequence and maps to the linker region between WD1 and WD2 and to the very start of the WD2 propeller blade. In these alanine substitution arrays each amino acid residue was sequentially substituted with an alanine, as is illustrated in figure 7.2. The interaction of NAMPT with these RACK1 alanine substitution arrays was investigated by overlaying the cellulose membranes with 3.3 µg/ml of recombinant NAMPT. Bound protein was detected as described for RACK1 peptide arrays in section 7.3.1. The binding of NAMPT to each peptide was quantified by densitometry and presented as a percentage of the control un-mutated wild-type (WT) parent sequence (see figure 7.2). More than a 50% reduction in binding intensity is generally indicative that that particular, substituted amino acid, is involved in the interaction.

In the case of the Peptide 1 alanine substitution array, a strong reduction in NAMPT binding intensity was observed (55% reduction) when lysine 44 was substituted with alanine. This indicates that lysine 44 of the RACK1 protein is involved in the interaction between RACK1 and NAMPT. When arginine 47 was substituted for an alanine, a 45% depletion in binding was observed. This reduction was just shy of the arbitrary 50% reduction which is recognised to indicate interaction involvement. However, in the case of the 'Peptide 2' alanine substitution array, a dramatic reduction in binding intensity (94% reduction) was observed when arginine 47 was substituted with alanine. Thus together, these results strongly indicate that arginine 47 of the RACK1 sequence is involved in mediating the interaction between RACK1 and NAMPT. In this Peptide 2 array, a > 50% reduction in binding intensity was also observed when tyrosine 52 (54% reduction in binding intensity) or arginine 60 (55% reduction in binding intensity) was substituted with an alanine. In contrast, in the Peptide 1 array substitution of tyrosine 52 did not lead to a reduction in binding intensity. Thus, whether tyrosine 52 is involved in the RACK1 – NAMPT interaction is inconclusive. Arginine 60 was not included in the Peptide 1 sequence.

When the results obtained from both Peptide 1 and Peptide 2 are considered together, there is strong evidence that arginine 47 of the RACK1 sequence is involved in, and required for, the interaction between RACK1 and NAMPT. There is also a strong indication that lysine 44 and arginine 60 are also involved in the interaction.

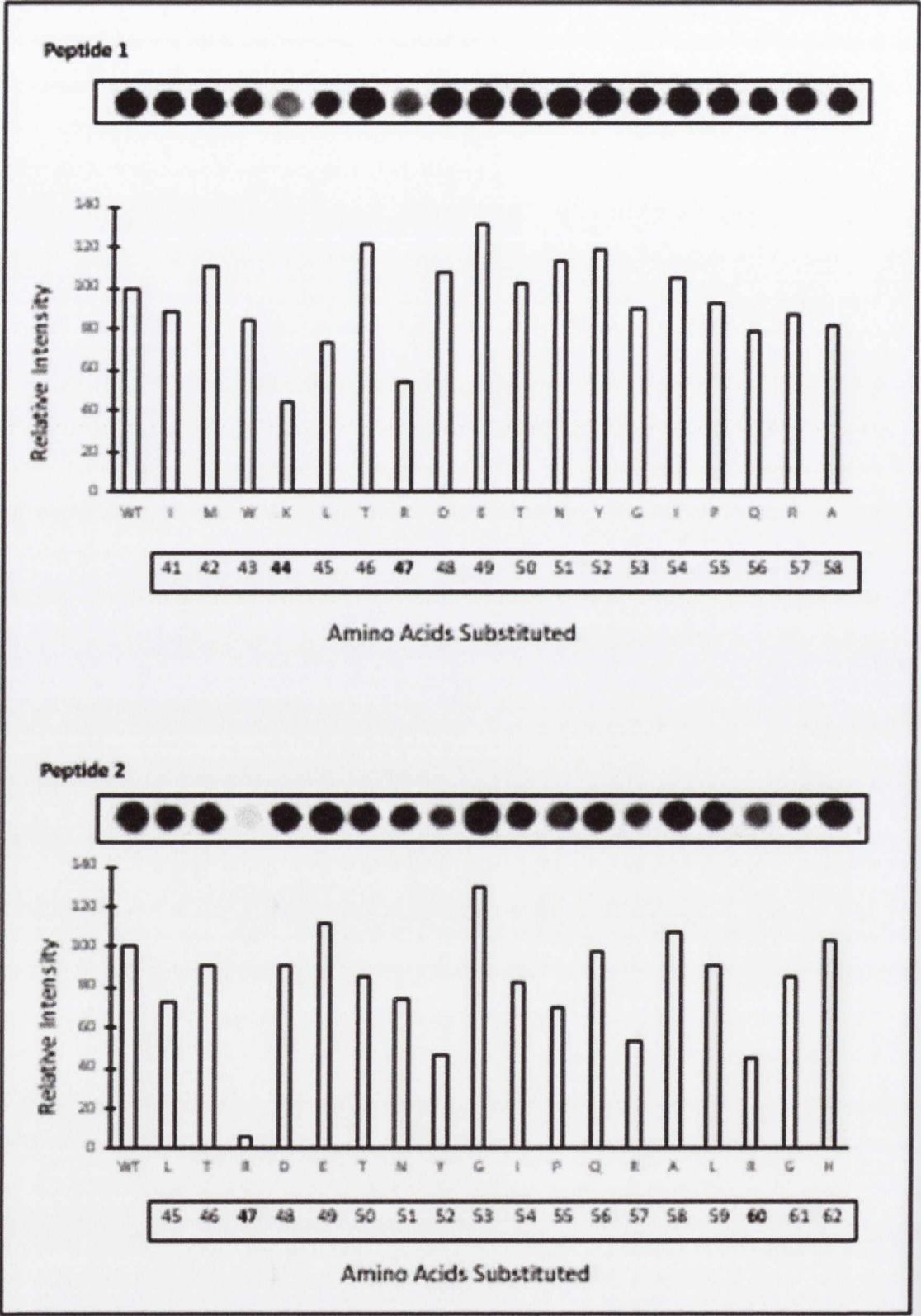


Figure 7.2: Identification of NAMPT binding sites on RACK1.Specific alanine substitution arrays of a region mapping to the end of WD1, the linker region between WD1 and WD2, and

the very start of WD2, of the RACK1 sequence. The binding of GST-NAMPT to each alanine substituted peptide was quantified by densitometry and displayed as a percentage of the control parent wildtype sequence, with an underlay identifying the amino acid, which was substituted with an alanine. More than 50% reduction in binding intensity is indicative of involvement in the interaction. Results very strongly indicate that arginine 47 in the RACK1 sequence is involved in interacting with NAMPT. Results also indicate that lysine 44 and arginine 60 in the RACK1 sequence are likely to be involved in mediating the RACK1-NAMPT interaction.

7.3.3 NAMPT is present in the cytoplasm and nucleus of FLO-1 cells.

The subcellular localisation of NAMPT was examined in FLO-1 cells using immunofluorescent staining and confocal microscopy. NAMPT was found present in both the cytoplasm and nucleus. The cells were also costained for RACK1. In general, the two proteins exhibited quite different staining patterns. However a few small areas of partial RACK1 – NAMPT colocalisation were observed in the cytoplasm of some cells, indicating that a subset of RACK1 may associate with a subset of NAMPT at certain times in FLO-1 cells.

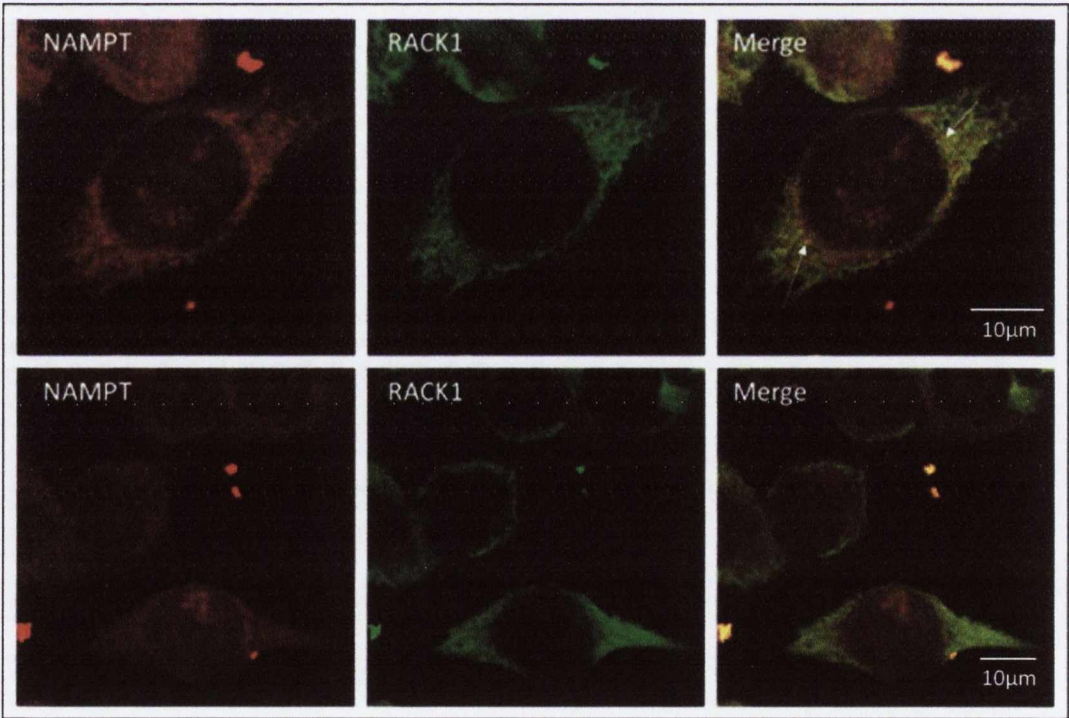


Figure 7.3 NAMPT is present in the cytoplasm and nucleus of FLO-1 cells. FLO-1 cells were fixed and staining as described in chapter 2, section X. NAMPT positive immunostaining was observed in areas of both the cytoplasm and nucleus. RACK1 positive immunostaining was confined to the cytoplasm and in general, it exhibited a different staining pattern to that of NAMPT. However, in some cells, a small amount of colocalisation between RACK1 and NAMPT was observed in the cytoplasm, as is highlighted by the white arrows in the top panel.

7.3.4 NAMPT depletion reduces FLO-1 cell proliferation and viability.

Having shown that NAMPT interacts with RACK1, we now sought to determine if NAMPT has a functional role in FLO-1 cells. To investigate if NAMPT is involved in FLO-1 cell proliferation and viability, we depleted NAMPT from FLO-1 cells using an siRNA approach. Cell proliferation was monitored in real time using an impedance-based xCELLigence proliferation assay system. This system is described in more detail in chapter 2. Our results indicate that NAMPT depletion significantly reduces FLO-1 cell proliferation, as is illustrated in figure 7.4 (B) (i-ii). Cell viability was also examined using MTT assays and was found to be significantly reduced in NAMPT depleted cells; see figure 7.4 (A).

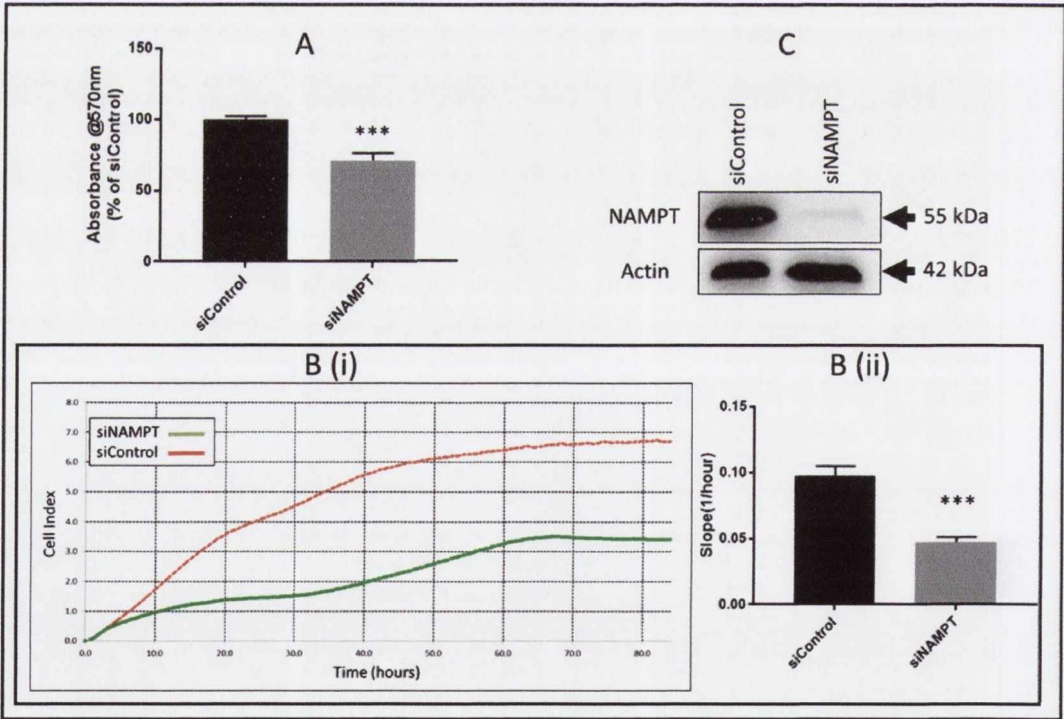


Figure 7.4: NAMPT depletion reduces proliferation and viability of FLO-1 cells.

NAMPT was depleted from FLO-1 cells using siRNA. (A) An MTT assay was performed to measure cell metabolic activity, as an indicator of viability and proliferative ability. NAMPT-

depleted FLO-1 cells exhibited a significant reduction in viability/proliferation in comparison to control cells. *** $P \leq 0.001$, unpaired t test. $N=5$. Data presented are mean $\pm$ SEM. (B) Cell proliferation was also assessed in real time using the xCELLigence RTCA platform. Cells were seeded into xCELLigence E plates and proliferation was measured over 80 hours and recorded in terms of cell index. (B)(i) A representative graph of cell index values for control and NAMPT depleted cells recorded in real time over a period of 80 hours. (B) (ii) The rate of proliferation as determined by analysing the slope of the line between the 8 and 48 hour interval. *** $P \leq 0.001$, unpaired t test. $N=3$. Data presented are mean $\pm$ SEM. (C) Representative Western blot showing NAMPT depletion from FLO-1 cells using siRNA.

7.3.5 NAMPT depletion reduces FLO-1 cell migration

We next investigated if NAMPT is involved in FLO-1 cell migration. NAMPT expression was depleted from FLO-1 cells using siRNA. 2D cell migration was then assessed using scratch wound assays in control and NAMPT depleted cells. The rate of cell migration into the wound was slower in NAMPT depleted cells in comparison to control cells (see figure 7.5). The xCELLigence RTCA platform was used to assess 3D cell migration. Serum starved cells were placed into an upper, fibronectin-coated chamber CIM chamber and cell migration down into a lower, chamber containing cell culture medium with 10% serum, was monitored in real time. This revealed a significant reduction in the 3D migration of NAMPT depleted FLO-1 cells (figure 7.5). It should be remembered that NAMPT depletion dramatically reduces FLO-1 cell proliferation and viability (see figure 7.4). Therefore it may be that the observed reductions in 2D and 3D cell migration in NAMPT-depleted cells may be due to the reduced viability of NAMPT depleted cells as opposed to a specific inhibition of migration.

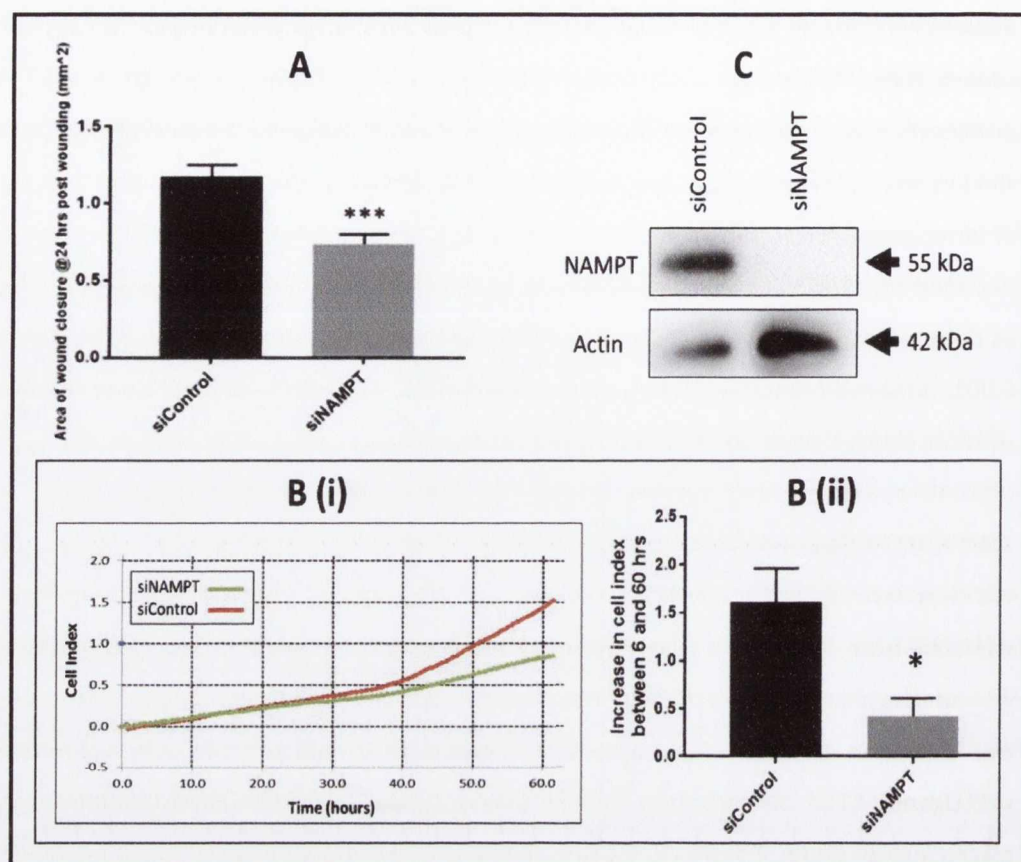


Figure 7.5: NAMPT depletion reduces 2D and 3D migration of FLO-1 cells. NAMPT was depleted from FLO-1 cells using siRNA. (A) Scratch wound assays were used to assess 2D cell migration. Cells were grown to confluence and then scratch wounded as described in chapter 2, section X. The size of the wounded area into which the cells migrated was measured after 24hours. NAMPT-depleted cells migrated more slowly and had filled in less wound area after 24hours. *** $P \leq 0.001$, $N=3$, unpaired t test. Data presented are mean $\pm$ SEM. (B) The xCELLigence RTCA platform was used to assess 3D cell migration. Serum starved cells were placed into an upper, fibronectin-coated chamber CIM chamber and cell migration down into a lower, chamber containing cell culture medium with 10% serum, was monitored in real time and recorded in the form of cell index values. (B)(i) A representative graph of cell index values for control and NAMPT depleted cells recorded in real time over a period of 60 hours. (B) (ii) The change in cell index between 6 and 60 hours. * $P \leq 0.05$. $N=3$, unpaired t-test. Data presented are mean $\pm$ SEM. (C) Representative Western blot showing NAMPT depletion from FLO-1 cells using siRNA.

7.3.6 A second, higher molecular weight form of NAMPT is observed in FLO-1 cell lysates analysed by SDS-PAGE.

Using SDS PAGE and Western blotting techniques immunoreactive NAMPT was detected in FLO-1 cell lysates. As expected, NAMPT was observed at approximately 55kDa. However a second band, at approximately 70kDa was also detected by the anti NAMPT antibody. When FLO-1 cells were depleted of NAMPT using an siRNA approach, the intensity of this band decreased indicating that it was a form of NAMPT (see figure 7.7). If this 70KDa band corresponds to dimeric NAMPT, it is plausible that its intensity could be reduced or obliterated if the samples were prepared for SDS PAGE using a buffer with a stronger reducing agent. We first observed this 70kDa form of NAMPT when using laemmli sample buffer with β -mercaptoethanol as a reducing agent. However DTT has been described as a stronger reducing agent than β -mercaptoethanol (Aitken et al., 2008). Samples were therefore prepared using a different SDS-PAGE sample buffer containing DTT and resolved on an SDS PAGE gel. When this DTT containing-SDS-PAGE sample buffer was used, the intensity of the 70kDa band was significantly reduced in comparison to when laemmli sample buffer containing β -mercaptoethanol had been used (figure 7.6). However, when DTT was excluded from this SDS-PAGE sample buffer, the intensity of the 70kDa band was also reduced, indicating that DTT was not responsible for the intensity reduction observed (figure 7.6). It is therefore unclear if this 70kDa form of NAMPT is dimeric NAMPT or if it is a form of NAMPT which has been post-transcriptionally modified, perhaps in preparation for its secretion from the cell.

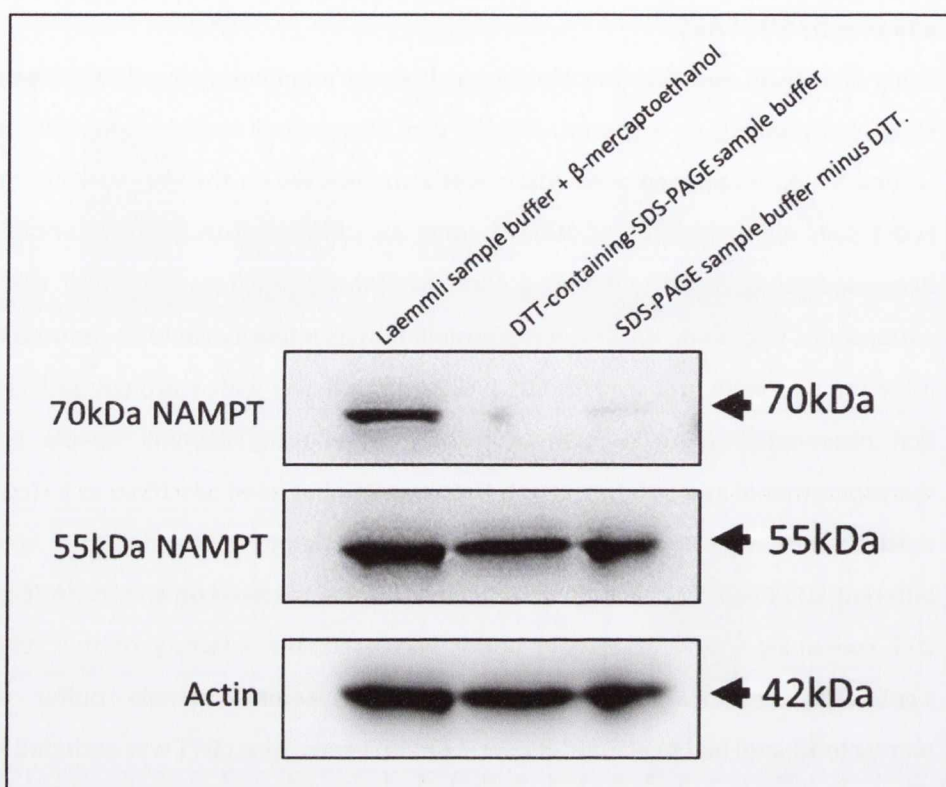


Figure 7.6: Detection of 70kDa NAMPT by Western blotting is influenced by the sample buffer used when preparing lysates for SDS PAGE.

7.3.7 RACK1 depletion results in decreased levels of high molecular weight NAMPT. Having shown that RACK1 interacts with NAMPT, we next investigated if RACK1 can influence the protein levels of 55kDa and 70kDa NAMPT in FLO-1 cells. RACK1 was depleted from FLO-1 cells using an siRNA approach. NAMPT protein levels were then analysed in control and RACK1 depleted cells. RACK1 depletion did not significantly affect the levels of 55kDa NAMPT. However, a significant reduction in the level of high molecular weight NAMPT (70kDa) was observed in RACK1 depleted cells.

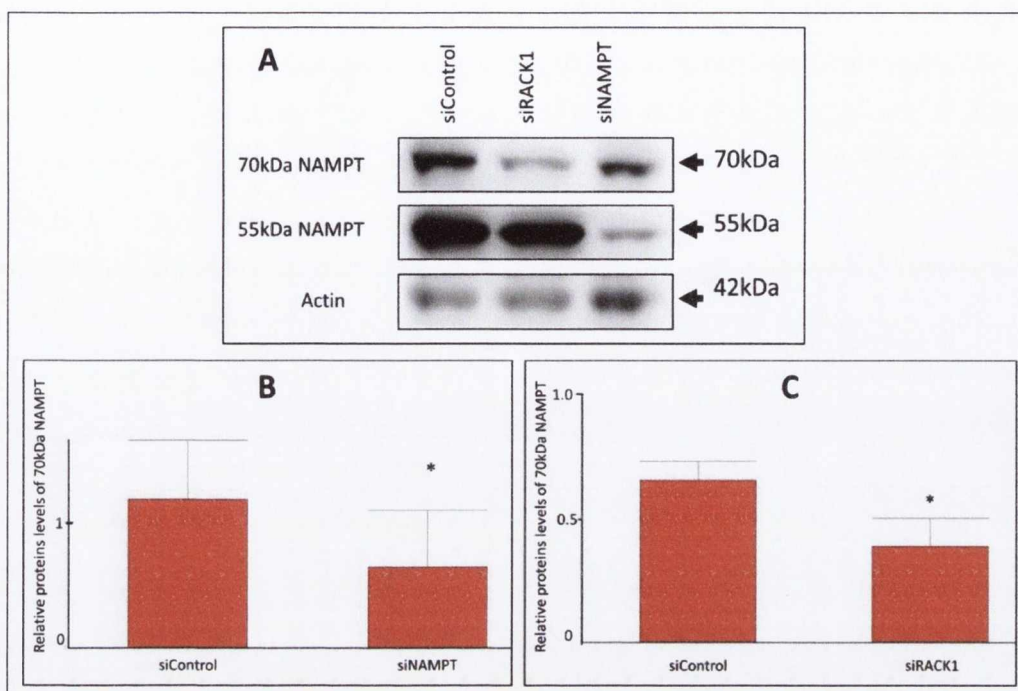


Figure 7.7: RACK1 depletion results in decreased levels of high molecular weight NAMPT. Levels of 70kDa NAMPT are reduced in FLO-1 cells treated with either RACK1 targeting siRNA or NAMPT targeting siRNA. This is illustrated in (A) by a representative Western blot. (B) 70kDa NAMPT protein bands were quantitated from Western blots using densitometry and normalised against actin. Data are presented as the mean $\pm$ SEM from four independent experiments. The level of 70kDa NAMPT is significantly reduced when cells are treated with NAMPT targeting siRNA (siNAMPT) in comparison to cells treated with non-targeting siRNA (siControl). * $P < 0.05$, paired t-test. (C) 70kDa NAMPT protein bands were quantitated from Western blots using densitometry and normalised against actin. Data are presented as the mean $\pm$ SEM from four independent experiments. The level of 70kDa NAMPT is significantly reduced when cells are treated with RACK1 targeting siRNA (siRACK1) in comparison to cells treated with non-targeting siRNA (siControl). * $P < 0.05$, paired t-test.

7.3.8 The effects of IL-1 β stimulation on NAMPT protein levels in FLO-1 cells.

As reported above, we have shown that RACK1 influences the levels of 70kDa NAMPT in FLO-1 cells. This high molecular weight form of NAMPT could potentially be a modified form of the protein which is about to be secreted from the cell. A previous study in HUVEC cells has demonstrated that IL-1 β stimulation (10 ng/mL; 18 h) promotes NAMPT secretion and also

increases levels of intracellular NAMPT (Romacho et al., 2013). RACK1 deficiency increases IL-1 β secretion in some cell types (Zhao et al., 2015). We therefore wished to investigate if IL-1 β played a role in this modulation of 70kDa NAMPT by RACK1. FLO-1 cells were treated with IL-1 β (10 ng/mL; 18 h) and NAMPT protein levels were then assessed by Western blotting (figure 7.8). No differences in the level of 55kDa NAMPT was found between control and IL-1 β treated cells. The experiment was performed three times. In one replicate, the level of 70kDa NAMPT appeared to increase in IL-1 β treated cells. However this increase was not observed in the other replicates of the experiment. Thus our results are inconclusive and the experiment should be repeated.

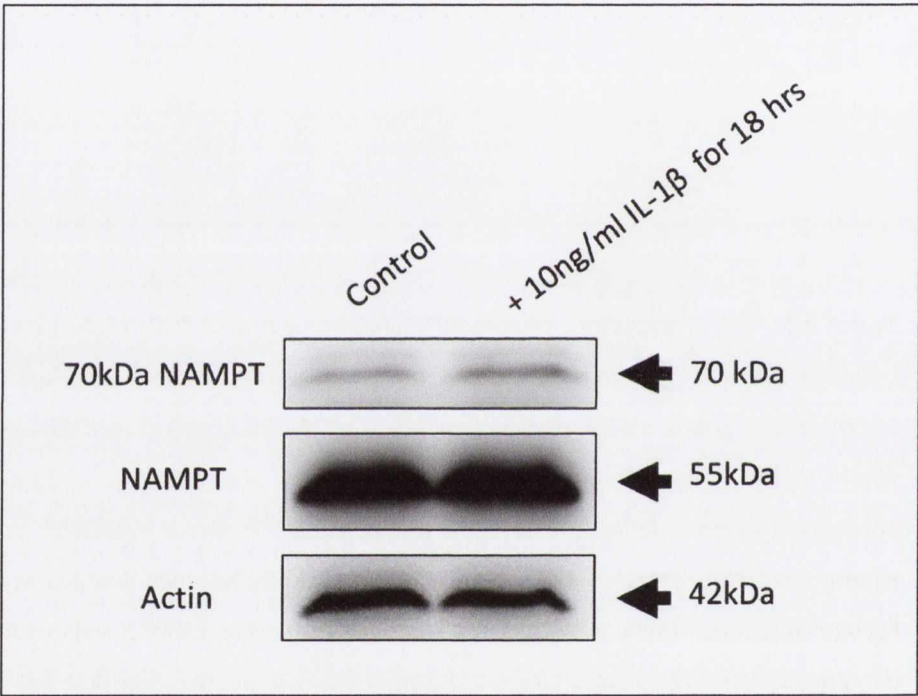


Figure 7.8: The impact of IL-1 β stimulation on NAMPT protein levels in FLO-1 cells.

FLO-1 cells were treated with IL-1 β at a concentration of 10 ng/mL for 18 hours. Cells were then lysed and NAMPT protein levels were assessed by Western blotting. No difference in the level of 55kDa NAMPT was found between control and IL-1 β treated cells. The experiment was performed three times. In one replicate, the result of which is shown in the blot above, the level of 70kDa NAMPT appeared to increase in IL-1 β treated cells. However this increase was not observed in the other two replicates of the experiment. Thus our results are inconclusive.

7.3.9 NAMPT depletion dramatically reduces NAD levels in FLO-1 cells.

Many cancer cells are particularly reliant on NAMPT for NAD production, as was discussed in the introduction to this chapter. However, the role of NAMPT in oesophageal adenocarcinoma cells has not been examined previously. Therefore we sought to investigate if FLO-1 cells require NAMPT to maintain their NAD levels. NAMPT was depleted from FLO-1 cells using an siRNA approach. NAD and levels were assessed in control and NAMPT depleted cells using a commercially available NAD/NADH Colourmetric Assay Kit (Abcam). As can be seen in figure 7.9, NAD levels was dramatically reduced in NAMPT depleted cells. This indicates that FLO-1 cells are highly reliant on NAMPT for NAD biosynthesis.

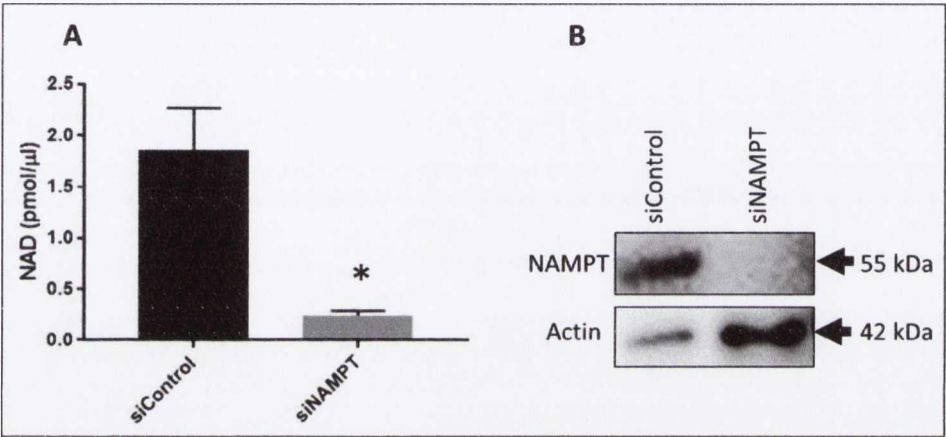


Figure 7.9: NAMPT depletion reduces NAD levels in FLO-1 cells. NAMPT was depleted from FLO-1 cells using an siRNA approach. NAD concentration was measured in control and NAMPT depleted cells using an NAD/NADH Colourmetric Assay Kit (Abcam). (A) NAD concentration, in pmol/ul, in control and NAMPT depleted cells. Data are presented as the mean $\pm$ SEM and were analysed using Student's t-test. * $P < 0.05$. $N = 3$. (B) Representative Western blot showing siRNA mediated depletion of NAMPT from FLO-1 cells.

7.3.10 RACK1 depletion does not affect NAD levels and the NAD/NADH ratio in FLO-1 cells.

We have shown that NAMPT is required to maintain NAD levels in FLO-1 cells. We therefore concluded that measurement of NAD levels in FLO-1 cells could serve as a useful tool to examine NAMPT activity. Having shown that RACK1 both interacts with NAMPT, and affects levels of 70kDa NAMPT, we next examined if RACK1 depletion influences NAD levels in FLO-1 cells.

RACK1 was depleted from FLO-1 cells using an siRNA approach and NAD concentration was measured in control and RACK1 depleted cells using the same method as that employed in section 7.3.9. As can be seen in figure 7.10 no significant difference in NAD concentration was observed between control and RACK1 depleted cells. Thus the interaction of RACK1 with NAMPT does not appear to influence its enzymatic role in the NAD biosynthesis pathway. However NAMPT does have a variety of other functions and thus RACK1 may play a role in controlling some other function of NAMPT. Our finding that RACK1 influences levels of 70kDa NAMPT, but does not influence NAD levels, suggests that this 70kDa form of NAMPT, which we observed in FLO-1 cells, may have a function that is separate from the function of NAMPT as an enzyme in the NAD biosynthesis pathway.

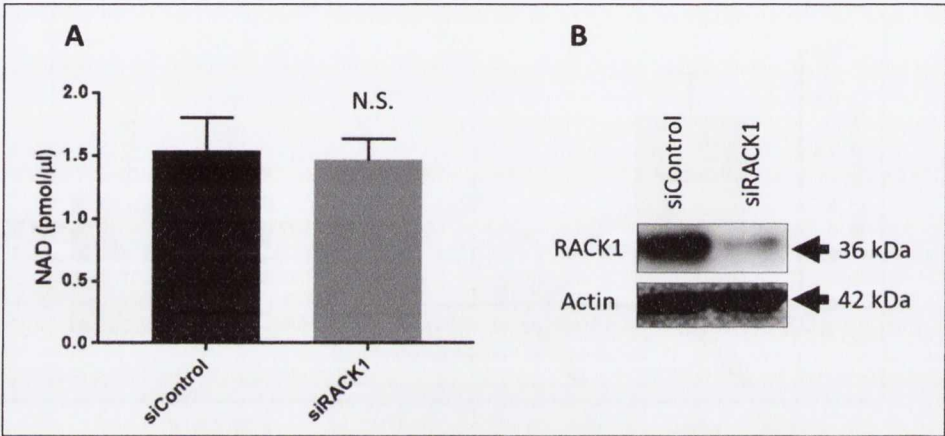


Figure 7.10: RACK1 depletion has no significant effect on NAD concentration in FLO-1 cells. RACK1 was depleted from FLO-1 cells using an siRNA approach. NAD concentration was measured in control and RACK1 depleted cells using an NAD/NADH Colourmetric Assay Kit (Abcam). (A) NAD concentration, in pmol/ul, in control and RACK1 depleted cells. Data are presented as the mean $\pm$ SEM and were analysed using Student's t-test. N.S.; not significant; $P > 0.05$. $N=3$. (B) Representative Western blot showing siRNA mediated depletion of RACK1 from FLO-1 cells.

7.4 Discussion

In chapter 6, we identified NAMPT as a potential RACK1 interaction partner using a proteomics strategy. To our knowledge, no previous interaction between RACK1 and NAMPT has been reported in the literature. In this current chapter, we therefore sought to examine the interaction between RACK1 and NAMPT in more detail and to also determine whether NAMPT interacts directly with RACK1, or if it simply forms part of a RACK1 containing multi-protein

complex. The function of NAMPT has not been previously examined in oesophageal adenocarcinoma. Therefore we also set out to investigate the functional significance of this protein in oesophageal adenocarcinoma cells. Furthermore, we sought to determine the functional implications of the RACK1-NAMPT interaction in oesophageal adenocarcinoma cell biology.

Using a RACK1 peptide array we determined that NAMPT interacts directly with RACK1. Furthermore we identified regions spanning RACK1's propeller blades WD1 and WD2 as putative binding sites for NAMPT. We then determined the amino acids on RACK1 involved in NAMPT interaction using alanine substitution arrays. Our results strongly indicate that NAMPT interacts with arginine 47 in the linker region between WD1 and WD2 of RACK1. They also indicate that NAMPT interacts with lysine 44 in the WD1 region of the RACK1 protein and arginine 60 in the linker region between WD1 and WD2.

Arginine and lysine are both positively charged basic amino acids under physiological conditions and are very often exposed to protein surfaces (Sokalingam et al., 2012). Thus, it makes sense that we identified arginine and lysine residues as putative mediators of the interaction between RACK1 and NAMPT. Indeed, other studies (including our own unpublished data) have identified various lysine and arginine residues in the RACK1 sequence as mediators of its interaction with partner proteins (Neasta et al., 2012). RACK1's scaffolding properties are mediated by the presence of its seven WD-repeats which serve as binding sites for multiple different proteins. Thus it is not surprising that we identified lysine 44 on WD1 as a residue on RACK1 with which NAMPT interacts.

Analysis of schematic representations of the RACK1 structure, that detail the organisation of its WD-repeats, indicate that both lysine 44 and arginine 47 are positioned so as to be available for interaction with a partner proteins (Adams et al., 2011). Thus it is very plausible these residues do indeed mediate a NAMPT- RACK1 interaction.

We examined the localisation of NAMPT in FLO-1 cells and also examined if NAMPT colocalises with RACK1. The subcellular localisation of NAMPT appears to be, at least in part, cell type specific. A large number of studies in various cell types have reported that NAMPT localises to the cytoplasm. However NAMPT also had cell type specific localisation to the nucleus and can also be found in mitochondria (Stein and Imai, 2012, Bi and Che, 2010). Our own immunofluorescent staining in FLO-1 cells indicated that that NAMPT was present in both the cytoplasm and nucleus of FLO-1 cells. We also co-stained FLO-1 cells with RACK1. We observed a small amount of partial colocalisation of RACK1 and NAMPT. Thus a small subset of total cellular RACK1 may interact with a subset of cellular NAMPT. Indeed, it is well known that

various subsets of RACK1 occur in different parts of the cell, where they carry out different functions and can undergo both transient and constitutive interactions with an array of different interaction partners(Adams et al., 2011).

The expression and function of NAMPT has not been previously examined in oesophageal adenocarcinoma. Therefore we also set out to investigate the functional significance of this protein in oesophageal adenocarcinoma cells. We found that NAMPT depletion from FLO-1 cells dramatically reduced their proliferation and viability, as assessed by MTT and impedance-based xCELLigence proliferation assay. Thus it seems clear that FLO-1 cells are highly dependent on NAMPT and thus the potential role of RACK1 in regulating NAMPT is of significant interest in the context of oesophageal adenocarcinoma.

We had previously shown, in chapter 5, that RACK1 depletion results in reduced migration of FLO-1 cells. We therefore examined if NAMPT depletion also reduces FLO-1 cell migration. Scratch wound assay experiments revealed that migration was significantly reduced in NAMPT depleted FLO-1 cells in comparison to controls. However, as discussed above, NAMPT depletion dramatically reduced FLO-1 cell viability and proliferation. Thus, it is possible that the observed reduction in migration is simply due to the dramatic reduction in cell viability which occurs with NAMPT depletion.

NAMPT is the rate-limiting enzyme in the mammalian NAD salvage pathway(Dahl et al., 2012) NAD is an important cofactor and second messenger and is essential for cell survival. It can also be synthesised de novo from tryptophan or produced from nicotinic acid through an alternative NAPRT1-dependent pathway (Dahl et al., 2012). However cancer cells do not efficiently utilize the de novo NAD synthesis and frequently do not express NAPRT-1(O'Brien et al., 2013, Sampath et al., 2015). Therefore many cancer cells are highly reliant on NAMPT for NAD production. To test if this is also the case with FLO-1 cells, we depleted their NAMPT expression using siRNA and measured their NAD levels. This revealed a dramatic reduction in cellular NAD levels following NAMPT depletion. Thus FLO-1 cells appear to be highly reliant on NAMPT for NAD synthesis. Indeed, our observance that NAMPT depletion also significantly reduces FLO-1 cell viability, proliferation and migration indicates that these cells are highly reliant on NAMPT to maintain normal cellular functions.

Measurement of cellular NAD levels can thus serve as a useful indicator of NAMPT function in cancer cells which rely on NAMPT for production of this metabolite. In light of our discovery that RACK1 directly interacts with NAMPT, we wished to investigate if RACK1 can influence NAMPT activity. We therefore examined NAD levels in control and RACK1 depleted FLO-1 cells using a commercially available NAD assay kit. Our results found no significant difference between RACK1 depleted and control cells. While this indicates that RACK1 may not have a

huge impact on the function of NAMPT as an NAD producing enzyme, it is important to note that NAMPT has many, varied functions and thus it is possible that RACK1 may influence an NAD production-independent function of this protein. Furthermore, we used an siRNA approach to deplete RACK1 from FLO-1 cells. While we achieved a very significant reduction (70 - 80%) in RACK1 levels using this technique it is important to note that some RACK1 did remain. Furthermore, RACK1 is highly expressed in FLO-1 cells, as was reported in chapter 5. Thus, we cannot rule out the possibility that RACK1 may influence the activity of NAMPT as an NAD biosynthetic enzyme. It may be that the RACK1 which remained in our RACK1 depleted cells was sufficient to enable proper NAMPT mediated NAD production.

Interestingly, we observed a high molecular weight form of NAMPT (with an apparent molecular weight of approximately 70kDa) when we analysed FLO-1 lysates by Western Blotting. This form of NAMPT was observed in addition to 55kDa NAMPT. Cell treatment with NAMPT targeting siRNA successfully reduced levels of this 70kDa protein, indicating that it was indeed NAMPT rather than an unspecific band detected by the anti-NAMPT antibody.

We hypothesised that if this 70kDa band corresponded to dimeric NAMPT, it might be possible to reduce its intensity or destroy it by using a stronger reducing agent prior to analysis by SDS PAGE and Western blotting. We had initially observed this 70kDa form of NAMPT following sample preparation using laemmli sample buffer containing β -mercaptoethanol as a reducing agent. However DTT has been described as a stronger reducing agent than β -mercaptoethanol (Aitken et al., 2008). Samples were therefore prepared in SDS-PAGE sample buffer containing DTT and resolved on an SDS PAGE gel. When this DTT-SDS-PAGE sample buffer was used, the intensity of the 70kDa band was significantly reduced in comparison to when laemmli sample buffer containing β -mercaptoethanol had been used (figure 7.6). However, when DTT was excluded from this SDS-PAGE sample buffer, the intensity of the 70kDa band was also reduced, indicating that DTT was not responsible for the intensity reduction observed (figure 7.6). It is therefore unclear if this 70kDa form of NAMPT is dimeric NAMPT. Furthermore, previous studies have reported that dimeric NAMPT, from mouse serum, has an apparent molecular weight of 100kDa when analysed by non-reducing SDS-PAGE (Kieswich et al., 2016). A previous study has indicated that extracellular NAMPT has a slightly higher molecular weight than the intracellular form (Revollo et al., 2007). Another group have observed a transient shift in the molecular mass of NAMPT in response to certain stimuli (their preliminary observations)(Revollo et al., 2004). Thus, the 70kDa form of NAMPT which we observed in FLO-1 lysates could possibly represent a sub pool of the protein which has been modified in response to a certain stimulus or which is about to be secreted from the cell.

Interestingly, when we depleted RACK1 from FLO-1 cells, we observed a significant reduction in the level of high molecular weight NAMPT (70kDa). In contrast, RACK1 depletion did not significantly influence the levels of 55kDa NAMPT. Thus RACK1 appears to specifically influence this high molecular weight form of NAMPT. IL-1 β can increase NAMPT secretion in HUVEC cells (Romacho et al., 2013). Interestingly, RACK1 deficiency increases IL-1 β secretion in some cell types (Zhao et al., 2015). If the 70kDa form of NAMPT which we observed represents a form of the protein which is about to be secreted, we hypothesised that RACK1 may regulate it through an IL-1 β dependent mechanism. To test this, we treated FLO-1 cells with IL-1 β (10 ng/mL; 18 h) and examined levels of both 70kDa NAMPT and 55kDa NAMPT (figure 7.8). We observed no difference, in the level of 55kDa NAMPT between control and IL-1 β treated cells. We observed an increase in the level of 70kDa NAMPT in IL-1 β treated cells in one experiment but did not observe this increase in a second and third replicate experiments. Thus our results are inconclusive and the experiment will have to be repeated.

Further work is required to elucidate the functional implications of the interaction between NAMPT and RACK1 in FLO-1 cells. It is interesting that RACK1 appears to regulate the protein levels of 70kDa NAMPT. Further studies, to determine the nature and function of this high molecular weight form of NAMPT, may provide clues on how RACK1 might possibly influence some aspect of NAMPT function.

Our peptide mapping experiments reveal that lysine 44, in the WD1 blade of RACK1, and arginine 47 and arginine 60, in the linker region between WD1 and WD2 of the RACK1 sequence, are required to mediate its interaction with NAMPT. This could be further verified by overexpressing RACK1 truncation mutants (which lack WD1 and WD2) and investigating if this abrogates the RACK1-NAMPT interaction. It would also be useful to determine if the interaction between NAMPT and RACK1 could be abrogated by the overexpression of RACK1 mutants where lysine 44, arginine 47 and arginine 60 are replaced with alanine residues.

The RACK1-NAMPT interaction could be evaluated further again by preventing binding with RACK1-derived peptides specific for the NAMPT binding site. Previous studies have made use of RACK1 derived peptides to examine the interaction between RACK1 and other binding partners such as the 14-3-3 scaffolding protein (Neasta et al., 2012). RACK1-derived peptides are advantageous over RACK1 targeting siRNA as they can block interactions with selected proteins only. This is particularly valuable in elucidating the functional implications of the protein specific interactions of scaffolding molecules, which have multiple interaction partners.

7.5 Conclusion

We have found that RACK1 interacts directly with NAMPT in FLO-1 cells. We have identified amino acid residues of RACK1 that are necessary for its interaction with NAMPT. Furthermore we have found that NAMPT plays a significant, functional role in FLO-1 cells. Thus the functional implications of the interaction between RACK1 and NAMPT are of interest in the setting of oesophageal adenocarcinoma. RACK1 appears to influence the protein levels of a high molecular weight form of NAMPT. However further studies are required to determine the nature of this form of NAMPT, and to fully elucidate the implications of the interaction between RACK1 and NAMPT in FLO-1 cells.

Chapter 8: General Discussion

Oesophageal cancer is the eight most common cancer worldwide and is a highly fatal disease, being the sixth most common cause of cancer death in the world(Thrift, 2016). There are two main subtypes of oesophageal cancer, namely oesophageal squamous cell carcinoma and oesophageal adenocarcinoma. Oesophageal squamous cell carcinoma is the most common subtype worldwide but there has been a huge shift in the epidemiology of oesophageal cancer in Western populations with the result that oesophageal adenocarcinoma is now the most common subtype in Europe, North America and Australia(Thrift, 2016). Indeed, since the early 1970s, oesophageal adenocarcinoma incidence has risen dramatically in most Western populations; in fact, from 1973 to 2001, it was one of the fastest rising cancers in many developed countries. The rate of increase has now slowed but the incidence of oesophageal adenocarcinoma is still rising and will pose a significant health burden in future years(Thrift, 2016).

Epidemiological studies strongly implicate gastro-oesophageal reflux disease as the main causative factor for oesophageal adenocarcinoma. A ground-breaking, nationwide, population-based case-control study in Sweden found that the odds of oesophageal adenocarcinoma increased about eight-fold for people with recurrent symptoms of gastro-oesophageal reflux disease in comparison to individuals with less frequent symptoms (Lagergren et al., 1999). Obesity, in particular abdominal obesity, has also been associated with oesophageal adenocarcinoma, as has male gender, cigarette smoking and white ethnicity (Thrift, 2016).

Oesophageal adenocarcinoma arises from a precursor lesion called Barrett's oesophagus, a condition where the normal stratified squamous epithelium lining of the oesophagus is replaced by a specialized, intestinal-like columnar epithelium. This columnar epithelium is salmon coloured while the normal stratified squamous epithelium is a pale pearly colour (Garud et al., 2010). The presence of goblet cells in the columnar epithelium is a diagnostic criterion for Barrett's oesophagus in the USA although it is not included in the British Society of Gastroenterology guideline(Pennathur et al., 2013). Barrett's oesophagus is considered to follow a progression sequence from intestinal metaplasia to low grade dysplasia to high grade dysplasia and finally, in a subset of patients, to oesophageal adenocarcinoma(Garud et al., 2010).

The development of Barrett's oesophagus is strongly associated with chronic gastro-oesophageal reflux disease. Gastro-oesophageal refluxate contains a mixture of harmful bile acids, acid and other chemicals which damage the oesophagus. It is thought that the squamous oesophageal cells are replaced by columnar cells in response to this damage during the healing process and that the development of Barrett's oesophagus is a protective adaptation (Pennathur et al., 2013, Garud et al., 2010). Further studies are required to fully understand the mechanisms which underlie the link between severe gastro-oesophageal reflux disease and oesophageal adenocarcinoma but it is hypothesized that reflux-induced injury to the oesophageal epithelium induces a cascade of cytokine responses that result in inflammation and cell proliferation and thereby initiate the metaplasia–dysplasia–adenocarcinoma sequence (Yoshida, 2007, Thrift, 2016).

In vitro studies have demonstrated that chronic exposure to 5 minute pulses of acid (at pH 4) and bile acid (deoxycholic acid; B4) for 60 weeks can progressively induce a columnar phenotype, genetic and epigenetic changes, chromosomal aberrations and malignant transformation in benign Barrett's oesophageal cell lines (Bajpai et al., 2012, Bajpai et al., 2013, Bajpai et al., 2008, Das et al., 2011). However, while gastro-oesophageal reflux disease, and other risk factors for Barrett's oesophagus such as abdominal obesity and tobacco use, are relatively common in the general population, the reported prevalence of Barrett's oesophagus is only 1-2% in the general population and is approximately 10-15% in people who are endoscopically examined for reflux symptoms (Pennathur et al., 2013, Rubenstein and Thrift, 2015, Thrift, 2016). Furthermore, the conversion rate of Barrett's oesophagus to oesophageal adenocarcinoma is less than 1% per year; the risk of adenocarcinoma in Barrett's patients has been estimated to be 0.5% per year by some while one study calculated it to be just 0.12% per year (Pennathur et al., 2013, Bajpai et al., 2014).

Therefore, improving our ability to predict which patients will or will not progress to oesophageal adenocarcinoma is an important challenge. At present, patients with a diagnosis of Barrett's oesophagus are subjected to repeated endoscopy surveillance, which for the majority of patients is unnecessary. Meanwhile, most oesophageal adenocarcinoma patients are initially asymptomatic and present to the clinic at a very advanced stage, with metastatic disease and a very poor prognosis (Thrift, 2016, Byrne et al., 2015). An improved understanding of the aetiology of oesophageal adenocarcinoma and of the molecular events which drive it should aid in the development of biomarkers to predict patients most at risk of the malignancy and in the development of therapeutics to treat it. Several genome-wide association studies and whole genome expression array profiling projects of Barrett's oesophagus and oesophageal adenocarcinoma have identified a range of risk loci for oesophageal

adenocarcinoma and have provided some insight into the potential biological processes which contribute to this malignancy (Nancarrow et al., 2011, Gharahkhani et al., 2016). Further studies and functional investigations are still required to elucidate the precise biological mechanisms underlying oesophageal adenocarcinoma but studies to date suggest considerable variation with regards to the expression of specific gene products and the disease stage at which they are important (Nancarrow et al., 2011). As is the case with other cancers, the biological processes which lead to the development of oesophageal adenocarcinoma are likely to be multi-factorial. Indeed in many cases, cancer, as well as many other diseases, may be viewed as network perturbations involving not one molecular malfunction but the malfunctioning of complex cellular networks (Del Sol et al., 2010).

If cancer can be viewed as a perturbation of cellular networks, the RACK1 scaffolding protein may be viewed as a convenor of cellular networks. RACK1 interacts with numerous proteins (Gandin et al., 2013b). As an adaptor or scaffolding protein it integrates inputs from distinct signalling pathways and plays key roles in cellular networks which coordinate important cell processes such as proliferation, migration, adhesion, cell survival, apoptosis, transcription and translation. The seven WD repeats of RACK1 provide multiple protein docking sites and facilitate interaction with numerous proteins, either directly or indirectly as part of multi-protein complexes (Adams et al., 2011). RACK1 can also form heterodimers with another WD-repeat protein, G β . The RACK1-G β heterodimer makes possible efficient cross-talk between signal transduction pathways mediated by G-protein-coupled receptors (GPCRs) and by ligand-gated ion channels, specifically between the cAMP/PKA pathway and the N-methyl D-aspartate receptor (NMDAR) (Chen et al., 2004, Chen et al., 2005, Thornton et al., 2004, Dell et al., 2002).

RACK1 can also create multi-protein signalling platforms by interacting with other scaffolding proteins. A study by Neasta and colleagues found that RACK1 can interact directly with the 14-3-3 ζ scaffolding protein. 14-3-3 ζ is required for RACK1 translocation to the nucleus and for the subsequent activation of BDNF transcription in response to activation of the cAMP/PKA pathway. As RACK1 and 14-3-3 ζ are two multifunctional scaffolding proteins that coordinate a wide range of signalling events, it is likely that their interaction also regulates other essential cellular functions (Neasta et al., 2012). RACK1 also functions as a homodimer which enables it to expand its binding partners and lets it simultaneously interact with proteins, such as Fyn and NR2B, which share the same binding site on RACK1 (Adams et al., 2011).

There are an estimated 100 million individual proteins involved in signal transduction pathways within a cellular milieu and scaffolding proteins like RACK1 play critical roles in organising such proteins into linear and branching signalling networks (Ron et al., 2013). By

assembling and compartmentalising numerous signalling and structural molecules, RACK1 provides a platform for their targeted positioning and appropriate, effective interactions. When a signalling protein is bound to RACK1, its local concentration is increased. In healthy cells, the expression level of RACK1 is normally tightly controlled as even subtle changes in its expression could have dramatic effects on important signalling pathways and on protein complex assembly(Adams et al., 2011, Ron et al., 2013).

In general, the influence of RACK1 on its interaction partners can be categorized into four types: modifying the activity of its interaction partners, modulating the stability of its partners, shuttling partners from one subcellular location to another and changing intermolecular interactions, i.e. enhancing association or dissociation. The list of biological events in which RACK1 has been found to participate, through interaction with its binding partners, continues to grow and is incredibly wide-ranging, including diverse processes such as cell migration, adhesion, proliferation, angiogenesis, viral infection, neural development, addiction and circadian rhythm(Adams et al., 2011, Li and Xie, 2014). RACK1 has also been associated with a range of diseases. Aberrations in RACK1 homeostasis have been associated with renal failure, brain development disorders, pulmonary arterial hypertension, muscle atrophy, dysfunctional sperm development, addiction, cardiac hypertrophy and heart failure(Adams et al., 2011, Ron et al., 2013).

As is discussed in detail in other chapters, (in particular in chapter 1, sections 1.3.2 and 1.3.3, and in chapter 5, section 5.1) altered RACK1 expression has been associated with a variety of cancers. As alluded to above, altered RACK1 expression has negative consequences for the properties and activity of RACK1 interaction partners and, depending on the interaction partners involved and the cell or tissue type, can have either pro-carcinogenic or tumour suppressive consequences(Adams et al., 2011, Li and Xie, 2014).

Therefore, the interactions of RACK1 with its binding partners represent a rich source of potential therapeutic targets for the treatment of cancer and other diseases. In the last twenty years there have been great improvements in the development of small molecule inhibitors which target specific protein-protein interactions as a therapeutic strategy (Arkin et al., 2014). Such molecules represent a particularly attractive means of targeting RACK1 therapeutically as they offer an alternative to targeting RACK1 non-specifically. (The ubiquitous expression of RACK1 and multi-functional nature is likely to mean that targeting it non-specifically would have far too many adverse effects).

Well-designed molecules which specifically target the interaction of RACK1 with a particular binding partner implicated in a certain pathology have the potential to act as very good therapeutics. Some progress has already been made in this regard. For example, cell

permeable peptides which function to hinder the interaction between a specific subset of RACK1 and its binding partner PDE4D5 have successfully inhibited metastasis and direction sensing of cancer cells without adversely affecting the many other critical cellular functions of RACK1 (Smith et al., 2007, Serrels et al., 2010, Serrels et al., 2011). Furthermore, a 23-amino acid peptide, named delcasertib, which selectively disrupts the binding of activated protein kinase C δ to RACK1 has been created. Delcasertib is highly specific and does not affect activation or localization of other protein kinase C isozymes (Lincoff et al., 2014). The activation of protein kinase C δ during reperfusion initiates the molecular events for cell death which ultimately result in inflammation and damage to the heart or brain during a stroke (Cunningham et al., 2017). Delcasertib successfully reduced infarct size during ischaemia/reperfusion in animal models and it also showed promise as a potential therapeutic to reduce myocardial necrosis and improve reperfusion in a pilot study during primary percutaneous coronary intervention (PCI) for ST elevation myocardial infarction (STEMI) (Bates et al., 2008, Inagaki et al., 2003, Ikeno et al., 2007, Lincoff et al., 2014). Unfortunately, however, results of a subsequent randomized control trial found that intravenous infusion of delcasertib during PCI for acute STEMI did not reduce biomarkers of myocardial injury (Lincoff et al., 2014).

Nevertheless, such studies illustrate the potential offered by inhibitors of specific RACK1 protein-protein interactions. An improved understanding of the significance of the interaction of RACK1 with particular binding partners in health and disease, combined with fine structural elucidation of the interaction interface between RACK1 and these partners should lead to the development of some highly specific molecules which target key interactions and offer exciting therapeutic potential. Some important first steps in preparing the groundwork for the development of such molecules would be to identify and characterise the binding partners with which RACK1 interacts in a disease of interest. In particular, those binding partners, whose interaction with RACK1 is of particular functional relevance to that disease would need to be identified and the precise role of their interaction with RACK1 elucidated. Bearing this in mind, research for this PhD did not examine RACK1 alone in oesophageal adenocarcinoma cells but attempted to identify and characterise proteins which interact with RACK1 in this cancer type.

The first potential RACK1 interaction partner which we examined in oesophageal adenocarcinoma was non muscle myosin heavy chain IIA (NMHCIIA). Upon conducting a mass spectrometry based proteomics screen of RACK1 co-immunoprecipitating proteins isolated from T-lymphocytes our group had previously discovered a novel interaction between NMHCIIA and RACK1. Peptide array experiments found that this interaction between the two

proteins was direct. Furthermore, it was found that, when T-lymphocyte migration was stimulated, RACK1 translocated to the lamellipodial leading edge in association with NMHCIIA, indicating that RACK1 may control the subcellular localisation of NMHCIIA during cell migration.

Cell migration is an important contributor to the metastatic process and metastasis is responsible for a huge number of oesophageal adenocarcinoma related deaths (Alizadeh et al., 2014, Pennathur et al., 2013). Therefore, we were particularly interested in investigating if RACK1 and NMHCIIA also interact in oesophageal adenocarcinoma cells and in investigating if their potential interaction was of functional significance to oesophageal adenocarcinoma cell migration. In chapter 3, we therefore examined the subcellular localisation and potential colocalisation of RACK1 and NMHCIIA in resting cell lines that represented each stage of the non-cancerous oesophagus to Barrett's metaplasia to Barrett's dysplasia-adenocarcinoma cancer progression sequence and in both resting and migrating SKGT-4 oesophageal adenocarcinoma cells. A small amount of perinuclear RACK1-NMHCIIA colocalisation was observed in resting HET-1A non-cancerous oesophageal cells, QhTERT Barrett's metaplastic cells and GohTERT Barrett's dysplastic cells, and in both resting and migrating SKGT-4 adenocarcinoma cells. Therefore, in these cells at least, this RACK1-NMHCIIA colocalisation was not specific to either cancer staging or to resting or migrating cell states and provided no indication that any potential association between the two proteins was of functional significance to either cancer progression or cell migration.

Upon examining migrating SKGT-4 cells, we observed that their mode of cell migration was very different to that employed by the migrating T-lymphocytes in which leading edge RACK1-NMHCIIA localization occurs. When lymphocytes are stimulated to migrate, they undergo a dramatic change in morphology and the cell polarizes with the formation of a tail (uropod) at the back of the cell and an obvious membrane protrusion, such as a lamellipodium or filopodium, at the cell front (Dupré et al., 2015). Lymphocytes migrate individually as single cells. In contrast, we observed that SKGT-4 cells migrate collectively, in cell sheets where contacts between adjacent cells are maintained. Migrating SKGT-4 cells do not display obvious, individual lamellipodia. In contrast to SKGT-4 cells however, some cancer cells, such as MDA-MB-231 breast adenocarcinoma cells, do migrate as single cells and display leading edge protrusions. NMHCIIA has been shown to localize to the leading edge of migrating MDA-MB-231 cells (Kim and Helfman, 2003). Interestingly, when we examined the localisation of RACK1 and NMHCIIA in migrating MDA-MB-231 cells we observed that the two proteins did display some colocalisation at the leading edge and also in the trailing cell tail, as had been also observed in T-lymphocytes. We therefore hypothesized that RACK1 and NMHCIIA may

colocalize (and potentially interact in a manner that is of functional relevance to cell migration) in oesophageal adenocarcinoma cells that, like MDA-MB-231 cells, utilise single cell migration. We therefore analysed RACK1 and NMHCIIA localisation in migrating FLO-1 oesophageal adenocarcinoma cells. FLO-1 cells, while they do migrate collectively in sheets and do not polarize as readily as MDA-MB-231 cells, also exhibit some single cell migration where cells display front-rear polarization and leading edge protrusions. Like in both T-lymphocytes and MDA-MB-231 cells, we observed NMHCIIA localization at the leading edge and the trailing tail of individually migrating FLO-1 cells. However, RACK1 did not colocalize with this leading edge NMHCIIA in migrating FLO-1 cells. We also expressed fluorescently tagged recombinant versions of RACK1 and NMHCIIA in FLO-1 cells and, using FRET assays, found no evidence of an interaction between the two recombinant proteins in either resting or migrating cells. These observations regarding the different localization/colocalization patterns of RACK and NMHCIIA in different cell types provide an interesting example how RACK1 subcellular localization and association with other proteins can vary depending on cell type; in this example this variation might be due, at least in part, to the different modes of migration employed by different cell types. These observations also highlight the importance of taking cellular context into account when making any assumptions about RACK1 function and underscore the need for an analysis of RACK1 in each individual cancer type and subtype. To our knowledge, RACK1 has not been previously examined in an oesophageal adenocarcinoma setting. Therefore, in this thesis we examine RACK1 in oesophageal adenocarcinoma cells for the first time and expand the range of cancers in which RACK1 has been studied.

In chapter 5 we report that RACK1 protein expression levels generally increase with increasing disease progression in a panel of cell lines representing the normal- to Barrett's metaplasia to Barrett's dysplasia to adenocarcinoma sequence. These cell lines were not isogenic so no definite conclusion can be drawn from this observation but it does indicate that RACK1 expression may increase in association with oesophageal adenocarcinoma disease progression and that RACK1 may play some role in driving this cancer progression sequence. As discussed above, gastro-oesophageal reflux disease is a key risk factor for oesophageal adenocarcinoma. Gastro-oesophageal reflux contains a mixture of acid, bile acids and other chemicals which damage the oesophagus and promote inflammation (Duggan et al., 2010, Jenkins et al., 2004, Jenkins et al., 2006). Included in this mixture is deoxycholic acid, an unconjugated bile acid which has been particularly implicated in carcinogenesis and which has been found to modulate the expression and function of a number of genes and proteins in oesophageal cells (Duggan et al., 2010, Byrne et al., 2012, Huo et al., 2011, Jenkins et al., 2004)

If DCA were to modulate the interactions of RACK1 with its binding partners, it would provide some insight into a potential mechanistic link between DCA, RACK1 and oesophageal adenocarcinoma progression. If DCA treatment were to bring about a change in RACK1 subcellular localisation it would hint at the possibility that DCA may alter the RACK1 associated proteome. However, upon treating both SKGT-4 and FLO-1 oesophageal adenocarcinoma cells with physiologically relevant concentrations of DCA we observed no change in RACK1 subcellular localisation. Nevertheless, RACK1 has a rather diffuse distribution throughout the cytoplasm in these cells and it is quite likely that changes in RACK1 binding partners could occur within the cytoplasm without an obvious visual change occurring to its overall staining pattern. Therefore, it is still quite possible DCA may modulate the RACK1 interactome and this would be an interesting further study to pursue. For example, one could conduct a mass-spectrometry based proteomics screen to compare and contrast the RACK1-associated proteome of DCA treated versus untreated control cells. While we observed no DCA-mediated change in RACK1 subcellular localisation, DCA did reduce overall RACK1 protein levels in both SKGT-4 and FLO-1 cells. This decrease in RACK1 protein levels following DCA treatment may be due to DCA induced oxidative stress which can have negative effects on protein translation and can also influence protein turnover (Duggan et al., 2010, Huo et al., 2011) (Vogel et al., 2011). As discussed above, RACK1 expression is normally tightly regulated in healthy cells. Therefore, any decrease in RACK1 protein levels which might occur in oesophageal cells *in vivo*, as a consequence of gastro-oesophageal reflux mediated exposure to DCA, could have serious implications for affected cells. A decrease in RACK1 expression is likely to result in increased competition for RACK1 among its interaction partners and could have a range of different consequences depending on the outcome of such competition and on the particular changes which occur to the RACK1 interactome.

When analysing RACK1 protein levels in oesophageal cells, we found that SKGT-4 cells had much lower RACK1 expression levels than that found in both FLO-1 and OE33 oesophageal adenocarcinoma cells. Using siRNA we depleted RACK1 from FLO-1 and SKGT-4 oesophageal cells. RACK1 depletion from SKGT-4 cells did not have a significant impact on cell proliferation. In contrast, RACK1 depletion from FLO-1 cells significantly decreased cell proliferation. It may be that SKGT-4 cells represent a form of oesophageal adenocarcinoma in which RACK1 does not play a particularly important role in cell proliferation (and, potentially in other cellular functions too) but that FLO-1 cells, in contrast, represent a subtype of oesophageal adenocarcinoma for which RACK1 is more functionally important. This difference between the two cell types is also interesting in light of our observation that DCA reduces RACK1 expression in both SKGT-4 and FLO-1 cells. One possible explanation for the differences we observed

between SKGT-4 and FLO-1 cells might be that SKGT-4 cells, prior to their isolation from a patient, and following repeated exposure to refluxate, adapted to a DCA mediated decrease in RACK1 levels – but that this did not occur in FLO-1 cells.

Interestingly however, we found that RACK1 depletion reduced two dimensional migration of both SKGT-4 and FLO-1 cells in scratch wound assays, indicating that SKGT-4 cells still require RACK1 for at least some of their cellular functions. However, given that RACK1 appears to play a greater role in FLO-1 cells, we chose this cell line as our model for a more detailed analysis of RACK1 function.

We also analysed the impact of RACK1 depletion on three dimensional cell migration; that is cell migration from a fibronectin-coated upper chamber, down through the small pores of a filter into a lower chamber into which a chemoattractant was placed. We found that RACK1 depletion also reduced this form of cell migration in FLO-1 cells.

Having found that RACK1 is of significant functional importance to a number of FLO-1 cellular processes, we sought to analyse the RACK1-associated proteome in FLO-1 cells. As discussed above, RACK1 functions not as an individual protein but as a convenor of protein-protein interactions and networks and thus an analysis of its associated proteins is crucial for a deep understanding of its function in any given cellular setting. Furthermore, as was also discussed above, RACK1 binding partners offer promise as therapeutic targets in cancer and other diseases. We therefore immunoprecipitated RACK1 from FLO-1 cell lysates and its co-immunoprecipitating proteins were analysed by mass spectrometry. We found that the RACK1 associated proteome in FLO-1 oesophageal adenocarcinoma cells is greatly enriched with nucleic acid binding proteins, and in particular, with ribosomal proteins. This finding adds to the credibility of our mass spectrometry results as RACK1 has been previously identified as a ribosomal associated protein(Adams et al., 2011). Indeed, as is discussed in more detail in chapter 6 (section 6.4), RACK1 plays important roles in translation and has been identified as an important constituent of the ribosomal machinery. X-ray crystallography studies have revealed RACK1 bound to the 40S eukaryotic ribosomal subunit (Adams et al., 2011). Acting as a 'ribosomal recruiter' RACK1 has been shown to recruit various factors to the ribosome and it may also recruit the ribosome itself to certain locations within the cell(Nielsen et al., 2017). The potential role of RACK1 in oesophageal adenocarcinoma ribosomal biology is of interest given the role which dysregulated translation plays in carcinogenesis.

Translation is usually tightly controlled in the healthy cell and involves the precisely choreographed interaction of ribosomes, auxiliary factors, tRNAs and mRNA. Dysregulation of the translation process has been considered as a hallmark of cancer and is associated with altered cancer energetics and with aberrant survival, angiogenesis and proliferation(Bhat et al.,

2015). Many of the most frequently observed cancer-associated mutations are found in pathways that regulate or feed into the translation machinery. Furthermore, a variety of translation initiation factors have been found to be amplified or dysregulated in a range of cancers. This is of particular interest in relation to RACK1 which has been shown to regulate the activity of a number of translation initiation factors. For example, RACK1, in association with PKC β II, regulates the phosphorylation of eukaryotic initiation factor 4E (eIF4E) which, in hepatocellular carcinoma, leads to the preferential translation of potent factors involved in growth and survival. In this setting of hepatocellular carcinoma, ribosomal RACK1 appears to promote both chemoresistance and growth (Ruan et al., 2012). RACK1 has also been shown to regulate PKC mediated phosphorylation of eukaryotic initiation factor 6 (eIF6) which regulates ribosomal subunit association (Ceci et al., 2003, Sengupta et al., 2004). Our mass spectrometry mediated proteomics screen of RACK1 co-immunoprecipitating proteins in FLO-1 cells identified a number of eukaryotic translation initiation factors (eIFs) as potential RACK1 associated proteins within the context of oesophageal adenocarcinoma. Our MaxQuant/Perseus generated list of 116 potential RACK1 co-immunoprecipitating proteins (generated using a stringency of $P=0.05$) included four eIFs, namely eIF3A, eIF3D, eIF4G1 and eIF2S3 while our list of 66 proteins deemed most probable to co-immunoprecipitate with RACK1 (generated using a higher stringency of $P=0.01$) included eIF3A (see figures 6.9 and 6.10). eIF3A overexpression has been observed in a number of cancers including oesophageal squamous cell carcinoma which makes its potential interaction with RACK1 even more interesting (Bhat et al., 2015, Chen and Burger, 1999).

Vigilin is another interesting RNA binding protein which our proteomic screen identified as a potential RACK1 interaction partner. The yeast homologue of vigilin, Scp160p, has been shown to interact with RACK1 in yeast but our finding that the two proteins may also interact in human cells is novel. Vigilin copurifies in a complex with elongation factor 1 A and transfer RNA and has been implicated in a range of processes including translational control and the transport and metabolism of RNA. Interestingly, it has also been implicated in carcinogenesis (Mobin et al., 2016). Following identification of vigilin in our proteomics screen of RACK interactions partners we also showed that subsets of RACK1 and vigilin colocalise in FLO-1 cells. However, as is described in chapter 6, section 6.3.5.1, results of our experiments to verify that RACK1 and vigilin co-immunoprecipitate in FLO-1 cells were inconclusive and thus further work will be required to establish if the two proteins interact and to explore the functional consequences of such an interaction, if it exists. Indeed further work to validate and functionally characterise the potential interactions between RACK1 and the various ribosomal proteins which we have identified in our screen could be fruitful in terms of identifying

potential therapeutic targets for oesophageal adenocarcinoma. Targeting translation in cancer is particularly attractive for a number of reasons. Cancer cells within any one tumour or metastasis can exhibit huge genetic variation (Gerlinger et al., 2012). Such intra-tumour heterogeneity poses a serious obstacle to the success of targeted therapies in treating cancer as although any particular drug may successfully obliterate tumour cells with a specific mutation, cells that are driven by different oncogenic pathways survive. However, the translation machinery integrates almost all oncogenic signals and therefore targeting this machinery (possibly by targeting crucial RACK1 interactions with key components of this machinery) offers some promise for solving the problems posed by intra-tumour heterogeneity (Bhat et al., 2015). Furthermore, the activity of most components of the translation machinery is increased in cancer cells. As a result, it is thought that cancer cells become addicted to elevated levels of protein synthesis (Silvera et al., 2010, Ruggero, 2013). This phenomenon potentially offers a therapeutic opportunity to selectively target cancer cells (Bhat et al., 2015).

One of the most exciting results of our proteomics screen of RACK1 co-immunoprecipitates was the identification of nicotinamide phosphoribosyltransferase (NAMPT) as a potential RACK1 interaction partner.

NAMPT is a multifaceted protein which is thought to function as both a nicotinamide adenine dinucleotide (NAD) biosynthetic enzyme and as a cytokine. NAMPT is involved in the modulation of important processes involved in the pathogenesis of obesity and related metabolic disorders through its influence on the oxidative stress response, inflammation, lipid and glucose metabolism, insulin resistance and cellular growth and apoptosis (Garten et al., 2015). NAMPT can play an important role in cancer cell metabolism and is overexpressed in a wide range of cancer tissues (Garten et al., 2015).

To our knowledge, an interaction between RACK1 and NAMPT has not been identified before. We successfully verified that NAMPT co-immunoprecipitates with RACK1 using co-immunoprecipitation experiments and Western Blotting. We then went on to show, using RACK1 peptide arrays, that RACK1 and NAMPT interact directly and that their interaction is likely to occur in the region spanning WD1 and WD2 of the RACK1 sequence. We also identified a number of amino acids on the RACK1 sequence which are likely to mediate the interaction between RACK1 and NAMPT, namely lysine 44 in WD1 and arginine 47 and arginine 60 in the linker region between WD1 and WD2 of RACK1. Our finding that a number of residues in the RACK1 sequence are involved in its interaction with NAMPT is not surprising as previous research has demonstrated that RACK1 interacts with its partners through a number of contact sites (Adams et al., 2011). The identification of such contact sites is crucial for the

development of a deeper understanding of the precise nature of the interaction of RACK1 with its partner proteins. The development of a detailed 'map' describing exactly how RACK1 and a partner protein such as NAMPT fit together establishes the ground work for the development of small molecule drugs which could specifically target the interaction between RACK1 and NAMPT. Such drugs could potentially offer therapeutic promise if the interaction between RACK1 and NAMPT was found to be of significant functional importance to oesophageal adenocarcinoma or to other malignancies.

We have found that NAMPT itself is of major functional importance to FLO-1 oesophageal adenocarcinoma cells. NAMPT depletion using siRNA significantly reduces FLO-1 cell proliferation, viability and migration and dramatically decreases their levels of NAD, an essential coenzyme. NAD is involved in cellular redox reactions and is a substrate for NAD-dependent enzymes (Garten et al., 2015).

The high energy demands of cancer cells necessitates that they turnover NAD rapidly. NAD-dependent enzymes, such as poly (ADP-ribose) polymerases (PARPs), mono-ADP ribosyltransferases (MARTs) and sirtuins, which are required for DNA repair, genome stability and proliferation, are also highly active in cancer cells (Schuster et al., 2015, Sampath et al., 2015). Our finding that FLO-1 cells depleted of NAMPT exhibit a dramatic reduction in NAD levels indicates that they are highly reliant on NAMPT's activity as an NAD biosynthetic enzyme. This is in accordance with previous research which has shown that many tumour cells are particularly reliant on NAMPT to meet their NAD requirements.

NAD can be synthesised de novo from tryptophan or recycled from nicotinamide through the NAMPT-dependent salvage pathway. Alternatively, nicotinic acid can be converted to NAD through a nicotinic acid phosphoribosyltransferase domain containing 1 (NAPRT1) dependent pathway (Sampath et al., 2015). However, cancer cells do not efficiently utilize the de novo synthesis pathway, frequently do not express NAPRT-1 (O'Brien et al., 2013) and have low nicotinic acid levels (Kirkland, 2009). These facts, in addition to our finding that NAMPT depletion dramatically reduces NAD levels in FLO-1 cells, indicates that they are highly reliant on NAMPT and make it an attractive potential therapeutic target for oesophageal adenocarcinoma.

We therefore sought to elucidate the functional consequences of the interaction between RACK1 and NAMPT in oesophageal adenocarcinoma and to determine if their interaction would be worth targeting for therapeutic benefit. As discussed earlier, one of the ways in which RACK1 influences its interaction partners is through the modulation of their activity. RACK1 can act as either a negative or positive regulator of its partners' activity. For example, RACK1 binding to Src has been reported to inhibit Src activity while the activity of MKK3/6 is

enhanced upon RACK1 binding (Chang et al., 1998, Chang et al., 2001, Chang et al., 2002, Wang et al., 2015b). Interestingly, in the case of the latter, RACK1 directly binds to and enhances the kinase activity of MKK3/6 without affecting MKK3/6 phosphorylation(Wang et al., 2015b).

We were interested in investigating if RACK1 influences the enzymatic, NAD biosynthetic activity of NAMPT and therefore we measured NAD levels in RACK1 depleted and control FLO-1 cells. However we found no significant difference in NAD levels between RACK1 depleted and control cells, which indicated that RACK1 may not control NAMPT's NAD biosynthetic activity. However we did find that RACK1 influences the protein levels of a higher molecular weight variant of NAMPT. We observed this high molecular weight form of NAMPT, which has a molecular weight of approximately 70kDa, when analysing FLO-1 lysates by SDS PAGE and Western blotting. This form of NAMPT was observed in addition to 55kDa NAMPT. We have found that the levels of this 70kDa form of NAMPT can be reduced using NAMPT targeting siRNA, indicating that it is a form of NAMPT.

A previous study by Wang and colleagues has also identified a 70kDa band corresponding to NAMPT on electrophoretic gels. In comparison to 55KDa NAMPT, they have found that this 70kDa form of NAMPT is preferentially bound by a chemical compound (P7C3) shown to be capable of enhancing the activity of the purified, recombinant NAMPT enzyme. This 70kDa band could be resolved to 55kDa by urea, suggesting the existence of a heterodimer(Wang et al., 2014). This study raises a number of questions on the nature of NAMPT activation. Where on the NAMPT structure does P7C3 bind? Does P7C3 displace the function of an inhibitory metabolite within the cell? Is the activity of the NAMPT enzyme regulated by post translational modification? Our discovery that RACK1 depletion reduces the levels of 70kDa NAMPT raises the possibility that RACK1 is in some way involved in the answers to such questions. It is intriguing to speculate that this 70kDa version of NAMPT might represent a RACK1-NAMPT heterodimer. On the other hand, this speculation that RACK1 may play a role in regulating NAMPT activity conflicts with our finding that RACK1 depletion, using siRNA, does not reduce cellular NAD levels. siRNA mediated protein depletion works by preventing the synthesis of new proteins. Could it be that the sub-pool of RACK1 which potentially controls NAMPT activity, and which potentially forms a heterodimer with NAMPT, has a longer half-life and is thus initially less affected by a reduction in the synthesis of new RACK1? Without doubt many questions remain regarding the functional consequences of the interaction between RACK1 and NAMPT and future work is required to answer same.

References

- ABDEL-LATIF, M. M. M., O'RIORDAN, J., WINDLE, H. J., CARTON, E., RAVI, N., KELLEHER, D. & REYNOLDS, J. V. 2004. NF- κ B activation in esophageal adenocarcinoma: relationship to Barrett's metaplasia, survival, and response to neoadjuvant chemoradiotherapy. *Annals of surgery*, 239, 491.
- ABERCROMBIE, M., JOAN, E., HEAYSMAN, M. & PEGRUM, S. M. 1970. The locomotion of fibroblasts in culture: II. "Ruffling". *Experimental cell research*, 60, 437-444.
- ADAMS, D. R., RON, D. & KIELY, P. A. 2011. RACK1, A multifaceted scaffolding protein: Structure and function. *Cell Commun Signal*, 9, 22.
- ADYA, R., TAN, B. K., CHEN, J. & RANDEVA, H. S. 2008a. Nuclear factor-kb induction by visfatin in human vascular endothelial cells. *Diabetes care*, 31, 758-760.
- ADYA, R., TAN, B. K., PUNN, A., CHEN, J. & RANDEVA, H. S. 2008b. Visfatin induces human endothelial VEGF and MMP-2/9 production via MAPK and PI3K/Akt signalling pathways: novel insights into visfatin-induced angiogenesis. *Cardiovascular research*, 78, 356-365.
- AGRAWAL, N., JIAO, Y., BETTEGOWDA, C., HUTFLESS, S. M., WANG, Y., DAVID, S., CHENG, Y., TWADDELL, W. S., LATT, N. L. & SHIN, E. J. 2012. Comparative genomic analysis of esophageal adenocarcinoma and squamous cell carcinoma. *Cancer discovery*, 2, 899-905.
- AITKEN, C. E., MARSHALL, R. A. & PUGLISI, J. D. 2008. An oxygen scavenging system for improvement of dye stability in single-molecule fluorescence experiments. *Biophysical journal*, 94, 1826-1835.
- ALIZADEH, A. M., SHIRI, S. & FARSINEJAD, S. 2014. Metastasis review: from bench to bedside. *Tumor Biology*, 35, 8483-8523.
- ALLOTT, E. H., LYSAGHT, J., CATHCART, M. C., DONOHOE, C. L., CUMMINS, R., MCGARRIGLE, S. A., KAY, E., REYNOLDS, J. V. & PIDGEON, G. P. 2013. MMP9 expression in oesophageal adenocarcinoma is upregulated with visceral obesity and is associated with poor tumour differentiation. *Molecular carcinogenesis*, 52, 144-154.
- ARKIN, M. R., TANG, Y. & WELLS, J. A. 2014. Small-Molecule Inhibitors of Protein-Protein Interactions: Progressing toward the Reality. *Chemistry & Biology*, 21.
- ASHBURNER, M., BALL, C. A., BLAKE, J. A., BOTSTEIN, D., BUTLER, H., CHERRY, J. M., DAVIS, A. P., DOLINSKI, K., DWIGHT, S. S. & EPPIG, J. T. 2000. Gene Ontology: tool for the unification of biology. *Nature genetics*, 25, 25-29.
- AUDRITO, V., SERRA, S., BRUSA, D., MAZZOLA, F., ARRUGA, F., VAISITTI, T., COSCIA, M., MAFFEI, R., ROSSI, D. & WANG, T. 2015. Extracellular nicotinamide phosphoribosyltransferase (NAMPT) promotes M2 macrophage polarization in chronic lymphocytic leukemia. *Blood*, 125, 111-123.

- BABBIN, B. A., KOCH, S., BACHAR, M., CONTI, M.-A., PARKOS, C. A., ADELSTEIN, R. S., NUSRAT, A. & IVANOV, A. I. 2009. Non-muscle myosin IIA differentially regulates intestinal epithelial cell restitution and matrix invasion. *The American journal of pathology*, 174, 436-448.
- BAJPAI, M., AVIV, H. & DAS, K. M. 2012. Prolonged exposure to acid and bile induces chromosome abnormalities that precede malignant transformation of benign Barrett's epithelium. *Molecular cytogenetics*, 5, 43.
- BAJPAI, M., DAS, K. M., LEFFERTS, J., LISOVSKY, M., MASHIMO, H., PHILLIPS, W. A., SRIVASTAVA, A. & TO, H. 2014. Molecular epidemiology of and genetic susceptibility to esophageal cancer. *Annals of the New York Academy of Sciences*, 1325, 40-48.
- BAJPAI, M., KESSEL, R., BHAGAT, T., NISCHAL, S., YU, Y., VERMA, A. & DAS, K. M. 2013. High resolution integrative analysis reveals widespread genetic and epigenetic changes after chronic in-vitro acid and bile exposure in barrett's epithelium cells. *Genes, Chromosomes and Cancer*, 52, 1123-1132.
- BAJPAI, M., LIU, J., GENG, X., SOUZA, R. F., AMENTA, P. S. & DAS, K. M. 2008. Repeated exposure to acid and bile selectively induces colonic phenotype expression in a heterogeneous Barrett's epithelial cell line. *Laboratory Investigation*, 88, 643-651.
- BALDEWIJNS, M. M., VAN VLODROP, I. J. H., VERMEULEN, P. B., SOETEKOUW, P. M. M. B., VAN ENGELAND, M. & DE BRUÏNE, A. P. 2010. VHL and HIF signalling in renal cell carcinogenesis. *The Journal of pathology*, 221, 125-138.
- BAO, J., JANA, S. S. & ADELSTEIN, R. S. 2005. Vertebrate nonmuscle myosin II isoforms rescue small interfering RNA-induced defects in COS-7 cell cytokinesis. *Journal of Biological Chemistry*, 280, 19594-19599.
- BAO, J., MA, X., LIU, C. & ADELSTEIN, R. S. 2007. Replacement of nonmuscle myosin II-B with II-A rescues brain but not cardiac defects in mice. *Journal of Biological Chemistry*, 282, 22102-22111.
- BATES, E., BODE, C., COSTA, M., GIBSON, C. M., GRANGER, C., GREEN, C., GRIMES, K., HARRINGTON, R., HUBER, K. & KLEIMAN, N. 2008. Intracoronary KAI-9803 as an adjunct to primary percutaneous coronary intervention for acute ST-segment elevation myocardial infarction. *Circulation*, 117, 886-896.
- BEDDY, P., HOWARD, J., MCMAHON, C., KNOX, M., DE BLACAM, C., RAVI, N., REYNOLDS, J. V. & KEOGAN, M. T. 2010. Association of visceral adiposity with oesophageal and junctional adenocarcinomas. *British Journal of Surgery*, 97, 1028-1034.
- BELO, V. A., LUIZON, M. R., LACCHINI, R., MIRANDA, J. A., LANNA, C. M. M., SOUZA-COSTA, D. C. & TANUS-SANTOS, J. E. 2015. The effects of NAMPT haplotypes and metabolic risk factors on circulating visfatin/NAMPT levels in childhood obesity. *International Journal of Obesity*, 39, 130-135.
- BERNS, H., HENGERER, B., KIEFER, F. N. & BATTEGAY, E. J. 2000. RACK1 is up-regulated in angiogenesis and human carcinomas. *The FASEB Journal*, 14, 2549-2558.
- BESSON, A., WILSON, T. L. & YONG, V. W. 2002. The Anchoring Protein RACK1 Links Protein Kinase C ϵ to Integrin β Chains REQUIREMENT

- FOR ADHESION AND MOTILITY. *Journal of Biological Chemistry*, 277, 22073-22084.
- BETAPUDI, V., LICATE, L. S. & EGELHOFF, T. T. 2006. Distinct roles of nonmuscle myosin II isoforms in the regulation of MDA-MB-231 breast cancer cell spreading and migration. *Cancer research*, 66, 4725-4733.
- BHAT, M., ROBICHAUD, N., HULEA, L., SONENBERG, N., PELLETIER, J. & TOPISIROVIC, I. 2015. Targeting the translation machinery in cancer. *Nature reviews Drug discovery*, 14, 261-278.
- BI, T.-Q. & CHE, X.-M. 2010. Nampt/PBEF/visfatin and cancer. *Cancer biology & therapy*, 10, 119-125.
- BIELENBERG, D. R. & ZETTER, B. R. 2015. The contribution of angiogenesis to the process of metastasis. *The Cancer Journal*, 21, 267-273.
- BLAKEMORE, A. I. F., MEYRE, D., DELPLANQUE, J., VATIN, V., LECOEUR, C., MARRE, M., TICHET, J., BALKAU, B., FROGUEL, P. & WALLEY, A. J. 2009. A rare variant in the visfatin gene (NAMPT/PBEF1) is associated with protection from obesity. *Obesity*, 17, 1549-1553.
- BUENSUCESO, C. S., WOODSIDE, D., HUFF, J. L., PLOPPER, G. E. & O'TOOLE, T. E. 2001. The WD protein Rack1 mediates protein kinase C and integrin-dependent cell migration. *Journal of Cell Science*, 114, 1691-1698.
- BYRNE, A.-M., BEKIARIS, S., DUGGAN, G., PRICHARD, D., KIRCA, M., FINN, S., REYNOLDS, J. V., KELLEHER, D. & LONG, A. 2015. Golgi phosphoprotein 2 (GOLPH2) is a novel bile acid-responsive modulator of oesophageal cell migration and invasion. *British journal of cancer*.
- BYRNE, A.-M., SHARMA, R., DUGGAN, G., KELLEHER, D. & LONG, A. 2012. Deoxycholic acid impairs glycosylation and fucosylation processes in esophageal epithelial cells. *Glycobiology*, 22, 638-648.
- CAO, X.-X., XU, J.-D., XU, J.-W., LIU, X.-L., CHENG, Y.-Y., WANG, W.-J., LI, Q.-Q., CHEN, Q., XU, Z.-D. & LIU, X.-P. 2010a. RACK1 promotes breast carcinoma proliferation and invasion/metastasis in vitro and in vivo. *Breast cancer research and treatment*, 123, 375-386.
- CAO, X. X., XU, J. D., LIU, X. L., XU, J. W., WANG, W. J., LI, Q. Q., CHEN, Q., XU, Z. D. & LIU, X. P. 2010b. RACK1: A superior independent predictor for poor clinical outcome in breast cancer. *International journal of cancer*, 127, 1172-1179.
- CAO, X. X., XU, J. D., XU, J. W., LIU, X. L., CHENG, Y. Y., LI, Q. Q., XU, Z. D. & LIU, X. P. 2011. RACK1 promotes breast carcinoma migration/metastasis via activation of the RhoA/Rho kinase pathway. *Breast cancer research and treatment*, 126, 555.
- CARDILLO, T. M., TRISAL, P., ARROJO, R., GOLDENBERG, D. M. & CHANG, C.-H. 2013. Targeting both IGF-1R and mTOR synergistically inhibits growth of renal cell carcinoma in vitro. *BMC cancer*, 13, 1.
- CECI, M., GAVIRAGHI, C., GORRINI, C., SALA, L. A., OFFENHÄUSER, N., MARCHISIO, P. C. & BIFFO, S. 2003. Release of eIF6 (p27BBP) from the 60S subunit allows 80S ribosome assembly. *Nature*, 426, 579-584.
- CHANG, B. Y., CHIANG, M. & CARTWRIGHT, C. A. 2001. The interaction of Src and RACK1 is enhanced by activation of protein kinase C and tyrosine phosphorylation of RACK1. *Journal of Biological Chemistry*, 276, 20346-20356.

- CHANG, B. Y., CONROY, K. B., MACHLEDER, E. M. & CARTWRIGHT, C. A. 1998. RACK1, a receptor for activated C kinase and a homolog of the β subunit of G proteins, inhibits activity of src tyrosine kinases and growth of NIH 3T3 cells. *Molecular and Cellular Biology*, 18, 3245-3256.
- CHANG, B. Y., HARTE, R. A. & CARTWRIGHT, C. A. 2002. RACK1: a novel substrate for the Src protein-tyrosine kinase. *Oncogene*, 21, 7619-7629.
- CHANG, Y. H., CHANG, D. M., LIN, K. C., SHIN, S. J. & LEE, Y. J. 2011. Visfatin in overweight/obesity, type 2 diabetes mellitus, insulin resistance, metabolic syndrome and cardiovascular diseases: a meta-analysis and systemic review. *Diabetes/metabolism research and reviews*, 27, 515-527.
- CHEN, G. & BURGER, M. M. 1999. p150 expression and its prognostic value in squamous-cell carcinoma of the esophagus. *International journal of cancer*, 84, 95-100.
- CHEN, J.-Y., CHEN, J.-C. & WU, J.-L. 2003. Molecular cloning and functional analysis of zebrafish high-density lipoprotein-binding protein. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 136, 117-130.
- CHEN, L., MIN, L., WANG, X., ZHAO, J., CHEN, H., QIN, J., CHEN, W., SHEN, Z., TANG, Z. & GAN, Q. 2015. Loss of RACK1 Promotes Metastasis of Gastric Cancer by Inducing a miR-302c/IL8 Signaling Loop. *Cancer research*, 75, 3832-3841.
- CHEN, S., DELL, E. J., LIN, F., SAI, J. & HAMM, H. E. 2004. RACK1 regulates specific functions of G $\beta\gamma$. *Journal of Biological Chemistry*, 279, 17861-17868.
- CHEN, S., LIN, F. & HAMM, H. E. 2005. RACK1 binds to a signal transfer region of G $\beta\gamma$ and inhibits phospholipase C β 2 activation. *Journal of Biological Chemistry*, 280, 33445-33452.
- CHEN, S., LIN, F., SHIN, M. E., WANG, F., SHEN, L. & HAMM, H. E. 2008. RACK1 Regulates Directional Cell Migration by Acting on G $\beta\gamma$ at the Interface with Its Effectors PLC β and PI3K γ . *Molecular Biology of the Cell*, 19, 3909-3922.
- CHEN, Y., CHEN, M., WU, Z. & ZHAO, S. 2013. Ox-LDL induces ER stress and promotes the adipokines secretion in 3T3-L1 adipocytes. *PloS one*, 8, e81379.
- CHICUREL, M. E., SINGER, R. H., MEYER, C. J. & INGBER, D. E. 1998. Integrin binding and mechanical tension induce movement of mRNA and ribosomes to focal adhesions. *Nature*, 392, 730-733.
- CHOI, D.-S., YOUNG, H., MCMAHON, T., WANG, D. & MESSING, R. O. 2003. The mouse RACK1 gene is regulated by nuclear factor- κ B and contributes to cell survival. *Molecular pharmacology*, 64, 1541-1548.
- CHOW, W.-H., BLOT, W. J., VAUGHAN, T. L., RISCH, H. A., GAMMON, M. D., STANFORD, J. L., DUBROW, R., SCHOENBERG, J. B., MAYNE, S. T. & FARROW, D. C. 1998. Body mass index and risk of adenocarcinomas of the esophagus and gastric cardia. *Journal of the National Cancer Institute*, 90, 150-155.
- COX, E. A., BENNIN, D., DOAN, A. T., O'TOOLE, T. & HUTTENLOCHER, A. 2003. RACK1 regulates integrin-mediated adhesion, protrusion, and chemotactic cell migration via its Src-binding site. *Molecular biology of the cell*, 14, 658-669.

- COX, J. & MANN, M. 2009. Computational Principles of Determining and Improving Mass Precision and Accuracy for Proteome Measurements in an Orbitrap. *Journal of the American Society for Mass Spectrometry*, 20, 1477-1485.
- COYLE, S. M., GILBERT, W. V. & DOUDNA, J. A. 2009. Direct link between RACK1 function and localization at the ribosome in vivo. *Molecular and cellular biology*, 29, 1626-1634.
- CRAMER, L. P. 2010. Forming the cell rear first: breaking cell symmetry to trigger directed cell migration. *Nature Cell Biology*, 12, 628-632.
- CREEDON, H. & BRUNTON, V. G. 2012. Src kinase inhibitors: promising cancer therapeutics? *Critical Reviews™ in Oncogenesis*, 17.
- CROFT, D., MUNDO, A. F., HAW, R., MILACIC, M., WEISER, J., WU, G., CAUDY, M., GARAPATI, P., GILLESPIE, M. & KAMDAR, M. R. 2014. The Reactome pathway knowledgebase. *Nucleic acids research*, 42, D472-D477.
- CUNNINGHAM, A. D., QVIT, N. & MOCHLY-ROSEN, D. 2017. Peptides and peptidomimetics as regulators of protein–protein interactions. *Current Opinion in Structural Biology*, 44, 59-66.
- DAHL, T. B., HOLM, S., AUKRUST, P. & HALVORSEN, B. 2012. Visfatin/NAMPT: a multifaceted molecule with diverse roles in physiology and pathophysiology. *Annual review of nutrition*, 32, 229-243.
- DALAMAGA, M. 2012. Nicotinamide phosphoribosyl-transferase/visfatin: a missing link between overweight/obesity and postmenopausal breast cancer? Potential preventive and therapeutic perspectives and challenges. *Medical hypotheses*, 79, 617-621.
- DALAMAGA, M. & CHRISTODOULATOS, G. S. 2017. Visfatin, Obesity, and Cancer. *Adipocytokines, Energy Balance, and Cancer*. Springer.
- DANIELS, C. C., ROVNAK, J. & QUACKENBUSH, S. L. 2008. Walleye dermal sarcoma virus Orf B functions through receptor for activated C kinase (RACK1) and protein kinase C. *Virology*, 375, 550-560.
- DAS, K. M., KONG, Y., BAJPAI, M., KULKARNI, D., GENG, X., MISHRA, P., BANERJEE, D. & HIRSHFIELD, K. 2011. Transformation of benign Barrett's epithelium by repeated acid and bile exposure over 65 weeks: a novel in vitro model. *International journal of cancer*, 128, 274-282.
- DAVE, J. M., KANG, H., ABBEY, C. A., MAXWELL, S. A. & BAYLESS, K. J. 2013. Proteomic profiling of endothelial invasion revealed receptor for activated C kinase 1 (RACK1) complexed with vimentin to regulate focal adhesion kinase (FAK). *Journal of Biological Chemistry*, 288, 30720-30733.
- DE ALMEIDA, J. C. M., CHAVES, P., PEREIRA, A. D. & ALTORKI, N. K. 1997. Is Barrett's esophagus the precursor of most adenocarcinomas of the esophagus and cardia? A biochemical study. *Annals of surgery*, 226, 725.
- DE CORTE, V., BRUYNEEL, E., BOUCHERIE, C., MAREEL, M., VANDEKERCKHOVE, J. & GETTEMANS, J. 2002. Gelsolin-induced epithelial cell invasion is dependent on Ras–Rac signaling. *The EMBO journal*, 21, 6781-6790.
- DE HOOG, C. L., FOSTER, L. J. & MANN, M. 2004. RNA and RNA binding proteins participate in early stages of cell spreading through spreading initiation centers. *Cell*, 117, 649-662.

- DEL SOL, A., BALLING, R., HOOD, L. & GALAS, D. 2010. Diseases as network perturbations. *Current opinion in biotechnology*, 21, 566-571.
- DEL VECCHIO, I., ZUCCOTTI, A., PISANO, F., CANNEVA, F., LENZKEN, S. C., ROUSSET, F., CORSINI, E., GOVONI, S. & RACCHI, M. 2009. Functional mapping of the promoter region of the GNB2L1 human gene coding for RACK1 scaffold protein. *Gene*, 430, 17-29.
- DELL, E. J., CONNOR, J., CHEN, S., STEBBINS, E. G., SKIBA, N. P., MOCHLY-ROSEN, D. & HAMM, H. E. 2002. The $\beta\gamma$ subunit of heterotrimeric G proteins interacts with RACK1 and two other WD repeat proteins. *Journal of Biological Chemistry*, 277, 49888-49895.
- DENG, B., FANG, J., ZHANG, X., QU, L., CAO, Z. & WANG, B. 2015. Role of gelsolin in cell proliferation and invasion of human hepatocellular carcinoma cells. *Gene*, 571, 292-297.
- DENG, Y. Z., YAO, F., LI, J. J., MAO, Z. F., HU, P. T., LONG, L. Y., LI, G., JI, X. D., SHI, S. & GUAN, D. X. 2012. RACK1 suppresses gastric tumorigenesis by stabilizing the β -catenin destruction complex. *Gastroenterology*, 142, 812-823.
- DERYCKE, L., STOVE, C., VERCOUTTER-EDOUART, A.-S., DE WEVER, O., DOLLÉ, L., COLPAERT, N., DEPYPERE, H., MICHALSKI, J.-C. & BRACKE, M. 2011. The role of non-muscle myosin IIA in aggregation and invasion of human MCF-7 breast cancer cells. *International Journal of Developmental Biology*, 55, 835.
- DEVREOTES, P. & HORWITZ, A. R. 2015. Signaling networks that regulate cell migration. *Cold Spring Harbor perspectives in biology*, 7, a005959.
- DOAN, A. T. & HUTTENLOCHER, A. 2007. RACK1 regulates Src activity and modulates paxillin dynamics during cell migration. *Experimental cell research*, 313, 2667-2679.
- DODSON, R. E. & SHAPIRO, D. J. 1997. Vigilin, a ubiquitous protein with 14 K homology domains, is the estrogen-inducible vitellogenin mRNA 3'-untranslated region-binding protein. *Journal of Biological Chemistry*, 272, 12249-12252.
- DUGGAN, S. P., BEHAN, F. M., KIRCA, M., SMITH, S., REYNOLDS, J. V., LONG, A. & KELLEHER, D. 2010. An integrative genomic approach in oesophageal cells identifies TRB3 as a bile acid responsive gene, downregulated in Barrett's oesophagus, which regulates NF- κ B activation and cytokine levels. *Carcinogenesis*, 31, 936-945.
- DUPRÉ, L., HOUMADI, R., TANG, C. & REY-BARROSO, J. 2015. T Lymphocyte migration: an action movie starring the actin and associated actors. *Frontiers in immunology*, 6.
- DURÁN, J. M., VALDERRAMA, F., CASTEL, S., MAGDALENA, J., TOMÁS, M., HOSOYA, H., RENAUI-PIQUERAS, J., MALHOTRA, V. & EGEA, G. 2003. Myosin motors and not actin comets are mediators of the actin-based Golgi-to-endoplasmic reticulum protein transport. *Molecular biology of the cell*, 14, 445-459.
- DUTKOWSKI, J., KRAMER, M., SURMA, M. A., BALAKRISHNAN, R., CHERRY, J. M., KROGAN, N. J. & IDEKER, T. 2013. A gene ontology inferred from molecular networks. *Nature biotechnology*, 31, 38-45.
- DVORAK, K., PAYNE, C. M., CHAVARRIA, M., RAMSEY, L., DVORAKOVA, B., BERNSTEIN, H., HOLUBEC, H., SAMPLINER, R. E., GUY, N. & CONDON, A. 2007. Bile acids in combination with low pH induce

- oxidative stress and oxidative DNA damage: relevance to the pathogenesis of Barrett's oesophagus. *Gut*, 56, 763-771.
- DWANE, S., DURACK, E., O'CONNOR, R. & KIELY, P. A. 2014. RACK1 promotes neurite outgrowth by scaffolding AGAP2 to FAK. *Cellular signalling*, 26, 9.
- ETIENNE-MANNEVILLE, S. 2008. Polarity proteins in migration and invasion. *Oncogene*, 27, 6970-6980.
- EVEN-RAM, S., DOYLE, A. D., CONTI, M. A., MATSUMOTO, K., ADELSTEIN, R. S. & YAMADA, K. M. 2007. Myosin IIA regulates cell motility and actomyosin-microtubule crosstalk. *Nature cell biology*, 9, 299-309.
- FRAME, M. C., FINCHAM, V. J., CARRAGHER, N. O. & WYKE, J. A. 2002. v-Src's hold over actin and cell adhesions. *Nature Reviews Molecular Cell Biology*, 3, 233-245.
- FREY, S., POOL, M. & SEEDORF, M. 2001. Scp160p, an RNA-binding, polysome-associated protein, localizes to the endoplasmic reticulum of *Saccharomyces cerevisiae* in a microtubule-dependent manner. *Journal of Biological Chemistry*, 276, 15905-15912.
- FRIEBE, D., NEEF, M., KRATZSCH, J., ERBS, S., DITTRICH, K., GARTEN, A., PETZOLD-QUINQUE, S., BLÜHER, S., REINEHR, T. & STUMVOLL, M. 2011. Leucocytes are a major source of circulating nicotinamide phosphoribosyltransferase (NAMPT)/pre-B cell colony (PBEF)/visfatin linking obesity and inflammation in humans. *Diabetologia*, 54, 1200-1211.
- FRIEDL, P. & GILMOUR, D. 2009. Collective cell migration in morphogenesis, regeneration and cancer. *Nature reviews Molecular cell biology*, 10, 445-457.
- FRIEDL, P. & WOLF, K. 2003. Tumour-cell invasion and migration: diversity and escape mechanisms. *Nature Reviews Cancer*, 3, 362-374.
- GALLÍ, M., VAN GOOL, F., RONGVAUX, A., ANDRIS, F. & LEO, O. 2010. The nicotinamide phosphoribosyltransferase: a molecular link between metabolism, inflammation, and cancer. *Cancer research*, 70, 8-11.
- GANDHI, T. K. B., ZHONG, J., MATHIVANAN, S., KARTHICK, L., CHANDRIKA, K. N., MOHAN, S. S., SHARMA, S., PINKERT, S., NAGARAJU, S. & PERIASWAMY, B. 2006. Analysis of the human protein interactome and comparison with yeast, worm and fly interaction datasets. *Nature genetics*, 38, 285-293.
- GANDIN, V., GUTIERREZ, G. J., BRILL, L. M., VARSANO, T., FENG, Y., AZA-BLANC, P., AU, Q., MCLAUGHLAN, S., FERREIRA, T. A. & ALAIN, T. 2013a. Degradation of newly synthesized polypeptides by ribosome-associated RACK1/c-Jun N-terminal kinase/eukaryotic elongation factor 1A2 complex. *Molecular and cellular biology*, 33, 2510-2526.
- GANDIN, V., SENFT, D., TOPISIROVIC, I. & ZE'EV, A. R. 2013b. RACK1 function in cell motility and protein synthesis. *Genes & cancer*, 4, 369-377.
- GARTEN, A., PETZOLD, S., KÖRNER, A., IMAI, S.-I. & KIESS, W. 2009. Nampt: linking NAD biology, metabolism and cancer. *Trends in Endocrinology & Metabolism*, 20, 130-138.
- GARTEN, A., SCHUSTER, S., PENKE, M., GORSKI, T., DE GIORGIS, T. & KIESS, W. 2015. Physiological and pathophysiological roles of NAMPT and NAD metabolism. *Nature Reviews Endocrinology*, 11, 535-546.

- GARUD, S. S., KEILIN, S., CAI, Q. & WILLINGHAM, F. F. 2010. Review: Diagnosis and management of Barrett's esophagus for the endoscopist. *Therapeutic advances in gastroenterology*, 3, 227-238.
- GELIN-LICHT, R., PALIWAL, S., CONLON, P., LEVCHENKO, A. & GERST, J. E. 2012. Scp160-dependent mRNA trafficking mediates pheromone gradient sensing and chemotropism in yeast. *Cell reports*, 1, 483-494.
- GERBASI, V. R., WEAVER, C. M., HILL, S., FRIEDMAN, D. B. & LINK, A. J. 2004. Yeast Asc1p and mammalian RACK1 are functionally orthologous core 40S ribosomal proteins that repress gene expression. *Molecular and Cellular Biology*, 24, 8276-8287.
- GERLINGER, M., ROWAN, A. J., HORSWELL, S., LARKIN, J., ENDESFELDER, D., GRONROOS, E., MARTINEZ, P., MATTHEWS, N., STEWART, A. & TARPEY, P. 2012. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med*, 2012, 883-892.
- GHARAHKHANI, P., FITZGERALD, R. C., VAUGHAN, T. L., PALLES, C., GOCKEL, I., TOMLINSON, I., BUAS, M. F., MAY, A., GERGES, C. & ANDERS, M. 2016. Genome-wide association studies in oesophageal adenocarcinoma and Barrett's oesophagus: a large-scale meta-analysis. *The Lancet Oncology*, 17, 1363-1373.
- GOHLA, A. & BOKOCH, G. M. 2002. 14-3-3 regulates actin dynamics by stabilizing phosphorylated cofilin. *Current biology: CB*, 12, 1704-1710.
- GROLLA, A. A., TORRETTA, S., GNEMMI, I., AMORUSO, A., ORSOMANDO, G., GATTI, M., CALDARELLI, A., LIM, D., PENENGO, L. & BRUNELLESCHI, S. 2015. Nicotinamide phosphoribosyltransferase (NAMPT/PBEF/visfatin) is a tumoural cytokine released from melanoma. *Pigment Cell & Melanoma Research*, 28, 718-729.
- GUO, Y., WANG, W., WANG, J., FENG, J., WANG, Q., JIN, J., LV, M., LI, X., LI, Y. & MA, Y. 2013. Receptor for activated C kinase 1 promotes hepatocellular carcinoma growth by enhancing mitogen-activated protein kinase kinase 7 activity. *Hepatology*, 57, 140-151.
- GUPTON, S. L., ANDERSON, K. L., KOLE, T. P., FISCHER, R. S., PONTI, A., HITCHCOCK-DEGREGORI, S. E., DANUSER, G., FOWLER, V. M., WIRTZ, D. & HANEIN, D. 2005. Cell migration without a lamellipodium: translation of actin dynamics into cell movement mediated by tropomyosin. *J. Cell Biol*, 168, 619-631.
- HAIDER, D. G., SCHALLER, G., KAPIOTIS, S., MAIER, C., LUGER, A. & WOLZT, M. 2006a. The release of the adipocytokine visfatin is regulated by glucose and insulin. *Diabetologia*, 49, 1909-1914.
- HAIDER, D. G., SCHINDLER, K., SCHALLER, G., PRAGER, G., WOLZT, M. & LUDVIK, B. 2006b. Increased plasma visfatin concentrations in morbidly obese subjects are reduced after gastric banding. *The Journal of Clinical Endocrinology & Metabolism*, 91, 1578-1581.
- HARA, N., YAMADA, K., SHIBATA, T., OSAGO, H. & TSUCHIYA, M. 2011. Nicotinamide phosphoribosyltransferase/visfatin does not catalyze nicotinamide mononucleotide formation in blood plasma. *PLoS One*, 6, e22781.
- HE, X., WANG, J., MESSING, E. M. & WU, G. 2011. Regulation of receptor for activated C kinase 1 protein by the von Hippel-Lindau tumor suppressor

- in IGF-I-induced renal carcinoma cell invasiveness. *Oncogene*, 30, 535-547.
- HEISSLER, S. M. & MANSTEIN, D. J. 2013. Nonmuscle myosin-2: mix and match. *Cellular and Molecular Life Sciences*, 70, 1-21.
- HERMANTO, U., ZONG, C. S., LI, W. & WANG, L.-H. 2002. RACK1, an insulin-like growth factor I (IGF-I) receptor-interacting protein, modulates IGF-I-dependent integrin signaling and promotes cell spreading and contact with extracellular matrix. *Molecular and cellular biology*, 22, 2345-2365.
- HIGUERUELO, A. P., JUBB, H. & BLUNDELL, T. L. 2013. Protein-protein interactions as druggable targets: recent technological advances. *Current opinion in pharmacology*, 13, 791-796.
- HINDMAN, B., GOECKELER, Z., SIERROS, K. & WYSOLMERSKI, R. 2015. Non-Muscle Myosin II Isoforms Have Different Functions in Matrix Rearrangement by MDA-MB-231 Cells. *PloS one*, 10, e0131920.
- HIRSCHMANN, W. D., WESTENDORF, H., MAYER, A., CANNAROZZI, G., CRAMER, P. & JANSEN, R.-P. 2014. Scp160p is required for translational efficiency of codon-optimized mRNAs in yeast. *Nucleic acids research*, 42, 4043-4055.
- HU, F., TAO, Z., WANG, M., LI, G., ZHANG, Y., ZHONG, H., XIAO, H., XIE, X. & JU, M. 2013. RACK1 promoted the growth and migration of the cancer cells in the progression of esophageal squamous cell carcinoma. *Tumor Biology*, 34, 3893-3899.
- HUANG, B. X. & KIM, H.-Y. 2013. Effective identification of Akt interacting proteins by two-step chemical crosslinking, co-immunoprecipitation and mass spectrometry. *PloS one*, 8, e61430.
- HUANG, Y., PETERS, C. J., FITZGERALD, R. C. & GJERSET, R. A. 2009. Progressive silencing of p14ARF in oesophageal adenocarcinoma. *Journal of cellular and molecular medicine*, 13, 398-409.
- HUERTAS, D., CORTÉS, A., CASANOVA, J. & AZORÍN, F. 2004. Drosophila DDP1, a multi-KH-domain protein, contributes to centromeric silencing and chromosome segregation. *Current biology*, 14, 1611-1620.
- HUO, X., JUERGENS, S., ZHANG, X., REZAEI, D., YU, C., STRAUCH, E. D., WANG, J.-Y., CHENG, E., MEYER, F. & WANG, D. H. 2011. Deoxycholic acid causes DNA damage while inducing apoptotic resistance through NF- κ B activation in benign Barrett's epithelial cells. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 301, G278-G286.
- IKENO, F., INAGAKI, K., REZAEI, M. & MOCHLY-ROSEN, D. 2007. Impaired perfusion after myocardial infarction is due to reperfusion-induced δ PKC-mediated myocardial damage. *Cardiovascular research*, 73, 699-709.
- INAGAKI, K., CHEN, L., IKENO, F., LEE, F. H., IMAHASHI, K.-I., BOULEY, D. M., REZAEI, M., YOCK, P. G., MURPHY, E. & MOCHLY-ROSEN, D. 2003. Inhibition of δ -protein kinase C protects against reperfusion injury of the ischemic heart in vivo. *Circulation*, 108, 2304-2307.
- JANNOT, G., BAJAN, S., GIGUÈRE, N. J., BOUASKER, S., BANVILLE, I. H., PIQUET, S., HUTVAGNER, G. & SIMARD, M. J. 2011. The ribosomal protein RACK1 is required for microRNA function in both *C. elegans* and humans. *EMBO reports*, 12, 581-586.
- JENKINS, G. J. S., D'SOUZA, F. R., SUZEN, S. H., ELTAHIR, Z. S., JAMES, S. A., PARRY, J. M., GRIFFITHS, P. A. & BAXTER, J. N. 2006.

- Deoxycholic acid at neutral and acid pH, is genotoxic to oesophageal cells through the induction of ROS: the potential role of anti-oxidants in Barrett's oesophagus. *Carcinogenesis*, 28, 136-142.
- JENKINS, G. J. S., HARRIES, K., DOAK, S. H., WILMES, A., GRIFFITHS, A. P., BAXTER, J. N. & PARRY, J. M. 2004. The bile acid deoxycholic acid (DCA) at neutral pH activates NF- κ B and induces IL-8 expression in oesophageal cells in vitro. *Carcinogenesis*, 25, 317-323.
- KALLURI, R. & WEINBERG, R. A. 2009. The basics of epithelial-mesenchymal transition. *The Journal of clinical investigation*, 119, 1420-1428.
- KARATAS, H., TOWNSEND, E. C., CAO, F., CHEN, Y., BERNARD, D., LIU, L., LEI, M., DOU, Y. & WANG, S. 2012. High-affinity, small-molecule peptidomimetic inhibitors of MLL1/WDR5 protein-protein interaction. *Journal of the American Chemical Society*, 135, 669-682.
- KATOH, Y. & KATOH, M. 2008. Hedgehog signaling, epithelial-to-mesenchymal transition and miRNA (review). *International journal of molecular medicine*, 22, 271.
- KIELY, M., ADAMS, D. R., HAYES, S. L., O'CONNOR, R., BAILLIE, G. S. & KIELY, P. A. 2016. RACK1 stabilises the activity of PP2A to regulate the transformed phenotype in mammary epithelial cells. *Cellular Signalling*.
- KIELY, P. A., BAILLIE, G. S., BARRETT, R., BUCKLEY, D. A., ADAMS, D. R., HOUSLAY, M. D. & O'CONNOR, R. 2009. Phosphorylation of RACK1 on tyrosine 52 by c-Abl is required for insulin-like growth factor I-mediated regulation of focal adhesion kinase. *Journal of Biological Chemistry*, 284, 20263-20274.
- KIELY, P. A., BAILLIE, G. S., LYNCH, M. J., HOUSLAY, M. D. & O'CONNOR, R. 2008. Tyrosine 302 in RACK1 is essential for insulin-like growth factor-I-mediated competitive binding of PP2A and β 1 integrin and for tumor cell proliferation and migration. *Journal of Biological Chemistry*, 283, 22952-22961.
- KIELY, P. A., LEAHY, M., O'GORMAN, D. & O'CONNOR, R. 2005. RACK1-mediated integration of adhesion and insulin-like growth factor I (IGF-I) signaling and cell migration are defective in cells expressing an IGF-I receptor mutated at tyrosines 1250 and 1251. *Journal of Biological Chemistry*, 280, 7624-7633.
- KIELY, P. A., O'GORMAN, D., LUONG, K., RON, D. & O'CONNOR, R. 2006. Insulin-like growth factor I controls a mutually exclusive association of RACK1 with protein phosphatase 2A and β 1 integrin to promote cell migration. *Molecular and cellular biology*, 26, 4041-4051.
- KIELY, P. A., SANT, A. & O'CONNOR, R. 2002. RACK1 is an insulin-like growth factor 1 (IGF-1) receptor-interacting protein that can regulate IGF-1-mediated Akt activation and protection from cell death. *Journal of Biological Chemistry*, 277, 22581-22589.
- KIESWICH, J., SAYERS, S. R., SILVESTRE, M. F., HARWOOD, S. M., YAQOOB, M. M. & CATON, P. W. 2016. Monomeric eNAMPT in the development of experimental diabetes in mice: a potential target for type 2 diabetes treatment. *Diabetologia*, 59, 2477-2486.
- KIM, E. J. & HELFMAN, D. M. 2003. Characterization of the metastasis-associated protein, S100A4 Roles of calcium binding and dimerization in cellular localization and interaction with myosin. *Journal of Biological Chemistry*, 278, 30063-30073.

- KIRKLAND, J. B. 2009. Niacin status, NAD distribution and ADP-ribose metabolism. *Current pharmaceutical design*, 15, 3-11.
- KRALISCH, S., KLEIN, J., LOSSNER, U., BLUHER, M., PASCHKE, R., STUMVOLL, M. & FASSHAUER, M. 2005. Hormonal regulation of the novel adipocytokine visfatin in 3T3-L1 adipocytes. *Journal of Endocrinology*, 185, R1-R8.
- KRUSE, C., GRÜNWELLER, A., WILLKOMM, K. D., PFEIFFER, T., HARTMANN, K. R. & MÜLLER, K. P. 1998. tRNA is entrapped in similar, but distinct, nuclear and cytoplasmic ribonucleoprotein complexes, both of which contain vigilin and elongation factor 1 α . *Biochemical Journal*, 329, 615-621.
- KRUSE, C., WILLKOMM, D. K., GRÜNWELLER, A., VOLLBRANDT, T., SOMMER, S., BUSCH, S., PFEIFFER, T., BRINKMANN, J., HARTMANN, R. K. & MÜLLER, P. K. 2000. Export and transport of tRNA are coupled to a multi-protein complex. *Biochemical Journal*, 346, 107-115.
- KUROHA, K., AKAMATSU, M., DIMITROVA, L., ITO, T., KATO, Y., SHIRAHIGE, K. & INADA, T. 2010. Receptor for activated C kinase 1 stimulates nascent polypeptide-dependent translation arrest. *EMBO reports*, 11, 956-961.
- LAGERGREN, J., BERGSTROM, R., LINDGREN, A. & NYREN, O. 1999. Symptomatic gastroesophageal reflux as a risk factor for esophageal adenocarcinoma. *New England journal of medicine*, 340, 825-831.
- LARSSON, C. 2006. Protein kinase C and the regulation of the actin cytoskeleton. *Cellular signalling*, 18, 276-284.
- LEBER, M. F. & EFFERTH, T. 2009. Molecular principles of cancer invasion and metastasis (review). *International journal of oncology*, 34, 881.
- LEE, B. Y., TIMPSON, P., HORVATH, L. G. & DALY, R. J. 2015. FAK signaling in human cancer as a target for therapeutics. *Pharmacology & therapeutics*, 146, 132-149.
- LEPAGE, C., DROUILLARD, A., JOUVE, J.-L. & FAIVRE, J. 2013. Epidemiology and risk factors for oesophageal adenocarcinoma. *Digestive and Liver Disease*, 45, 625-629.
- LEWIS-SARAVALLI, S., CAMPBELL, S. & CLAING, A. 2013. ARF1 controls Rac1 signaling to regulate migration of MDA-MB-231 invasive breast cancer cells. *Cellular signalling*, 25, 1813-1819.
- LI, G., JI, X. D., GAO, H., ZHAO, J. S., XU, J. F., SUN, Z. J., DENG, Y. Z., SHI, S., FENG, Y. X. & ZHU, Y. Q. 2012a. EphB3 suppresses non-small-cell lung cancer metastasis via a PP2A/RACK1/Akt signalling complex. *Nature communications*, 3, 667.
- LI, H., BAI, E., ZHANG, Y., JIA, Z., HE, S. & FU, J. 2017. Role of Nampt and Visceral Adiposity in Esophagogastric Junction Adenocarcinoma. *Journal of Immunology Research*, 2017.
- LI, J., GUO, Y., FENG, X., WANG, Z., WANG, Y., DENG, P., ZHANG, D., WANG, R., XIE, L. & XU, X. 2012b. Receptor for activated C kinase 1 (RACK1): a regulator for migration and invasion in oral squamous cell carcinoma cells. *Journal of cancer research and clinical oncology*, 138, 563-571.
- LI, J. J. & XIE, D. 2014. RACK1, a versatile hub in cancer. *Oncogene*.

- LI, X., XIAO, Y., FAN, S., XIAO, M., WANG, X., CHEN, X., LI, C., ZONG, G., ZHOU, G. & WAN, C. 2016. RACK1 overexpression associates with pancreatic ductal adenocarcinoma growth and poor prognosis. *Experimental and Molecular Pathology*, 101, 176-186.
- LI, Z.-H. & BRESNICK, A. R. 2006. The S100A4 metastasis factor regulates cellular motility via a direct interaction with myosin-IIA. *Cancer research*, 66, 5173-5180.
- LID, R. 2001. WD-repeat proteins: Structure characteristics, biological function, and their involvement in human diseases. *Cell Mol Life Sci*, 58, 2085-2097.
- LINCOFF, A. M., ROE, M., AYLWARD, P., GALLA, J., RYNKIEWICZ, A., GUETTA, V., ZELIZKO, M., KLEIMAN, N., WHITE, H. & MCERLEAN, E. 2014. Inhibition of delta-protein kinase C by delcasertib as an adjunct to primary percutaneous coronary intervention for acute anterior ST-segment elevation myocardial infarction: results of the PROTECTION AMI Randomized Controlled Trial. *European heart journal*, ehu177.
- LINK, A. J., ENG, J., SCHIELTZ, D. M., CARMACK, E., MIZE, G. J., MORRIS, D. R., GARVIK, B. M. & YATES, J. R. 1999. Direct analysis of protein complexes using mass spectrometry. *Nature biotechnology*, 17, 676-682.
- LIU, T., YE, Y., ZHANG, X., ZHU, A., YANG, Z., FU, Y., WEI, C., LIU, Q., ZHAO, C. & WANG, G. 2016. Downregulation of non-muscle myosin IIA expression inhibits migration and invasion of gastric cancer cells via the c-Jun N-terminal kinase signaling pathway. *Molecular medicine reports*, 13, 1639-1644.
- LOOBY, E., ABDEL-LATIF, M. M. M., ATHIÉ-MORALES, V., DUGGAN, S., LONG, A. & KELLEHER, D. 2009. Deoxycholate induces COX-2 expression via Erk1/2-, p38-MAPK and AP-1-dependent mechanisms in esophageal cancer cells. *BMC cancer*, 9, 190.
- LOOBY, E., LONG, A., KELLEHER, D. & VOLKOV, Y. 2005. Bile acid deoxycholate induces differential subcellular localisation of the PKC isoenzymes $\beta 1$, ϵ and δ in colonic epithelial cells in a sodium butyrate insensitive manner. *International journal of cancer*, 114, 887-895.
- LU, F., ZHANG, C., WU, W.-J. & WU, Y.-M. 2012. RACK1 downregulation suppresses migration and proliferation of neuroblastoma cell lines. *Oncology reports*, 27, 1646-1652.
- LV, Q.-L., HUANG, Y.-T., WANG, G.-H., LIU, Y.-L., HUANG, J., QU, Q., SUN, B., HU, L., CHENG, L. & CHEN, S.-H. 2016. Overexpression of RACK1 Promotes Metastasis by Enhancing Epithelial-Mesenchymal Transition and Predicts Poor Prognosis in Human Glioma. *International Journal of Environmental Research and Public Health*, 13, 1021.
- LYNAM-LENNON, N., CONNAUGHTON, R., CARR, E., MONGAN, A.-M., NAOIMH, J. O., PORTER, R. K., BRENNAN, L., PIDGEON, G. P., LYSAGHT, J. & REYNOLDS, J. V. 2014. Excess visceral adiposity induces alterations in mitochondrial function and energy metabolism in esophageal adenocarcinoma. *BMC Cancer*, 14, 907-907.
- LYSAGHT, J., VAN DER STOK, E. P., ALLOTT, E. H., CASEY, R., DONOHOE, C. L., HOWARD, J. M., MCGARRIGLE, S. A., RAVI, N., REYNOLDS, J. V. & PIDGEON, G. P. 2011. Pro-inflammatory and tumour proliferative properties of excess visceral adipose tissue. *Cancer letters*, 312, 62-72.
- MA, X., JANA, S. S., CONTI, M. A., KAWAMOTO, S., CLAYCOMB, W. C. & ADELSTEIN, R. S. 2010. Ablation of nonmuscle myosin II-B and II-C

- reveals a role for nonmuscle myosin II in cardiac myocyte karyokinesis. *Molecular biology of the cell*, 21, 3952-3962.
- MALLIRI, A. & COLLARD, J. G. 2003. Role of Rho-family proteins in cell adhesion and cancer. *Current opinion in cell biology*, 15, 583-589.
- MAMIDIPUDI, V. & CARTWRIGHT, C. A. 2009. A novel pro-apoptotic function of RACK1: suppression of Src activity in the intrinsic and Akt pathways. *Oncogene*, 28, 4421-4433.
- MAMIDIPUDI, V., DHILLON, N. K., PARMAN, T., MILLER, L. D., LEE, K. C. & CARTWRIGHT, C. A. 2007. RACK1 inhibits colonic cell growth by regulating Src activity at cell cycle checkpoints. *Oncogene*, 26, 2914-2924.
- MAMIDIPUDI, V., ZHANG, J., LEE, K. C. & CARTWRIGHT, C. A. 2004. RACK1 regulates G1/S progression by suppressing Src kinase activity. *Molecular and cellular biology*, 24, 6788-6798.
- MAYOR, R. & ETIENNE-MANNEVILLE, S. 2016. The front and rear of collective cell migration. *Nature Reviews Molecular Cell Biology*.
- MCQUAID, K. R., LAINE, L., FENNERTY, M. B., SOUZA, R. & SPECHLER, S. J. 2011. Systematic review: the role of bile acids in the pathogenesis of gastro-oesophageal reflux disease and related neoplasia. *Alimentary pharmacology & therapeutics*, 34, 146-165.
- MENDELSON, B. A., LI, A. M., VARGAS, C. A., RIEHMAN, K., WATSON, A. & FRIDOVICH-KEIL, J. L. 2003. Genetic and biochemical interactions between SCP160 and EAP1 in yeast. *Nucleic acids research*, 31, 5838-5847.
- MIERKE, C. T. 2013. Physical break-down of the classical view on cancer cell invasion and metastasis. *European journal of cell biology*, 92, 89-104.
- MILLER, C. T., LIN, L., CASPER, A. M., LIM, J., THOMAS, D. G., ORRINGER, M. B., CHANG, A. C., CHAMBERS, A. F., GIORDANO, T. J. & GLOVER, T. W. 2006. Genomic amplification of MET with boundaries within fragile site FRA7G and upregulation of MET pathways in esophageal adenocarcinoma. *Oncogene*, 25, 409-418.
- MILLER, L. D., LEE, K. C., MOCHLY-ROSEN, D. & CARTWRIGHT, C. A. 2004. RACK1 regulates Src-mediated Sam68 and p190RhoGAP signaling. *Oncogene*, 23, 5682-5686.
- MITRA, S. K., HANSON, D. A. & SCHLAEPFER, D. D. 2005. Focal adhesion kinase: in command and control of cell motility. *Nature reviews Molecular cell biology*, 6, 56-68.
- MOBIN, M. B., GERSTBERGER, S., TEUPSER, D., CAMPANA, B., CHARISSE, K., HEIM, M. H., MANOHARAN, M., TUSCHL, T. & STOFFEL, M. 2016. The RNA-binding protein vigilin regulates VLDL secretion through modulation of Apob mRNA translation. *Nature communications*, 7, 12848.
- MORGAN, S. & MEUILLET, E. 2009. Abstract# 5048: Deoxycholic acid induces endocytosis of the insulin-like growth factor-I receptor in colorectal cancer cells. *Cancer Research*, 69, 5048-5048.
- MOSCHEN, A. R., KASER, A., ENRICH, B., MOSHEIMER, B., THEURL, M., NIEDEREGGER, H. & TILG, H. 2007. Visfatin, an adipocytokine with proinflammatory and immunomodulating properties. *The Journal of Immunology*, 178, 1748-1758.

- MYERS, K. R. & CASANOVA, J. E. 2008. Regulation of actin cytoskeleton dynamics by Arf-family GTPases. *Trends in cell biology*, 18, 184-192.
- NANCARROW, D. J., CLOUSTON, A. D., SMITHERS, B. M., GOTLEY, D. C., DREW, P. A., WATSON, D. I., TYAGI, S., HAYWARD, N. K., WHITEMAN, D. C. & AUSTRALIAN CANCER STUDY AND THE STUDY OF DIGESTIVE, H. 2011. Whole genome expression array profiling highlights differences in mucosal defense genes in Barrett's esophagus and esophageal adenocarcinoma. *PLoS One*, 6, e22513.
- NAPIER, K. J., SCHEERER, M. & MISRA, S. 2014. Esophageal cancer: A Review of epidemiology, pathogenesis, staging workup and treatment modalities. *World J Gastrointest Oncol*, 6, 112-20.
- NEASTA, J., FIORENZA, A., HE, D.-Y., PHAMLUONG, K., KIELY, P. A. & RON, D. 2016. Activation of the cAMP Pathway Induces RACK1-Dependent Binding of β -Actin to BDNF Promoter. *PloS one*, 11, e0160948.
- NEASTA, J., KIELY, P. A., HE, D.-Y., ADAMS, D. R., O'CONNOR, R. & RON, D. 2012. Direct interaction between scaffolding proteins RACK1 and 14-3-3 ζ regulates brain-derived neurotrophic factor (BDNF) transcription. *Journal of Biological Chemistry*, 287, 322-336.
- NEHRA, D., HOWELL, P., WILLIAMS, C. P., PYE, J. K. & BEYNON, J. 1999. Toxic bile acids in gastro-oesophageal reflux disease: influence of gastric acidity. *Gut*, 44, 598-602.
- NIELSEN, M. H., FLYGAARD, R. K. & JENNER, L. B. 2017. Structural analysis of ribosomal RACK1 and its role in translational control. *Cellular Signalling*.
- NORMAN, J. C., JONES, D., BARRY, S. T., HOLT, M. R., COCKCROFT, S. & CRITCHLEY, D. R. 1998. ARF1 mediates paxillin recruitment to focal adhesions and potentiates Rho-stimulated stress fiber formation in intact and permeabilized Swiss 3T3 fibroblasts. *J Cell Biol*, 143, 1981-1995.
- O'BRIEN, T., OEH, J., XIAO, Y., LIANG, X., VANDERBILT, A., QIN, A., YANG, L., LEE, L. B., LY, J. & COSINO, E. 2013. Supplementation of nicotinic acid with NAMPT inhibitors results in loss of in vivo efficacy in NAPRT1-deficient tumor models. *Neoplasia*, 15, 1314-IN3.
- O'DONOVAN, H. C., KIELY, P. A. & O'CONNOR, R. 2007. Effects of RACK1 on cell migration and IGF-I signalling in cardiomyocytes are not dependent on an association with the IGF-IR. *Cellular signalling*, 19, 2588-2595.
- O'RIORDAN, J. M., ABDEL-LATIF, M. M., RAVI, N., MCNAMARA, D., BYRNE, P. J., MCDONALD, G. S. A., KEELING, P. W. N., KELLEHER, D. & REYNOLDS, J. V. 2005. Proinflammatory cytokine and nuclear factor kappa-B expression along the inflammation–metaplasia–dysplasia–adenocarcinoma sequence in the esophagus. *The American journal of gastroenterology*, 100, 1257-1264.
- O'SULLIVAN, K. E., PHELAN, J. J., O'HANLON, C., LYSAGHT, J., O'SULLIVAN, J. N. & REYNOLDS, J. V. 2014. The role of inflammation in cancer of the esophagus. *Expert review of gastroenterology & hepatology*, 8, 749.
- ONISHI, I., LIN, P. J. C., DIERING, G. H., WILLIAMS, W. P. & NUMATA, M. 2007. RACK1 associates with NHE5 in focal adhesions and positively regulates the transporter activity. *Cellular signalling*, 19, 194-203.

- OTI, M., SNEL, B., HUYNEN, M. A. & BRUNNER, H. G. 2006. Predicting disease genes using protein-protein interactions. *Journal of medical genetics*, 43, 691-698.
- OUDERKIRK, J. L. & KRENDEL, M. 2014. Non-muscle myosins in tumor progression, cancer cell invasion, and metastasis. *Cytoskeleton*, 71, 447-463.
- PALSSON-MCDERMOTT, E. M. & O'NEILL, L. A. 2013. The Warburg effect then and now: from cancer to inflammatory diseases. *Bioessays*, 35, 965-973.
- PARSONS, J. T., MARTIN, K. H., SLACK, J. K., TAYLOR, J. M. & WEED, S. A. 2000. Focal adhesion kinase: a regulator of focal adhesion dynamics and cell movement. *Oncogene*, 19, 5606.
- PATEL, S. T., MISTRY, T., BROWN, J. E. P., DIGBY, J. E., ADYA, R., DESAI, K. M. & RANDEVA, H. S. 2010. A novel role for the adipokine visfatin/pre-B cell colony-enhancing factor 1 in prostate carcinogenesis. *Peptides*, 31, 51-57.
- PENG, H., GONG, P.-G., LI, J.-B., CAI, L.-M., YANG, L., LIU, Y.-Y., YAO, K.-T. & LI, X. 2016. The important role of the receptor for activated C kinase 1 (RACK1) in nasopharyngeal carcinoma progression. *Journal of translational medicine*, 14, 1.
- PENG, R., JIANG, B., MA, J., MA, Z., WAN, X., LIU, H., CHEN, Z., CHENG, Q. & CHEN, R. 2013. Forced downregulation of RACK1 inhibits glioma development by suppressing Src/Akt signaling activity. *Oncology reports*, 30, 2195-2202.
- PENNATHUR, A., GIBSON, M. K., JOBE, B. A. & LUKETICH, J. D. 2013. Oesophageal carcinoma. *The Lancet*, 381, 400-412.
- PICARDO, S. L., MAHER, S. G., O'SULLIVAN, J. N. & REYNOLDS, J. V. 2012. Barrett's to oesophageal cancer sequence: a model of inflammatory-driven upper gastrointestinal cancer. *Digestive surgery*, 29, 251-260.
- POHL, H., WROBEL, K., BOJARSKI, C., VODERHOLZER, W., SONNENBERG, A., RÖSCH, T. & BAUMGART, D. C. 2013. Risk factors in the development of esophageal adenocarcinoma. *The American journal of gastroenterology*, 108, 200-207.
- POLACHECK, W. J., ZERVANTONAKIS, I. K. & KAMM, R. D. 2013. Tumor cell migration in complex microenvironments. *Cellular and Molecular Life Sciences*, 70, 1335-1356.
- PONTI, A., MACHACEK, M., GUPTON, S. L., WATERMAN-STORER, C. M. & DANUSER, G. 2004. Two distinct actin networks drive the protrusion of migrating cells. *Science*, 305, 1782-1786.
- RENEHAN, A. G., TYSON, M., EGGER, M., HELLER, R. F. & ZWAHLEN, M. 2008. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. *The Lancet*, 371, 569-578.
- REVOLLO, J. R., GRIMM, A. A. & IMAI, S.-I. 2004. The NAD biosynthesis pathway mediated by nicotinamide phosphoribosyltransferase regulates Sir2 activity in mammalian cells. *Journal of Biological Chemistry*, 279, 50754-50763.
- REVOLLO, J. R., KÖRNER, A., MILLS, K. F., SATOH, A., WANG, T., GARTEN, A., DASGUPTA, B., SASAKI, Y., WOLBERGER, C. & TOWNSEND, R.

- R. 2007. Nampt/PBEF/visfatin regulates insulin secretion in β cells as a systemic NAD biosynthetic enzyme. *Cell metabolism*, 6, 363-375.
- RIDLEY, A. J. 2011. Life at the leading edge. *Cell*, 145, 1012-1022.
- RIDLEY, A. J., SCHWARTZ, M. A., BURRIDGE, K., FIRTEL, R. A., GINSBERG, M. H., BORISY, G., PARSONS, J. T. & HORWITZ, A. R. 2003. Cell migration: integrating signals from front to back. *Science*, 302, 1704-1709.
- ROBLES, M. S., BOYAULT, C., KNUTTI, D., PADMANABHAN, K. & WEITZ, C. J. 2010. Identification of RACK1 and protein kinase C α as integral components of the mammalian circadian clock. *Science*, 327, 463-466.
- ROMACHO, T., VILLALOBOS, L. A., CERCAS, E., CARRARO, R., SÁNCHEZ-FERRER, C. F. & PEIRÓ, C. 2013. Visfatin as a novel mediator released by inflamed human endothelial cells. *PloS one*, 8, e78283.
- RON, D., ADAMS, D. R., BAILLIE, G. S., LONG, A., O'CONNOR, R. & KIELY, P. A. 2013. RACK (1) to the future—a historical perspective. *Cell Communication and Signaling*, 11.
- RON, D., CHEN, C.-H., CALDWELL, J., JAMIESON, L., ORR, E. & MOCHLY-ROSEN, D. 1994. Cloning of an intracellular receptor for protein kinase C: a homolog of the beta subunit of G proteins. *Proceedings of the National Academy of Sciences*, 91, 839-843.
- RON, D., LUO, J. & MOCHLY-ROSEN, D. 1995. C2 region-derived peptides inhibit translocation and function of β protein kinase C in vivo. *Journal of Biological Chemistry*, 270, 24180-24187.
- RUAN, Y., SUN, L., HAO, Y., WANG, L., XU, J., ZHANG, W., XIE, J., GUO, L., ZHOU, L. & YUN, X. 2012. Ribosomal RACK1 promotes chemoresistance and growth in human hepatocellular carcinoma. *The Journal of clinical investigation*, 122, 2554-2566.
- RUBENSTEIN, J. H. & THRIFT, A. P. 2015. Risk factors and populations at risk: selection of patients for screening for Barrett's oesophagus. *Best Practice & Research Clinical Gastroenterology*, 29, 41-50.
- RUGGERO, D. 2013. Translational control in cancer etiology. *Cold Spring Harbor perspectives in biology*, 5, a012336.
- SADDI-ROSA, P., OLIVEIRA, C. S., CRISPIM, F., GIUFFRIDA, F. M. A., DE LIMA, V. C., VIEIRA, J. G., DORIA, A., VELHO, G. & REIS, A. F. 2013. Association of circulating levels of nicotinamide phosphoribosyltransferase (NAMPT/Visfatin) and of a frequent polymorphism in the promoter of the NAMPT gene with coronary artery disease in diabetic and non-diabetic subjects. *Cardiovascular diabetology*, 12, 1.
- SAITO, A., FUJII, G., SATO, Y., GOTOH, M., SAKAMOTO, M., TODA, G. & HIROHASHI, S. 2002. Detection of genes expressed in primary colon cancers by in situ hybridisation: overexpression of RACK 1. *Molecular Pathology*, 55, 34-39.
- SAMPATH, D., ZABKA, T. S., MISNER, D. L., O'BRIEN, T. & DRAGOVICH, P. S. 2015. Inhibition of nicotinamide phosphoribosyltransferase (NAMPT) as a therapeutic strategy in cancer. *Pharmacology & therapeutics*, 151, 16-31.
- SANDQUIST, J. C., SWENSON, K. I., DEMALI, K. A., BURRIDGE, K. & MEANS, A. R. 2006. Rho kinase differentially regulates phosphorylation

- of nonmuscle myosin II isoforms A and B during cell rounding and migration. *Journal of Biological Chemistry*, 281, 35873-35883.
- SAVAGNER, P. 2015. Chapter Eight-Epithelial-Mesenchymal Transitions: From Cell Plasticity to Concept Elasticity. *Current topics in developmental biology*, 112, 273-300.
- SAWICKA-GUTAJ, N., WALIGÓRSKA-STACHURA, J., ANDRUSIEWICZ, M., BICZYSKO, M., SOWIŃSKI, J., SKROBISZ, J. & RUCHAŁA, M. 2015. Nicotinamide phosphorybosyltransferase overexpression in thyroid malignancies and its correlation with tumor stage and with survivin/survivin DEx3 expression. *Tumor Biology*, 36, 7859-7863.
- SCHMITZ, A. A. P., GOVEK, E.-E., BÖTTNER, B. & VAN AELST, L. 2000. Rho GTPases: signaling, migration, and invasion. *Experimental cell research*, 261, 1-12.
- SCHRAMEK, D., SENDOEL, A., SEGAL, J. P., BERONJA, S., HELLER, E., ORISTIAN, D., REVA, B. & FUCHS, E. 2014. Direct in vivo RNAi screen unveils myosin IIa as a tumor suppressor of squamous cell carcinomas. *Science*, 343, 309-313.
- SCHUSTER, S., PENKE, M., GORSKI, T., GEBHARDT, R., WEISS, T. S., KIESS, W. & GARTEN, A. 2015. FK866-induced NAMPT inhibition activates AMPK and downregulates mTOR signaling in hepatocarcinoma cells. *Biochemical and biophysical research communications*, 458, 334-340.
- SEGAWA, K., FUKUHARA, A., HOSOGAI, N., MORITA, K., OKUNO, Y., TANAKA, M., NAKAGAWA, Y., KIHARA, S., FUNAHASHI, T. & KOMURO, R. 2006. Visfatin in adipocytes is upregulated by hypoxia through HIF1 α -dependent mechanism. *Biochemical and biophysical research communications*, 349, 875-882.
- SENGUPTA, J., NILSSON, J., GURSKY, R., SPAHN, C. M. T., NISSEN, P. & FRANK, J. 2004. Identification of the versatile scaffold protein RACK1 on the eukaryotic ribosome by cryo-EM. *Nature structural & molecular biology*, 11, 957-962.
- SENISTERRA, G., WU, H., ALLALI-HASSANI, A., WASNEY, G. A., BARSYTE-LOVEJOY, D., DOMBROVSKI, L., DONG, A., NGUYEN, K. T., SMIL, D. & BOLSHAN, Y. 2013. Small-molecule inhibition of MLL activity by disruption of its interaction with WDR5. *Biochemical Journal*, 449, 151-159.
- SERRELS, B., SANDILANDS, E. & FRAME, M. C. 2011. Signaling of the direction-sensing FAK/RACK1/PDE4D5 complex to the small GTPase Rap1. *Small GTPases*.
- SERRELS, B., SANDILANDS, E., SERRELS, A., BAILLIE, G., HOUSLAY, M. D., BRUNTON, V. G., CANEL, M., MACHESKY, L. M., ANDERSON, K. I. & FRAME, M. C. 2010. A complex between FAK, RACK1, and PDE4D5 controls spreading initiation and cancer cell polarity. *Current Biology*, 20, 1086-1092.
- SHACKELFORD, R. E., BUI, M. M., COPPOLA, D. & HAKAM, A. 2010. Over-expression of nicotinamide phosphoribosyltransferase in ovarian cancers. *Int J Clin Exp Pathol*, 3, 522-527.
- SHAHEEN, N. & RANSOHOFF, D. F. 2002. Gastroesophageal reflux, barrett esophagus, and esophageal cancer: scientific review. *Jama*, 287, 1972-1981.

- SHI, S., DENG, Y.-Z., ZHAO, J.-S., JI, X.-D., SHI, J., FENG, Y.-X., LI, G., LI, J.-J., ZHU, D. & KOEFFLER, H. P. 2012. RACK1 promotes non-small-cell lung cancer tumorigenicity through activating sonic hedgehog signaling pathway. *Journal of Biological Chemistry*, 287, 7845-7858.
- SILVERA, D., FORMENTI, S. C. & SCHNEIDER, R. J. 2010. Translational control in cancer. *Nature Reviews Cancer*, 10, 254-267.
- SINGH, S., SHARMA, A. N., MURAD, M. H., BUTTAR, N. S., EL-SERAG, H. B., KATZKA, D. A. & IYER, P. G. 2013. Central adiposity is associated with increased risk of esophageal inflammation, metaplasia, and adenocarcinoma: a systematic review and meta-analysis. *Clinical Gastroenterology and Hepatology*, 11, 1399-1412.
- SMITH, K. J., BAILLIE, G. S., HYDE, E. I., LI, X., HOUSLAY, T. M., MCCAHERILL, A., DUNLOP, A. J., BOLGER, G. B., KLUSSMANN, E. & ADAMS, D. R. 2007. ¹H NMR structural and functional characterisation of a cAMP-specific phosphodiesterase-4D5 (PDE4D5) N-terminal region peptide that disrupts PDE4D5 interaction with the signalling scaffold proteins, β arrestin and RACK1. *Cellular signalling*, 19, 2612-2624.
- SOKALINGAM, S., RAGHUNATHAN, G., SOUNDARARAJAN, N. & LEE, S.-G. 2012. A study on the effect of surface lysine to arginine mutagenesis on protein stability and structure using green fluorescent protein. *PLoS One*, 7, e40410.
- SONCINI, D., CAFFA, I., ZOPPOLI, G., CEA, M., CAGNETTA, A., PASSALACQUA, M., MASTRACCI, L., BOERO, S., MONTECUCCO, F. & SOCIALI, G. 2014. Nicotinamide phosphoribosyltransferase promotes epithelial-to-mesenchymal transition as a soluble factor independent of its enzymatic activity. *Journal of Biological Chemistry*, 289, 34189-34204.
- SONDEK, J., BOHM, A., LAMBRIGHT, D. G., HAMM, H. E. & SIGLER, P. B. 1996. Crystal structure of a GA protein β dimer at 2.1 Å resolution. *Nature*, 379, 369-374.
- SONJA, B., BITTINS, M., STEFFEN, F. & SEEDORF, M. 2004. Asc1p, a WD40-domain containing adaptor protein, is required for the interaction of the RNA-binding protein Scp160p with polysomes. *Biochemical Journal*, 380, 823-830.
- SPIERING, D. & HODGSON, L. 2011. Dynamics of the Rho-family small GTPases in actin regulation and motility. *Cell adhesion & migration*, 5, 170-180.
- STADLER, C., REXHEPAJ, E., SINGAN, V. R., MURPHY, R. F., PEPPERKOK, R., UHLÉN, M., SIMPSON, J. C. & LUNDBERG, E. 2013. Immunofluorescence and fluorescent-protein tagging show high correlation for protein localization in mammalian cells. *Nature methods*, 10, 315-323.
- STEIN, L. R. & IMAI, S.-I. 2012. The dynamic regulation of NAD metabolism in mitochondria. *Trends in Endocrinology & Metabolism*, 23, 420-428.
- STIRNIMANN, C. U., PETSALAKI, E., RUSSELL, R. B. & MÜLLER, C. W. 2010. WD40 proteins propel cellular networks. *Trends in biochemical sciences*, 35, 565-574.
- STONER, G. D., KAIGHN, M. E., REDDEL, R. R., RESAU, J. H., BOWMAN, D., NAITO, Z., MATSUKURA, N., YOU, M., GALATI, A. J. & HARRIS, C. C. 1991. Establishment and characterization of SV40 T-antigen

- immortalized human esophageal epithelial cells. *Cancer Research*, 51, 365-371.
- SUBAUSTE, M. C., VENTURA-HOLMAN, T., DU, L., SUBAUSTE, J. S., CHAN, S.-L., YU, V. C. & MAHER, J. F. 2009. RACK1 down-regulates levels of the pro-apoptotic protein Fem1b in apoptosis-resistant colon cancer cells. *Cancer biology & therapy*, 8, 2295-2303.
- SUN, J. & HEMLER, M. E. 2001. Regulation of MMP-1 and MMP-2 production through CD147/extracellular matrix metalloproteinase inducer interactions. *Cancer research*, 61, 2276-2281.
- SWAMINATHAN, G. & CARTWRIGHT, C. A. 2012. Rack1 promotes epithelial cell-cell adhesion by regulating E-cadherin endocytosis. *Oncogene*, 31, 376-389.
- TAN, B., YOUNG, D. A., LU, Z.-H., WANG, T., MEIER, T. I., SHEPARD, R. L., ROTH, K., ZHAI, Y., HUSS, K. & KUO, M.-S. 2013. Pharmacological inhibition of nicotinamide phosphoribosyltransferase (NAMPT), an enzyme essential for NAD⁺ biosynthesis, in human cancer cells metabolic basis and potential clinical implications. *Journal of Biological Chemistry*, 288, 3500-3511.
- THOMPSON, O. M., BERESFORD, S. A. A., KIRK, E. A., BRONNER, M. P. & VAUGHAN, T. L. 2010. Serum leptin and adiponectin levels and risk of Barrett's esophagus and intestinal metaplasia of the gastroesophageal junction. *Obesity*, 18, 2204-2211.
- THORNTON, C., TANG, K.-C., PHAMLUONG, K., LUONG, K., VAGTS, A., NIKANJAM, D., YAKA, R. & RON, D. 2004. Spatial and temporal regulation of RACK1 function and N-methyl-D-aspartate receptor activity through WD40 motif-mediated dimerization. *Journal of Biological Chemistry*, 279, 31357-31364.
- THRIFT, A. P. 2016. The epidemic of oesophageal carcinoma: Where are we now? *Cancer epidemiology*, 41, 88-95.
- THRIFT, A. P., SHAHEEN, N. J., GAMMON, M. D., BERNSTEIN, L., REID, B. J., ONSTAD, L., RISCH, H. A., LIU, G., BIRD, N. C. & WU, A. H. 2014. Obesity and risk of esophageal adenocarcinoma and Barrett's esophagus: a Mendelian randomization study. *Journal of the National Cancer Institute*, 106, dju252.
- TOGO, T. & STEINHARDT, R. A. 2004. Nonmuscle myosin IIA and IIB have distinct functions in the exocytosis-dependent process of cell membrane repair. *Molecular biology of the cell*, 15, 688-695.
- TREROTOLA, M., LI, J., ALBERTI, S. & LANGUINO, L. R. 2012. Trop-2 inhibits prostate cancer cell adhesion to fibronectin through the β 1 integrin-RACK1 axis. *Journal of cellular physiology*, 227, 3670-3677.
- TURRIZIANI, B., GARCIA-MUNOZ, A., PILKINGTON, R., RASO, C., KOLCH, W. & VON KRIEGSHEIM, A. 2014. On-beads digestion in conjunction with data-dependent mass spectrometry: a shortcut to quantitative and dynamic interaction proteomics. *Biology*, 3, 320-332.
- UNDERWOOD, T. J., DEROUET, M. F., WHITE, M. J., NOBLE, F., MOUTASIM, K. A., SMITH, E., DREW, P. A., THOMAS, G. J., PRIMROSE, J. N. & BLAYDES, J. P. 2010. A comparison of primary oesophageal squamous epithelial cells with HET-1A in organotypic culture. *Biology of the Cell*, 102, 635-644.

- VAN ZIJL, F., KRUPITZA, G. & MIKULITS, W. 2011. Initial steps of metastasis: cell invasion and endothelial transmigration. *Mutation Research/Reviews in Mutation Research*, 728, 23-34.
- VARA, J. Á. F., CASADO, E., DE CASTRO, J., CEJAS, P., BELDA-INIESTA, C. & GONZÁLEZ-BARÓN, M. 2004. PI3K/Akt signalling pathway and cancer. *Cancer treatment reviews*, 30, 193-204.
- VICENTE-MANZANARES, M., MA, X., ADELSTEIN, R. S. & HORWITZ, A. R. 2009. Non-muscle myosin II takes centre stage in cell adhesion and migration. *Nature reviews Molecular cell biology*, 10, 778-790.
- VOGEL, C., SILVA, G. M. & MARCOTTE, E. M. 2011. Protein expression regulation under oxidative stress. *Molecular & Cellular Proteomics*, 10, M111-009217.
- WALL, M. A., COLEMAN, D. E., LEE, E., IÑIGUEZ-LLUHI, J. A., POSNER, B. A., GILMAN, A. G. & SPRANG, S. R. 1995. The structure of the G protein heterotrimer $G_{i\alpha 1} \beta 1 \gamma 2$. *Cell*, 83, 1047-1058.
- WANG, G., HAN, T., NIJHAWAN, D., THEODOROPOULOS, P., NAIDOO, J., YADAVALLI, S., MIRZAEI, H., PIEPER, A. A., READY, J. M. & MCKNIGHT, S. L. 2014. P7C3 neuroprotective chemicals function by activating the rate-limiting enzyme in NAD salvage. *Cell*, 158, 1324-1334.
- WANG, G.-J., SHEN, N.-J., CHENG, L., FANG, Y., HUANG, H. & LI, K.-H. 2016. Visfatin triggers the in vitro migration of osteosarcoma cells via activation of NF- κ B/IL-6 signals. *European Journal of Pharmacology*, 791, 322-330.
- WANG, N., LIU, F., CAO, F., JIA, Y., WANG, J., MA, W., TAN, B., WANG, K., SONG, Q. & CHENG, Y. 2015a. RACK1 predicts poor prognosis and regulates progression of esophageal squamous cell carcinoma through its epithelial-mesenchymal transition. *Cancer Biology & Therapy*, 00-00.
- WANG, Q., ZHANG, Z., BLACKWELL, K. & CARMICHAEL, G. G. 2005. Vigilins bind to promiscuously A-to-I-edited RNAs and are involved in the formation of heterochromatin. *Current biology*, 15, 384-391.
- WANG, Q., ZHOU, S., WANG, J.-Y., CAO, J., ZHANG, X., WANG, J., HAN, K., CHENG, Q., QIU, G. & ZHAO, Y. 2015b. RACK1 antagonizes TNF- α -induced cell death by promoting p38 activation. *Scientific reports*, 5.
- WANG, Z., ZHANG, B., JIANG, L., ZENG, X., CHEN, Y., FENG, X., GUO, Y. & CHEN, Q. 2009. RACK1, an excellent predictor for poor clinical outcome in oral squamous carcinoma, similar to Ki67. *European Journal of Cancer*, 45, 490-496.
- WATSON, G. A., ZHANG, X., STANG, M. T., LEVY, R. M., DE OLIVEIRA, P. E. Q., GOODING, W. E., CHRISTENSEN, J. G. & HUGHES, S. J. 2006. Inhibition of c-Met as a therapeutic strategy for esophageal adenocarcinoma. *Neoplasia*, 8, 949-955.
- WEBER, V., WERNITZNIG, A., HAGER, G., HARATA, M., FRANK, P. & WINTERSBERGER, U. 1997. Purification and nucleic-acid-binding properties of a *Saccharomyces cerevisiae* protein involved in the control of ploidy. *The FEBS Journal*, 249, 309-317.
- WEHNER, P., SHNITSAR, I., URLAUB, H. & BORCHERS, A. 2011. RACK1 is a novel interaction partner of PTK7 that is required for neural tube closure. *Development*, 138, 1321-1327.
- WILLETT, M., POLLARD, H. J., VLASAK, M. & MORLEY, S. J. 2010. Localization of ribosomes and translation initiation factors to

- talin/ β 3-integrin-enriched adhesion complexes in spreading and migrating mammalian cells. *Biology of the Cell*, 102, 265-276.
- WINTERSBERGER, U., KÜHNE, C. & KARWAN, A. 1995. Scp160p, a new yeast protein associated with the nuclear membrane and the endoplasmic reticulum, is necessary for maintenance of exact ploidy. *Yeast*, 11, 929-944.
- WU, J., MENG, J., DU, Y., HUANG, Y., JIN, Y., ZHANG, J., WANG, B., ZHANG, Y., SUN, M. & TANG, J. 2013. RACK1 promotes the proliferation, migration and invasion capacity of mouse hepatocellular carcinoma cell line in vitro probably by PI3K/Rac1 signaling pathway. *Biomedicine & Pharmacotherapy*, 67, 313-319.
- XIA, Z. K., YUAN, Y. C., YIN, N., YIN, B. L., TAN, Z. P. & HU, Y. R. 2012. Nonmuscle myosin IIA is associated with poor prognosis of esophageal squamous cancer. *Diseases of the Esophagus*, 25, 427-436.
- XU, H., BARNES, G. T., YANG, Q., TAN, G., YANG, D., CHOU, C. J., SOLE, J., NICHOLS, A., ROSS, J. S. & TARTAGLIA, L. A. 2003. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *The Journal of clinical investigation*, 112, 1821-1830.
- XU, J., LAMOUILLE, S. & DERYNCK, R. 2009. TGF- β -induced epithelial to mesenchymal transition. *Cell research*, 19, 156-172.
- XU, Z., LI, P., WEI, D., WANG, Z., BAO, Y., SUN, J., QU, L. & WANG, L. 2016. NMMHC-IIA-dependent nuclear location of CXCR4 promotes migration and invasion in renal cell carcinoma. *Oncology Reports*, 36, 2681-2688.
- YAMAGUCHI, H. & CONDEELIS, J. 2007. Regulation of the actin cytoskeleton in cancer cell migration and invasion. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1773, 642-652.
- YANG, W. L., WEI, L., HUANG, W. Q., LI, R., SHEN, W. Y., LIU, J. Y., XU, J. M., LI, B. & QIN, Y. 2014. Vigilin is overexpressed in hepatocellular carcinoma and is required for HCC cell proliferation and tumor growth. *Oncology reports*, 31, 2328-2334.
- YANG, Y., WU, N., WANG, Z., ZHANG, F., TIAN, R., JI, W., REN, X. & NIU, R. 2016. Rack1 Mediates the Interaction of P-Glycoprotein with Anxa2 and Regulates Migration and Invasion of Multidrug-Resistant Breast Cancer Cells. *International Journal of Molecular Sciences*, 17, 1718.
- YOON, M. J., YOSHIDA, M., JOHNSON, S., TAKIKAWA, A., USUI, I., TOBE, K., NAKAGAWA, T., YOSHINO, J. & IMAI, S.-I. 2015. SIRT1-mediated eNAMPT secretion from adipose tissue regulates hypothalamic NAD⁺ and function in mice. *Cell metabolism*, 21, 706-717.
- YOSHIDA, N. 2007. Oxidative Stress in Digestive Disease Guest Editor: Yuji Naito Inflammation and Oxidative Stress in Gastroesophageal Reflux Disease. *Journal of Clinical Biochemistry and Nutrition*, 40, 13-23.
- ZHANG, C., YAN, D., WANG, S., XU, C., DU, W., NING, T., LIU, C., ZHANG, M., HOU, R. & CHEN, Z. 2015. Genetic polymorphisms of NAMPT related with susceptibility to esophageal Squamous cell carcinoma. *BMC gastroenterology*, 15, 1.
- ZHANG, H. Y., HORMI-CARVER, K., ZHANG, X., SPECHLER, S. J. & SOUZA, R. F. 2009. In benign Barrett's epithelial cells, acid exposure generates reactive oxygen species that cause DNA double-strand breaks. *Cancer research*, 69, 9083-9089.

ZHAO, Y., WANG, Q., QIU, G., ZHOU, S., JING, Z., WANG, J., WANG, W., CAO, J., HAN, K. & CHENG, Q. 2015. RACK1 promotes autophagy by enhancing the Atg14L-Beclin 1-Vps34-Vps15 complex formation upon phosphorylation by AMPK. *Cell reports*, 13, 1407-1417.

ZHONG, X., LI, M., NIE, B. & WU, F. 2013. Overexpressions of RACK1 and CD147 Associated with Poor Prognosis in Stage T1 Pulmonary Adenocarcinoma. *Annals of Surgical Oncology*, 20, 1044.

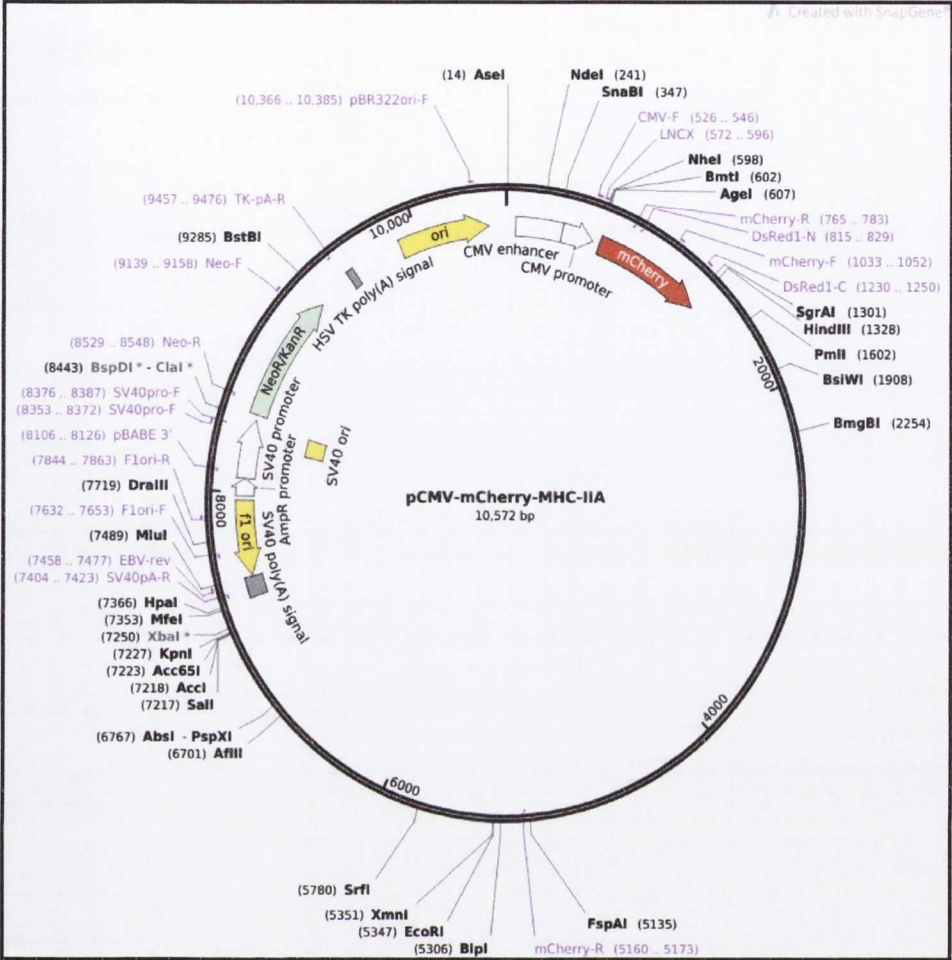
ZHOU, T., LI, C., ZHAO, W., WANG, X., WANG, F. & SHA, J. 2016. MaxReport: An enhanced proteomic result reporting tool for maxquant. *PloS one*, 11, e0152067.

Appendices

Appendix 1: siRNA and plasmids

Plasmid or siRNA used	Source
SMARTpool: ON-TARGETplus GNB2L1 siRNA	Dharmacon
ON-TARGETplus Non-targeting Control Pool	Dharmacon
SMARTpool: ON-TARGETplus MYH9 siRNA	Dharmacon
SMARTpool: ON-TARGETplus NAMPT siRNA	Dharmacon
pCMV-mCherry-MHC-IIA plasmid	addgene (deposited by Venkaiah Betapudi)
RACK1-EGFP plasmid	Kind gift from Dr Pat Kiely (University Limerick)
Plasmid encoding free mCherry	Kind gift from Prof Joel Swanson (University Michigan)
Plasmid encoding free EGFP	Kind gift from Prof Joel Swanson (University Michigan)

Appendix 1 Table 1 siRNAs and DNA plasmids.



Appendix 1 Figure 1: Plasmid map for pCMV-mCherry-MHC-IIA.

Appendix 2: Antibodies

Antibody	Source	Cat. No.	Dilution(s) used WB = Western Blotting IF= Immunofluorescent staining
Mouse anti-RACK1	BD Transduction	610177	1/3000 for WB 1/150 for IF
Rabbit anti-NMHCIIA	Biomedical Technologies, Inc.	BT-567	1/3000 for WB 1/100 for IF
Rabbit anti-NMHCIIIB	Cell Signalling Technology, Inc.	3404	1/2000 for WB
Rabbit anti-NMHCIIIC	Cell Signalling Technology, Inc.	8189	1/2000 for WB
Rabbit anti-HDLBP antibody	Proteintech Group	15406-1-AP	1/1000 for WB 1/50 for IF
Rabbit anti-visfatin	Proteintech Group	11776-1-AP	1/3000 for WB
Rabbit anti-visfatin antibody	Abcam	ab151235	1/100 for IF
Mouse Anti- β -Actin	Sigma	A5316	1/4000 for WB
Goat anti-mouse IgG polyclonal antibody HRP conjugated	Cell Signalling	7074	1/5000 for WB
Goat anti-rabbit IgG polyclonal antibody HRP conjugated	Cell Signalling	7076	1/5000 for WB
AlexaFluor goat anti- mouse 488	Molecular Probes	A-11001	1/200 for IF
AlexaFluor goat anti- rabbit 546	Molecular Probes	A-11003	1/200 for IF
AlexaFluor goat anti- rabbit 488	Molecular Probes	A-11008	1/200 for IF

Appendix 2; Table 1. Antibodies used during the PhD project

Appendix 3: Supplementary Data for Chapter 6

Appendix 3: Table 1: PEAKS analysis of data generated by mass spectrometry analysis of RACK1 IP and Control IP. This table lists proteins present in the Control IP

Accession	- 10lgP	Coverage	#Peptides	#Unique	Gene Name
P38646 GRP75_HUMAN	528.76	74	71	70	HSPA9
P60709 ACTB_HUMAN	475.62	89	35	1	ACTB
P08670 VIME_HUMAN	464.59	87	56	46	VIM
P11021 GRP78_HUMAN	463.26	59	52	49	HSPA5
P11142 HSP7C_HUMAN	460.17	61	46	28	HSPA8
P63261 ACTG_HUMAN	457.62	89	35	1	ACTG1
P07437 TBB5_HUMAN	451.8	73	29	6	TUBB
P68371 TBB4B_HUMAN	444.94	69	27	2	TUBB4B
P35579 MYH9_HUMAN	435.09	38	68	60	MYH9
Q13885 TBB2A_HUMAN	413.2	57	21	3	TUBB2A
Q9BVA1 TBB2B_HUMAN	413.2	57	21	3	TUBB2B
Q13509 TBB3_HUMAN	411.17	47	20	6	TUBB3
P14618-2 KPYM_HUMAN	390.57	72	31	1	PKM
P68104 EF1A1_HUMAN	389.55	61	24	12	EEF1A1
Q9BQE3 TBA1C_HUMAN	384.24	59	25	2	TUBA1C
P06733 ENOA_HUMAN	382.8	56	19	17	ENO1
P08107 HSP71_HUMAN	376.59	55	40	5	HSPA1A
P02768 ALBU_HUMAN	343.36	53	46	19	ALB
P04264 K2C1_HUMAN	343.07	49	35	30	KRT1
P54652 HSP72_HUMAN	336.76	29	21	4	HSPA2
P08238 HS90B_HUMAN	332.9	31	24	6	HSP90AB1
P68133 ACT5_HUMAN	327.22	36	20	1	ACTA1
P05787 K2C8_HUMAN	324.4	57	30	21	KRT8
P05787-2 K2C8_HUMAN	324.4	53	30	21	KRT8
P63267 ACTH_HUMAN	318.76	36	19	1	ACTG2
P62736 ACTA_HUMAN	318.76	36	19	1	ACTA2
P25705 ATPA_HUMAN	312.41	41	20	3	ATP5A1
P04406 G3P_HUMAN	311.46	52	13	12	GAPDH
P06576 ATPB_HUMAN	297.94	42	17	17	ATP5B
P62826 RAN_HUMAN	297.9	61	17	17	RAN
P17987 TCPA_HUMAN	293.27	36	20	20	TCP1
P35637-2 FUS_HUMAN	287.3	26	12	9	FUS
P35637 FUS_HUMAN	287.3	26	12	9	FUS
P34931 HS71L_HUMAN	286.85	25	17	3	HSPA1L
P31943 HNRH1_HUMAN	284.18	41	17	7	HNRNPH1
P62805 H4_HUMAN	284.17	60	11	11	HIST1H4A
P35908 K22E_HUMAN	284.04	31	18	12	KRT2
P04792 HSPB1_HUMAN	283.95	71	13	13	HSPB1
P35527 K1C9_HUMAN	281.33	37	20	19	KRT9
P13645 K1C10_HUMAN	271.73	48	23	17	KRT10
P07900-2 HS90A_HUMAN	271.11	14	13	3	HSP90AA1
Q6S8J3 POTEE_HUMAN	269.24	9	9	1	POTEE
P23528 COF1_HUMAN	260.82	78	9	6	CFI1
P13639 EF2_HUMAN	260.58	19	14	14	EEF2
P05783 K1C18_HUMAN	255.5	46	18	16	KRT18
P60842 IF4A1_HUMAN	254.15	20	7	5	EIF4A1
P07355 ANXA2_HUMAN	253.45	45	13	1	ANXA2
P22626-2 ROA2_HUMAN	251.87	30	11	9	HNRNPA2B1
P22626 ROA2_HUMAN	251.87	29	11	9	HNRNPA2B1
P23396 RS3_HUMAN	251.24	65	15	15	RPS3
Q06830 PRDX1_HUMAN	250.98	56	12	12	PRDX1
Q14764 MVP_HUMAN	247.65	34	19	19	MVP
P61247 RS3A_HUMAN	245.17	36	9	9	RPS3A
P26373 RL13_HUMAN	244.66	55	16	15	RPL13
P62241 RS8_HUMAN	244.18	59	12	12	RPS8
P62753 RS6_HUMAN	244.14	39	11	11	RPS6
P32969 RL9_HUMAN	243.17	40	8	8	RPL9
Q9BUF5 TBB6_HUMAN	242.58	22	9	1	TUBB6
Q09666 AHNK_HUMAN	237.68	3	13	13	AHNAK
P09382 LEG1_HUMAN	237.11	39	4	4	LGALS1
O00571-2 DDX3X_HUMAN	235.17	18	9	3	DDX3X
O00571 DDX3X_HUMAN	235.17	17	9	3	DDX3X
P36578 RL4_HUMAN	234.55	33	9	9	RPL4
P62081 RS7_HUMAN	231.16	46	7	7	RPS7
P49368-2 TCPG_HUMAN	226.37	24	9	9	CCT3
P49368 TCPG_HUMAN	226.37	23	9	9	CCT3
Q01844-6 EWS_HUMAN	224.99	17	7	7	EWSR1
Q01844-3 EWS_HUMAN	224.99	16	7	7	EWSR1
Q01844 EWS_HUMAN	224.99	16	7	7	EWSR1

Q01844-5 EWS_HUMAN	224.99	16	7	7 EWSR1
Q02878 RL6_HUMAN	224.6	31	8	8 RPL6
P29401 TKT_HUMAN	224.5	16	8	8 TKT
P29401-2 TKT_HUMAN	224.5	16	8	8 TKT
P31942 HNRH3_HUMAN	223.77	31	9	5 HNRNPH3
P62280 RS11_HUMAN	222.63	60	11	11 RPS11
P61978-3 HNRPK_HUMAN	218.32	31	10	10 HNRNPK
P61978 HNRPK_HUMAN	218.32	30	10	10 HNRNPK
P61978-2 HNRPK_HUMAN	218.32	30	10	10 HNRNPK
Q92804-2 RBP56_HUMAN	217.8	17	10	7 TAF15
Q92804 RBP56_HUMAN	217.8	17	10	7 TAF15
P62701 RS4X_HUMAN	217.69	45	11	6 RPS4X
P63244 GBLP_HUMAN	217.48	39	8	8 GNB2L1
P22102 PUR2_HUMAN	214.49	15	9	9 GART
O14979 HNRDL_HUMAN	214.44	15	8	5 HNRNPDL
P07477 TRY1_HUMAN	214.18	17	6	1 PRSS1
P52597 HNRPF_HUMAN	214.05	20	6	4 HNRNPF
P62424 RL7A_HUMAN	212.86	41	11	11 RPL7A
P27635 RL10_HUMAN	211.33	41	8	4 RPL10
P42766 RL35_HUMAN	209.85	41	9	8 RPL35
Q07020 RL18_HUMAN	208.21	38	7	7 RPL18
P10412 H14_HUMAN	207.49	27	8	1 HIST1H1E
P49411 EFTU_HUMAN	204.97	18	6	6 TUFM
P40227 TCPZ_HUMAN	204.94	26	10	10 CCT6A
P46776 RL27A_HUMAN	204.85	32	6	6 RPL27A
P60866 RS20_HUMAN	203.18	38	6	6 RPS20
P60866-2 RS20_HUMAN	203.18	32	6	6 RPS20
P18124 RL7_HUMAN	203.06	38	8	8 RPL7
P16403 H12_HUMAN	202.8	25	7	1 HIST1H1C
P62917 RL8_HUMAN	202.66	37	10	10 RPL8
P17844 DDX5_HUMAN	202.46	13	7	5 DDX5
P50454 SERPH_HUMAN	202.21	22	7	7 SERPINH1
P84098 RL19_HUMAN	196.61	42	10	9 RPL19
P62910 RL32_HUMAN	193.11	49	7	7 RPL32
Q00839 HNRPU_HUMAN	192.98	12	7	7 HNRNPU
P62249 RS16_HUMAN	190.9	54	10	10 RPS16
P12236 ADT3_HUMAN	190.83	20	7	1 SLC25A6
Q99880 H2B1L_HUMAN	189.88	36	5	2 HIST1H2BL
P08865 RSSA_HUMAN	189.75	36	7	7 RPSA
P50991-2 TCPD_HUMAN	188.79	17	6	6 CCT4
P50991 TCPD_HUMAN	188.79	16	6	6 CCT4
Q16777 H2A2C_HUMAN	188.47	58	8	2 HIST2H2AC
Q6F113 H2A2A_HUMAN	188.47	58	8	2 HIST2H2AA3
P62263 RS14_HUMAN	188.05	34	6	6 RPS14
P39023 RL3_HUMAN	187.79	31	10	10 RPL3
P46781 RS9_HUMAN	187.72	41	12	12 RPS9
Q9H3K6 BOLA2_HUMAN	187.11	53	4	4 BOLA2
Q8WUM4 PDC6I_HUMAN	186.47	12	9	9 PDCD6IP
P33993 MCM7_HUMAN	186.35	14	7	7 MCM7
P13647 K2C5_HUMAN	184.84	16	9	4 KRT5
P05141 ADT2_HUMAN	184.84	23	8	2 SLC25A5
P15880 RS2_HUMAN	183.77	33	8	8 RPS2
Q7L7L0 H2A3_HUMAN	182.09	58	8	2 HIST3H2A
P04908 H2A1B_HUMAN	182.09	58	8	2 HIST1H2AB
Q93077 H2A1C_HUMAN	182.09	58	8	2 HIST1H2AC
Q8N257 H2B3B_HUMAN	179.74	39	4	1 HIST3H2BB
Q92841-1 DDX17_HUMAN	178.95	8	5	3 DDX17
Q92841-2 DDX17_HUMAN	178.95	8	5	3 DDX17
Q92841-3 DDX17_HUMAN	178.95	8	5	3 DDX17
Q92841 DDX17_HUMAN	178.95	7	5	3 DDX17
P18621 RL17_HUMAN	178.84	39	7	7 RPL17
P18621-3 RL17_HUMAN	178.84	32	7	7 RPL17
P00338 LDHA_HUMAN	178.14	22	6	5 LDHA
P30050 RL12_HUMAN	174.66	43	5	5 RPL12
Q14103-4 HNRPD_HUMAN	174.18	20	6	3 HNRNPD
Q14103-3 HNRPD_HUMAN	174.18	18	6	3 HNRNPD
Q14103-2 HNRPD_HUMAN	174.18	17	6	3 HNRNPD
Q14103 HNRPD_HUMAN	174.18	16	6	3 HNRNPD
P10809 CH60_HUMAN	169.58	29	10	10 HSPD1
P83731 RL24_HUMAN	169.1	26	7	7 RPL24
P05388 RLA0_HUMAN	168.68	20	5	1 RPLP0

Q92734-2 TFG_HUMAN	167.32	25	5	5 TFG
Q92734 TFG_HUMAN	167.32	24	5	5 TFG
P60660-2 MYL6_HUMAN	165.28	48	6	4 MYL6
P60660 MYL6_HUMAN	165.28	48	6	4 MYL6
P43358 MAGA4_HUMAN	164.7	13	4	3 MAGEA4
P62750 RL23A_HUMAN	164.41	37	5	5 RPL23A
P46778 RL21_HUMAN	163.13	31	4	4 RPL21
Q71D13 H32_HUMAN	162.92	35	8	2 HIST2H3A
P84243 H33_HUMAN	162.92	35	8	2 H3F3A
P68431 H31_HUMAN	162.92	35	8	2 HIST1H3A
Q16695 H31T_HUMAN	162.92	35	8	2 HIST3H3
P78371 TCPB_HUMAN	162.44	15	5	5 CCT2
P45880-2 VDAC2_HUMAN	161.55	19	5	5 VDAC2
P38919 IF4A3_HUMAN	160.78	7	2	1 EIF4A3
Q99729-3 ROAA_HUMAN	160.34	19	5	1 HNRNPAB
Q99729-2 ROAA_HUMAN	160.34	17	5	1 HNRNPAB
P62266 RS23_HUMAN	157.04	43	6	6 RPS23
P41250 SYG_HUMAN	156.34	8	5	5 GARS
P01834 IGKC_HUMAN	155.81	55	4	4 IGKC
P22695 QCR2_HUMAN	155.61	18	5	5 UQCRC2
P19474 ROS2_HUMAN	155.21	18	6	6 TRIM21
P61254 RL26_HUMAN	152.96	39	6	6 RPL26
Q00610-2 CLH1_HUMAN	149.34	6	6	4 CLTC
Q00610 CLH1_HUMAN	149.34	5	6	4 CLTC
P62913-2 RL11_HUMAN	148.73	23	4	4 RPL11
P62913 RL11_HUMAN	148.73	23	4	4 RPL11
P26641 EF1G_HUMAN	148.46	14	5	5 EEF1G
P19338 NUCL_HUMAN	147.12	8	5	5 NCL
Q15084-3 PDIA6_HUMAN	146.01	9	3	3 PDIA6
Q15084 PDIA6_HUMAN	146.01	9	3	3 PDIA6
Q15084-2 PDIA6_HUMAN	146.01	8	3	3 PDIA6
Q99623-2 PHB2_HUMAN	145.54	20	4	4 PHB2
Q99623 PHB2_HUMAN	145.54	17	4	4 PHB2
P69905 HBA_HUMAN	145.47	28	3	3 HBA1
P46779 RL28_HUMAN	142.84	27	5	5 RPL28
B9A064 IGLL5_HUMAN	142.61	21	4	1 IGLL5
P02533 K1C14_HUMAN	140.93	13	7	1 KRT14
Q86X55-1 CARM1_HUMAN	140.08	8	3	3 CARM1
Q86X55 CARM1_HUMAN	140.08	7	3	3 CARM1
Q01813-2 K6PP_HUMAN	135.7	10	6	5 PFKP
P61353 RL27_HUMAN	135.65	38	4	4 RPL27
P26640 SYVC_HUMAN	135.49	3	3	3 VARS
Q9Y285 SYFA_HUMAN	134.39	5	2	2 FARSA
Q14152 EIF3A_HUMAN	133.74	4	4	4 EIF3A
P50914 RL14_HUMAN	133.68	11	2	2 RPL14
Q9Y3U8 RL36_HUMAN	131.74	22	3	3 RPL36
P62847-4 RS24_HUMAN	130.28	13	4	4 RPS24
P35268 RL22_HUMAN	128.77	26	4	4 RPL22
P41091 IF2G_HUMAN	128.43	17	5	1 EIF2S3
Q02543 RL18A_HUMAN	127.67	28	4	4 RPL18A
P14625 ENPL_HUMAN	127.46	6	3	2 HSP90B1
P62244 RS15A_HUMAN	127.29	22	3	3 RPS15A
P40429 RL13A_HUMAN	127.16	17	4	4 RPL13A
Q9BUJ2-4 HNRL1_HUMAN	125.92	6	3	3 HNRNPUL1
Q9BUJ2-2 HNRL1_HUMAN	125.92	6	3	3 HNRNPUL1
Q9BUJ2 HNRL1_HUMAN	125.92	6	3	3 HNRNPUL1
P06310 KV206_HUMAN	125.87	17	3	2 4 SV
P51991-2 ROA3_HUMAN	125	24	6	6 HNRNPA3
P51991 ROA3_HUMAN	125	22	6	6 HNRNPA3
P62888 RL30_HUMAN	123.59	24	2	2 RPL30
P47914 RL29_HUMAN	123.09	14	2	2 RPL29
P62854 RS26_HUMAN	120.62	44	4	4 RPS26
P01871 IGHM_HUMAN	120.28	10	4	1 IGHM
P50990-3 TCPQ_HUMAN	119.74	9	3	3 CCT8
P50990-2 TCPQ_HUMAN	119.74	8	3	3 CCT8
P50990 TCPQ_HUMAN	119.74	8	3	3 CCT8
P16401 H15_HUMAN	117.87	15	3	3 HIST1H1B
P22061 PIMT_HUMAN	117.57	19	2	2 PCMT1
P22061-2 PIMT_HUMAN	117.57	19	2	2 PCMT1
Q92945 FUBP2_HUMAN	115.59	9	4	4 KHSRP
P21796 VDAC1_HUMAN	113.96	11	3	2 VDAC1
P62899-3 RL31_HUMAN	112.86	26	4	4 RPL31

P62899	RL31_HUMAN	112.86	26	4	4	RPL31
P62899-2	RL31_HUMAN	112.86	25	4	4	RPL31
P62937	PPIA_HUMAN	112.85	16	2	2	PPIA
O95433	AH5A1_HUMAN	112.3	12	3	3	AH5A1
P28799-2	GRN_HUMAN	112.06	11	3	3	GRN
P28799	GRN_HUMAN	112.06	8	3	3	GRN
P83881	RL36A_HUMAN	111.73	30	4	4	RPL36A
O98900	RL36L_HUMAN	111.73	30	4	4	RPL36AL
P08493	CDK1_HUMAN	110.41	10	3	3	CDK1
Q86JA1	PRP39_HUMAN	109.85	6	3	3	PRPF39
O00159-2	MYO1C_HUMAN	109.36	6	4	4	MYO1C
O00159-3	MYO1C_HUMAN	109.36	6	4	4	MYO1C
O00159	MYO1C_HUMAN	109.36	6	4	4	MYO1C
P36542-2	ATP5C_HUMAN	108.31	12	3	3	ATP5C1
P36542	ATP5C_HUMAN	108.31	12	3	3	ATP5C1
Q14498-3	RBM39_HUMAN	108.31	8	3	3	RBM39
Q14498-2	RBM39_HUMAN	108.31	8	3	3	RBM39
Q14498	RBM39_HUMAN	108.31	8	3	3	RBM39
Q14204	DYH1_HUMAN	107.31	1	2	2	DYNCH1
P46777	RL5_HUMAN	106.05	13	3	3	RPL5
P46783	RS10_HUMAN	102.87	20	3	3	RPS10
P62277	RS13_HUMAN	102.13	27	3	3	RPS13
P09104	ENO2_HUMAN	101.26	6	2	1	ENO2
P05387	RLA2_HUMAN	100.88	41	3	3	RPLP2
P53992	SEC24C_HUMAN	100.15	3	2	2	SEC24C
P26599	PTBP1_HUMAN	99.55	4	1	1	PTBP1
P26599-2	PTBP1_HUMAN	99.55	4	1	1	PTBP1
P26599-3	PTBP1_HUMAN	99.55	4	1	1	PTBP1
P07195	LDHB_HUMAN	99.47	8	2	1	LDHB
P02786	TFR1_HUMAN	98.57	4	2	2	TFR1
P35030-5	TRY3_HUMAN	97.87	17	3	3	PR553
P35030-3	TRY3_HUMAN	97.87	16	3	3	PR553
P35030-2	TRY3_HUMAN	97.87	15	3	3	PR553
P35030-4	TRY3_HUMAN	97.87	15	3	3	PR553
P35030	TRY3_HUMAN	97.87	13	3	3	PR553
Q00325-2	MPCP_HUMAN	96.97	11	2	2	SLC25A3
P01857	IGHG1_HUMAN	96.12	9	3	1	IGHG1
P02100	HBBE_HUMAN	95.8	20	2	1	HBBE1
P62851	RS25_HUMAN	94.82	15	2	2	RPS25
P61019-2	RAB2A_HUMAN	94.28	22	3	1	RAB2A
P61019	RAB2A_HUMAN	94.28	20	3	1	RAB2A
P68871	HBB_HUMAN	93.46	16	2	1	HBB
O14980	XPO1_HUMAN	92.93	3	2	2	XPO1
Q13347	EIF3I_HUMAN	89.08	7	2	2	EIF3I
Q99832-3	TCPH_HUMAN	88.25	5	1	1	CCT7
Q99832	TCPH_HUMAN	88.25	5	1	1	CCT7
P04843	RPN1_HUMAN	87.86	5	2	2	RPN1
P08708	RS17_HUMAN	87.63	33	3	3	RPS17
P0CW22	RS17L_HUMAN	87.63	33	3	3	RPS17L
P46782	RS5_HUMAN	87	7	1	1	RPS5
Q9H984	SFXN1_HUMAN	86.36	4	1	1	SFXN1
P02545	LMNA_HUMAN	85.66	6	3	3	LMNA
Q9Y277	VDAC3_HUMAN	85.32	7	2	1	VDAC3
Q9Y277-2	VDAC3_HUMAN	85.32	7	2	1	VDAC3
P62906	RL10A_HUMAN	85.01	13	2	2	RPL10A
Q14737	PDCD5_HUMAN	82.16	10	1	1	PDCD5
P51149	RAB7A_HUMAN	81.3	11	2	2	RAB7A
P11216	PYGB_HUMAN	80.94	4	2	2	PYGB
P48643-2	TCPE_HUMAN	80.81	5	2	2	CCT5
P48643	TCPE_HUMAN	80.81	4	2	2	CCT5
P35232	PHB_HUMAN	80.37	4	1	1	PHB
O00148	DK39A_HUMAN	80.33	5	2	1	DDK39A
Q15149-7	PLEC_HUMAN	79.25	1	2	2	PLEC
Q15149-8	PLEC_HUMAN	79.25	1	2	2	PLEC
Q15149-9	PLEC_HUMAN	79.25	1	2	2	PLEC
Q15149-5	PLEC_HUMAN	79.25	1	2	2	PLEC
Q15149-4	PLEC_HUMAN	79.25	1	2	2	PLEC
Q15149-6	PLEC_HUMAN	79.25	1	2	2	PLEC
Q15149-3	PLEC_HUMAN	79.25	1	2	2	PLEC
Q15149-2	PLEC_HUMAN	79.25	1	2	2	PLEC
Q15149	PLEC_HUMAN	79.25	1	2	2	PLEC
P27816-6	MAP4_HUMAN	79.06	4	2	1	MAP4

Q9Y678 COPG1_HUMAN	78.58	3	2	1 COPG1
P62857 RS28_HUMAN	78.54	17	1	1 RPS28
P78527-2 PRKDC_HUMAN	78.12	1	2	2 PRKDC
P78527 PRKDC_HUMAN	78.12	1	2	2 PRKDC
P23588 IF4B_HUMAN	78.11	3	1	1 EIF4B
P07737 PROF1_HUMAN	77.05	13	1	1 PFN1
Q9NR30 DDX21_HUMAN	77.02	4	2	2 DDX21
Q15645 PCH2_HUMAN	76.8	6	2	2 TRIP13
P46940 IQGA1_HUMAN	76.71	1	2	2 IQGAP1
P52272-2 HNRPM_HUMAN	76.48	2	1	1 HNRNPM
P52272 HNRPM_HUMAN	76.48	2	1	1 HNRNPM
P07910-3 HNRPC_HUMAN	75.59	9	2	2 HNRNPC
P07910-4 HNRPC_HUMAN	75.59	8	2	2 HNRNPC
P07910-2 HNRPC_HUMAN	75.59	7	2	2 HNRNPC
P07910 HNRPC_HUMAN	75.59	7	2	2 HNRNPC
P61313 RL15_HUMAN	75.43	4	1	1 RPL15
P63220 RS21_HUMAN	74.88	33	2	2 RPS21
P29692-2 EF1D_HUMAN	73.15	2	1	1 EEF1D
P52292 IMA1_HUMAN	72.38	5	2	2 KPNA2
P04844-2 RPN2_HUMAN	71.4	7	2	2 RPN2
P04844 RPN2_HUMAN	71.4	6	2	2 RPN2
Q8TF72 SHRM3_HUMAN	71.19	1	2	2 SHROOM3
Q03468 ERCC6_HUMAN	70.63	1	1	1 ERCC6
P02775 CXCL7_HUMAN	70.26	20	2	2 PPBP
P37108 SRP14_HUMAN	69.81	10	1	1 SRP14
Q04637-3 IF4G1_HUMAN	69.14	1	1	1 EIF4G1
Q16555 DPYL2_HUMAN	67.7	5	1	1 DPYSL2
Q14444-2 CAPR1_HUMAN	67.5	2	1	1 CAPRIN1
Q14444 CAPR1_HUMAN	67.5	2	1	1 CAPRIN1
Q5TZA2 CROCC_HUMAN	67.22	0	1	1 CROCC
P62861 RS30_HUMAN	66.92	17	1	1 FAU
Q96JB1-2 DYH8_HUMAN	65.43	0	1	1 DNAH8
Q96JB1 DYH8_HUMAN	65.43	0	1	1 DNAH8
P62158 CALM_HUMAN	63.8	11	1	1 CALM1
P49207 RL34_HUMAN	63.46	7	1	1 RPL34
P00403 COX2_HUMAN	61.45	7	1	1 MT-CO2
P40763 STAT3_HUMAN	60.79	3	1	1 STAT3
P18077 RL35A_HUMAN	60.36	16	2	2 RPL35A
P05386 RLA1_HUMAN	60.1	14	1	1 RPLP1
Q9NZM1-3 MYOF_HUMAN	58.98	1	1	1 MYOF
Q9NZM1 MYOF_HUMAN	58.98	1	1	1 MYOF
P14866-2 HNRPL_HUMAN	57.2	3	1	1 HNRNPL
P14866 HNRPL_HUMAN	57.2	2	1	1 HNRNPL
A7E2Y1 MYH7B_HUMAN	56.6	1	1	1 MYH7B
Q13283 G3BP1_HUMAN	56.09	2	1	1 G3BP1
Q9Y295 DRG1_HUMAN	55.99	3	1	1 DRG1
Q92614-4 MY18A_HUMAN	55.88	1	1	1 MYO18A
Q92614 MY18A_HUMAN	55.88	1	1	1 MYO18A
P63173 RL38_HUMAN	55.74	19	1	1 RPL38
Q9Y383-3 LC7L2_HUMAN	53.84	2	1	1 LUC7L2
P23381-2 SYWC_HUMAN	52.9	4	1	1 WARS
P23381 SYWC_HUMAN	52.9	3	1	1 WARS
P61604 CH10_HUMAN	52.3	14	1	1 HSPE1
P06748-3 NPM_HUMAN	51.9	8	1	1 NPM1
P06748-2 NPM_HUMAN	51.9	8	1	1 NPM1
P06748 NPM_HUMAN	51.9	7	1	1 NPM1
Q8TD84-2 DSCL1_HUMAN	49.56	0	1	1 DSCAML1
Q8TD84 DSCL1_HUMAN	49.56	0	1	1 DSCAML1
P06744 G6PI_HUMAN	48.43	3	1	1 GPI
P06744-2 G6PI_HUMAN	48.43	3	1	1 GPI
Q9H223 EHD4_HUMAN	48.32	2	1	1 EHD4
Q9UPQ9-1 TNR6B_HUMAN	48.2	1	1	1 TNRC6B
Q9UPQ9 TNR6B_HUMAN	48.2	1	1	1 TNRC6B
P62273 RS29_HUMAN	47.07	14	1	1 RPS29
P18085 ARF4_HUMAN	47	6	1	1 ARF4
P61020-2 RAB5B_HUMAN	46.66	6	1	1 RAB5B
P20339 RAB5A_HUMAN	46.66	5	1	1 RAB5A
P61020 RAB5B_HUMAN	46.66	5	1	1 RAB5B
P51148 RAB5C_HUMAN	46.66	5	1	1 RAB5C
P51148-2 RAB5C_HUMAN	46.66	4	1	1 RAB5C

P22392 NDKB_HUMAN	46.65	8	1	1 NME2
P15531 NDKA_HUMAN	46.65	8	1	1 NME1
P15531-2 NDKA_HUMAN	46.65	7	1	1 NME1
P22392-2 NDKB_HUMAN	46.65	4	1	1 NME2
O94850 DEND_HUMAN	46.56	2	1	1 DDN
P04080 CYTB_HUMAN	46.22	12	1	1 CSTB
P61204 ARF3_HUMAN	46.14	6	1	1 ARF3
Q5T1M5-2 FKB15_HUMAN	45.99	1	1	1 FKBP15
Q5T1M5 FKB15_HUMAN	45.99	1	1	1 FKBP15
Q13243-3 SRSF5_HUMAN	44.5	3	1	1 SRSF5
Q13243 SRSF5_HUMAN	44.5	3	1	1 SRSF5
Q13247-3 SRSF6_HUMAN	44.5	3	1	1 SRSF6
Q13247 SRSF6_HUMAN	44.5	3	1	1 SRSF6
Q08170 SRSF4_HUMAN	44.5	2	1	1 SRSF4
Q13936-28 CAC1C_HUMAN	43.78	0	1	1 CACNA1C
Q13936-18 CAC1C_HUMAN	43.78	0	1	1 CACNA1C
Q9H2V7-3 SPNS1_HUMAN	43.26	2	1	1 SPNS1
Q9H2V7-4 SPNS1_HUMAN	43.26	2	1	1 SPNS1
Q9H2V7-2 SPNS1_HUMAN	43.26	2	1	1 SPNS1
Q9H2V7 SPNS1_HUMAN	43.26	2	1	1 SPNS1
Q66PJ3-7 AR6P4_HUMAN	41.56	7	1	1 ARL6IP4
Q66PJ3-5 AR6P4_HUMAN	41.56	5	1	1 ARL6IP4
Q66PJ3-4 AR6P4_HUMAN	41.56	4	1	1 ARL6IP4
Q66PJ3-3 AR6P4_HUMAN	41.56	4	1	1 ARL6IP4
Q66PJ3-2 AR6P4_HUMAN	41.56	4	1	1 ARL6IP4
Q66PJ3 AR6P4_HUMAN	41.56	4	1	1 ARL6IP4
Q9P1Y5 CAMP3_HUMAN	41.28	1	1	1 CAMSAP3
Q9P1Y5-2 CAMP3_HUMAN	41.28	1	1	1 CAMSAP3
Q96PE2 ARHGH_HUMAN	41.21	1	1	1 ARHGEF17
Q9GZV4 IF5A2_HUMAN	40.35	8	1	1 EIF5A2
Q6IS14 IF5A1_HUMAN	40.35	8	1	1 EIF5A1
P63241 IF5A1_HUMAN	40.35	8	1	1 EIF5A
P63241-2 IF5A1_HUMAN	40.35	7	1	1 EIF5A
Q9Y4F4 F179B_HUMAN	40.12	0	1	1 FAM179B
P02462-2 CO4A1_HUMAN	39.65	2	1	1 COL4A1
P02462 CO4A1_HUMAN	39.65	1	1	1 COL4A1
Q16678 CP1B1_HUMAN	38.75	2	1	1 CYP1B1
Q49A26-5 GLYR1_HUMAN	38.49	2	1	1 GLYR1
Q6ZNG0-2 ZN620_HUMAN	37.75	6	1	1 ZNF620
P61224-2 RAP1B_HUMAN	36.87	10	1	1 RAP1B
P61224-4 RAP1B_HUMAN	36.87	10	1	1 RAP1B
P61224-3 RAP1B_HUMAN	36.87	8	1	1 RAP1B
P61224 RAP1B_HUMAN	36.87	8	1	1 RAP1B
A6NIZ1 RP1BL_HUMAN	36.87	8	1	1 2 SV
P62834 RAP1A_HUMAN	36.87	8	1	1 RAP1A
P01605 KV113_HUMAN	36.36	8	1	1 1 SV
P01624 KV306_HUMAN	36.36	8	1	1 1 SV
P04434 KV310_HUMAN	36.36	8	1	1 4 SV
P04207 KV308_HUMAN	36.36	7	1	1 1 SV
P00558 PGK1_HUMAN	36.34	4	1	1 PGK1
Q9HCE9-2 ANO8_HUMAN	35.22	1	1	1 ANO8
P02765 FETUA_HUMAN	34.68	2	1	1 AHSF
Q96AE4 FUBP1_HUMAN	34.28	2	1	1 FUBP1
Q96AE4-2 FUBP1_HUMAN	34.28	2	1	1 FUBP1
P06276 CHLE_HUMAN	34.06	2	1	1 BCHE
Q16825 PTN21_HUMAN	33.99	1	1	1 PTPN21
Q6UXD5-5 SE6L2_HUMAN	33.97	2	1	1 SEZ6L2
Q96JF0 SIAT2_HUMAN	33.83	3	1	1 ST6GAL2
Q9Y2G0-2 EFR3B_HUMAN	33.6	4	1	1 EFR3B
Q9HAK2 COE2_HUMAN	33.41	2	1	1 EBF2
P63104-2 1433Z_HUMAN	32.93	7	1	1 YWHAZ
P63104 1433Z_HUMAN	32.93	5	1	1 YWHAZ
Q9BQL6 FERM1_HUMAN	32.83	2	1	1 FERMT1
P98196 AT11A_HUMAN	32.31	1	1	1 ATP11A
Q9UPV9 TRAK1_HUMAN	32.24	2	1	1 TRAK1
Q9HAU0-6 PKHA5_HUMAN	31.93	0	1	1 PLEKHA5

Appendix 3: Table 2: PEAKS analysis of data generated by mass spectrometry analysis of RACK1 IP and Control IP. This table lists proteins present in the RACK1 IP

Accession	-10lgP	Coverage	#Peptides	#Unique	Gene Name
P38646 GRP75_HUMAN		504.51	73	62	62 HSPA9
P14618 KPYP_HUMAN		489.07	76	42	4 PKM
P63244 GBLP_HUMAN		483.73	95	38	38 GNB2L1
P14618-2 KPYP_HUMAN		479.36	71	39	1 PKM
P35579 MYH9_HUMAN		474.62	48	94	79 MYH9
P08670 VIME_HUMAN		472.91	88	56	47 VIM
P60709 ACTB_HUMAN		458.8	79	32	1 ACTB
P11021 GRP78_HUMAN		454.62	60	48	45 HSPA5
P04264 K2C1_HUMAN		452.49	52	43	38 KRT1
P11142 HSP7C_HUMAN		447.71	59	42	28 HSPA8
P63261 ACTG_HUMAN		447.33	79	32	1 ACTG1
P07437 TBB5_HUMAN		442.28	74	30	5 TUBB
P68371 TBB4B_HUMAN		433.88	70	28	1 TUBB4B
P68104 EF1A1_HUMAN		425.62	64	32	20 EEF1A1
Q98QE3 TBA1C_HUMAN		413.4	77	27	2 TUBA1C
P06733 ENOA_HUMAN		408.17	69	24	22 ENO1
Q13885 TBB2A_HUMAN		402.16	56	23	2 TUBB2A
P25705 ATPA_HUMAN		398.71	45	22	3 ATP5A1
Q13509 TBB3_HUMAN		391	50	23	6 TUBB3
P04350 TBB4A_HUMAN		388.1	66	25	1 TUBB4A
P08107 HSP71_HUMAN		382.43	53	33	19 HSPA1A
P05787 K2C8_HUMAN		377.32	61	35	27 KRT8
P05787-2 K2C8_HUMAN		377.32	58	35	27 KRT8
P13645 K1C10_HUMAN		374.19	61	41	33 KRT10
P08238 H590B_HUMAN		358.01	46	33	16 HSP90AB1
P35527 K1C9_HUMAN		357.24	49	26	25 KRT9
P35908 K22E_HUMAN		356.64	54	34	24 KRT2
P05783 K1C18_HUMAN		351.15	59	27	24 KRT18
P39023 RL3_HUMAN		350.3	46	19	19 RPL3
Q00839 HNRPU_HUMAN		349.99	40	22	21 HNRNPU
P04792 HSPB1_HUMAN		346.58	91	18	18 HSPB1
P31943 HNRH1_HUMAN		337.88	42	17	7 HNRNPH1
P36578 RL4_HUMAN		337.02	41	17	17 RPL4
P68133 ACTS_HUMAN		335.76	34	18	1 ACTA1
P04406 G3P_HUMAN		333.15	53	14	14 GAPDH
P17987 TCPA_HUMAN		331.99	44	21	21 TCP1
P60842 IF4A1_HUMAN		329.83	43	19	11 EIF4A1
P54652 HSP72_HUMAN		327.82	29	17	4 HSPA2
P49368 TCPG_HUMAN		327.06	47	24	24 CCT3
P13639 EF2_HUMAN		322.93	32	23	23 EEF2
P10809 CH60_HUMAN		320.65	41	19	19 HSPD1
P29401 TKT_HUMAN		320.21	38	15	15 TKT
P29401-2 TKT_HUMAN		320.21	38	15	15 TKT
Q00341 VIGLN_HUMAN		319.43	25	24	24 HDLBP
P06576 ATPB_HUMAN		319.26	44	17	17 ATP5B
Q92734-2 TFG_HUMAN		318.25	39	12	11 TFG
Q92734 TFG_HUMAN		318.25	39	12	11 TFG
P35637-2 FUS_HUMAN		315.64	19	13	10 FUS
P35637 FUS_HUMAN		315.64	19	13	10 FUS
P22626 ROA2_HUMAN		312.39	50	16	14 HNRNPA2B1
Q09666 AHNK_HUMAN		311.41	10	41	41 AHNAK
O00159-2 MYO1C_HUMAN		308.81	24	19	19 MYO1C
O00159-3 MYO1C_HUMAN		308.81	24	19	19 MYO1C
O00159 MYO1C_HUMAN		308.81	23	19	19 MYO1C
P02533 K1C14_HUMAN		308.06	50	23	6 KRT14
Q32CM7 TBB8_HUMAN		306.87	29	12	1 TUBB8
P62241 RS8_HUMAN		303	60	18	18 RPS8
P62701 RS4X_HUMAN		300.74	58	15	9 RPS4X
P31942 HNRH3_HUMAN		298.45	40	10	8 HNRNPH3
P02538 K2C6A_HUMAN		298.01	43	21	1 KRT6A
P62826 RAN_HUMAN		297.96	56	15	15 RAN
P23396 RS3_HUMAN		295.19	77	24	24 RPS3
P08779 K1C16_HUMAN		295.14	46	20	7 KRT16
P61247 RS3A_HUMAN		293.11	52	15	15 RPS3A
P27635 RL10_HUMAN		292.21	51	16	9 RPL10
P07900-2 H590A_HUMAN		291.56	26	20	7 HSP90AA1
Q9BUE5 TBB6_HUMAN		291.36	33	14	3 TUBB6
O00571-2 DDX3X_HUMAN		289.9	27	15	4 DDX3X
O00571 DDX3X_HUMAN		289.9	26	15	4 DDX3X

P04259 K2C6B_HUMAN	288.34	42	21	1 KRT6B
P55795 HNRH2_HUMAN	287.22	27	11	2 HNRNPH2
Q01844-6 EWS_HUMAN	283.93	24	10	9 EWSR1
Q01844-3 EWS_HUMAN	283.93	22	10	9 EWSR1
Q01844 EWS_HUMAN	283.93	22	10	9 EWSR1
Q01844-5 EWS_HUMAN	283.93	22	10	9 EWSR1
Q06830 PRDX1_HUMAN	282.33	63	16	15 PRDX1
P33993 MCM7_HUMAN	280.64	30	15	15 MCM7
P15880 RS2_HUMAN	280.41	52	15	14 RPS2
P17844 DDX5_HUMAN	276.24	30	16	9 DDX5
P26373 RL13_HUMAN	274.99	55	17	17 RPL13
P62753 RS6_HUMAN	274.58	31	10	10 RPS6
P78527 PRKDC_HUMAN	274.12	8	24	24 PRKDC
P40227 TCPZ_HUMAN	273.71	29	12	10 CCT6A
P32969 RL9_HUMAN	273.31	52	8	8 RPL9
P18124 RL7_HUMAN	272.34	41	13	13 RPL7
P22102 PUR2_HUMAN	272.04	20	12	12 GART
P62805 H4_HUMAN	269.63	61	12	12 HIST1H4A
A5A3E0 POTEF_HUMAN	269.48	8	9	1 POTEF
Q92841-1 DDX17_HUMA	269.4	29	16	9 DDX17
Q92841-3 DDX17_HUMA	269.4	29	16	9 DDX17
Q92841 DDX17_HUMAN	269.4	26	16	9 DDX17
P62081 RS7_HUMAN	269.14	60	11	11 RPS7
P19474 ROS2_HUMAN	268.75	31	12	12 TRIM21
Q02878 RL6_HUMAN	268.1	33	10	10 RPL6
Q71RC2-3 LARP4_HUMA	265.91	27	14	14 LARP4
Q71RC2 LARP4_HUMAN	265.91	27	14	14 LARP4
Q71RC2-4 LARP4_HUMA	265.91	27	14	14 LARP4
P62424 RL7A_HUMAN	264.34	45	14	14 RPL7A
P50991 TCPD_HUMAN	263.3	24	9	9 CCT4
P62917 RL8_HUMAN	261.52	47	10	10 RPL8
P18621-3 RL17_HUMAN	261.22	40	12	12 RPL17
P46776 RL27A_HUMAN	260.28	33	8	8 RPL27A
P34931 HS71L_HUMAN	259.78	21	11	1 HSPA1L
P22695 QCR2_HUMAN	259.59	26	8	8 UQCRC2
Q05639 EF1A2_HUMAN	258.79	30	13	1 EE1A2
P23528 COF1_HUMAN	258.61	67	9	6 CFL1
P13647 K2C5_HUMAN	257.74	26	15	6 KRT5
Q14764 MVP_HUMAN	256.73	26	17	17 MVP
P62263 RS14_HUMAN	255.82	42	7	7 RPS14
P52597 HNRPF_HUMAN	254.56	30	9	7 HNRNPF
P62280 RS11_HUMAN	254.1	65	13	13 RPS11
P62910 RL32_HUMAN	250.65	50	9	9 RPL32
Q14152 EIF3A_HUMAN	249.46	15	17	17 EIF3A
P07355 ANXA2_HUMAN	249.25	37	10	1 ANXA2
P61978-3 HNRPK_HUMA	248.18	34	10	10 HNRNPK
P61978 HNRPK_HUMAN	248.18	32	10	10 HNRNPK
P61978-2 HNRPK_HUMA	248.18	32	10	10 HNRNPK
Q92804-2 RBP56_HUMA	247.31	22	11	8 TAF15
Q92804 RBP56_HUMAN	247.31	22	11	8 TAF15
P84077 ARF1_HUMAN	246.33	56	10	8 ARF1
P61204 ARF3_HUMAN	246.33	56	10	8 ARF3
P62750 RL23A_HUMAN	245.96	51	10	10 RPL23A
P50454 SERPH_HUMAN	245.16	28	8	8 SERPINH1
P62937 PPIA_HUMAN	244.26	56	8	8 PPIA
P08865 RSSA_HUMAN	244.04	41	8	8 RPSA
P62249 RS16_HUMAN	243.01	53	10	10 RPS16
Q9BUJ2-2 HNRL1_HUMA	242.09	20	12	11 HNRNPUL1
Q9BUJ2 HNRL1_HUMAN	242.09	18	12	11 HNRNPUL1
Q02543 RL18A_HUMAN	240.15	48	12	12 RPL18A
P02768 ALBU_HUMAN	239.34	24	16	16 ALB
P49411 EFTU_HUMAN	238.91	30	9	9 TUFM
P00338 LDHA_HUMAN	234.85	39	9	8 LDHA
O43242 PSMD3_HUMAN	234.15	25	11	11 PSMD3
P61254 RL26_HUMAN	234.13	52	11	11 RPL26
Q04695 K1C17_HUMAN	233.68	30	15	3 KRT17
P35580 MYH10_HUMAN	233.05	5	12	1 MYH10
P35580-2 MYH10_HUMA	233.05	5	12	1 MYH10
P35580-3 MYH10_HUMA	233.05	5	12	1 MYH10
P35580-4 MYH10_HUMA	233.05	5	12	1 MYH10
P22087 FBRL_HUMAN	231.47	29	8	7 FBL

P11586 C1TC_HUMAN	230.84	14	11	11 MTHFD1
Q8WUM4 PDC6I_HUMAN	229.95	17	13	13 PDCD6IP
P46778 RL21_HUMAN	229.65	46	10	9 RPL21
P41091 IF2G_HUMAN	224.02	30	10	10 EIF2S3
O15371 EIF3D_HUMAN	223.1	15	9	9 EIF3D
Q9NZM1-6 MYOF_HUMAN	222.48	9	11	8 MYOF
Q9NZM1-3 MYOF_HUMAN	222.48	9	11	8 MYOF
Q9NZM1 MYOF_HUMAN	222.48	8	11	8 MYOF
P05141 ADT2_HUMAN	222.23	34	10	3 SLC25A5
P48643 TCPE_HUMAN	222.05	25	11	3 CCT5
P09382 LEG1_HUMAN	221.96	53	6	6 LGALS1
P26641 EF1G_HUMAN	221.62	23	8	8 EF1G
P10412 H14_HUMAN	220.79	27	10	2 HIST1H1E
P46781 RS9_HUMAN	219.89	52	15	15 RPS9
P84098 RL19_HUMAN	219.59	27	8	8 RPL19
O14979-3 HNRDL_HUMAN	218.13	30	9	7 HNRNPDL
O14979-2 HNRDL_HUMAN	218.13	25	9	7 HNRNPDL
O14979 HNRDL_HUMAN	218.13	18	9	7 HNRNPDL
P17858 K6PL_HUMAN	217.95	15	8	5 PFKL
P41250 SYG_HUMAN	217.04	16	10	10 GARS
Q9NR30 DDX21_HUMAN	216.7	13	9	8 DDX21
Q00610-2 CLH1_HUMAN	216.48	7	7	5 CLTC
Q00610 CLH1_HUMAN	216.48	6	7	5 CLTC
P51991 ROA3_HUMAN	216.47	34	10	10 HNRNPA3
P12236 ADT3_HUMAN	215.03	34	10	3 SLC25A6
P16403 H12_HUMAN	214.86	25	8	1 HIST1H1C
P02545-3 LMNA_HUMAN	211.98	14	7	7 LMNA
P02545 LMNA_HUMAN	211.98	14	7	7 LMNA
Q13347 EIF3I_HUMAN	211.77	33	7	7 EIF3I
P60866 RS20_HUMAN	211.41	38	7	6 RPS20
P60866-2 RS20_HUMAN	211.41	32	7	6 RPS20
O95433 AHSA1_HUMAN	211.07	22	6	6 AHSA1
P46777 RL5_HUMAN	210.62	20	4	4 RPL5
Q99729-3 ROAA_HUMAN	210.38	29	8	7 HNRNPAB
Q99623 PHB2_HUMAN	209.93	29	8	8 PHB2
Q7L7L0 H2A3_HUMAN	207.51	58	10	2 HIST3H2A
P04908 H2A1B_HUMAN	207.51	58	10	2 HIST1H2AB
Q93077 H2A1C_HUMAN	207.51	58	10	2 HIST1H2AC
P62899-3 RL31_HUMAN	207.31	46	7	7 RPL31
P62899 RL31_HUMAN	207.31	45	7	7 RPL31
P62899-2 RL31_HUMAN	207.31	44	7	7 RPL31
Q12931 TRAP1_HUMAN	207.26	14	7	6 TRAP1
Q9H3K6 BOLA2_HUMAN	206.53	80	6	6 BOLA2
P35232 PHB_HUMAN	206.17	38	7	7 PHB
P43487 RANG_HUMAN	205.89	34	5	5 RANBP1
P60660-2 MYL6_HUMAN	205.77	53	6	3 MYL6
P60660 MYL6_HUMAN	205.77	53	6	3 MYL6
P02786 TFR1_HUMAN	205.41	13	8	8 TFR1
P11940-2 PABP1_HUMAN	203.61	18	8	1 PABPC1
P11940 PABP1_HUMAN	203.61	15	8	1 PABPC1
P45880-2 VDAC2_HUMAN	202.03	25	6	6 VDAC2
P28799 GRN_HUMAN	201.3	12	6	6 GRN
Q16777 H2A2C_HUMAN	201.17	58	8	2 HIST2H2AC
Q6F113 H2A2A_HUMAN	201.17	58	8	2 HIST2H2AA3
P38919 IF4A3_HUMAN	200.82	13	5	2 EIF4A3
P42766 RL35_HUMAN	200.45	42	8	8 RPL35
P61353 RL27_HUMAN	200.45	38	5	5 RPL27
Q07020 RL18_HUMAN	200.36	27	6	6 RPL18
P46779-3 RL28_HUMAN	200.18	46	11	11 RPL28
P46940 IQGA1_HUMAN	198.51	8	8	8 IQGAP1
P62266 RS23_HUMAN	196.6	49	6	6 RPS23
Q14204 DYHC1_HUMAN	196.46	3	8	8 DYNC1H1
Q86X55-1 CARM1_HUMAN	195.1	14	6	6 CARM1
Q86X55 CARM1_HUMAN	195.1	14	6	6 CARM1
P35268 RL22_HUMAN	194.89	46	5	5 RPL22
P53621 COPA_HUMAN	194.84	9	9	9 COPA
P78371 TCPB_HUMAN	191.16	17	6	6 CCT2
P52272-2 HNRPM_HUMAN	190.98	13	5	5 HNRNPM
P52272 HNRPM_HUMAN	190.98	12	5	5 HNRNPM
P04844 RPN2_HUMAN	190.95	13	5	5 RPN2
P07737 PROF1_HUMAN	190.51	39	5	5 PFN1

P61019-2 RAB2A_HUMAN	189.8	37	5	3 RAB2A
P61019 RAB2A_HUMAN	189.8	33	5	3 RAB2A
P61313 RL15_HUMAN	189.64	43	8	8 RPL15
P30050 RL12_HUMAN	188.78	43	5	5 RPL12
P22061 PIMT_HUMAN	188.66	30	5	5 PCMT1
P22061-2 PIMT_HUMAN	188.66	29	5	5 PCMT1
P09651-3 ROA1_HUMAN	188.13	26	5	1 HNRNPA1
P09651-2 ROA1_HUMAN	188.13	22	5	1 HNRNPA1
P09651 ROA1_HUMAN	188.13	19	5	1 HNRNPA1
P43490 NAMPT_HUMAN	186.63	22	7	7 NAMPT
Q99832-3 TCPH_HUMAN	184.84	11	5	5 CCT7
Q99832 TCPH_HUMAN	184.84	10	5	5 CCT7
P19338 NUCL_HUMAN	183.71	13	7	7 NCL
P26640 SYVC_HUMAN	183.5	10	9	9 VARS
Q969Q0 RL36L_HUMAN	183	55	9	9 RPL36AL
P62854 RS26_HUMAN	182.77	50	5	5 RPS26
P62277 RS13_HUMAN	181.66	34	6	6 RPS13
P62913 RL11_HUMAN	181.06	29	5	5 RPL11
P01834 IGKC_HUMAN	180.9	69	5	5 IGKC
P36542-2 ATPG_HUMAN	180.36	24	6	6 ATP5C1
P36542 ATPG_HUMAN	180.36	24	6	6 ATP5C1
P17661 DESM_HUMAN	179.48	10	6	1 DES
P62269 RS18_HUMAN	178.35	36	6	6 RPS18
P62847-4 RS24_HUMAN	175.91	13	4	4 RPS24
P52292 IMA1_HUMAN	174.57	16	6	6 KPNA2
P04080 CYTB_HUMAN	172.47	70	4	4 CSTB
B9A064 IGLL5_HUMAN	171.69	18	3	1 IGLL5
Q14568 HS902_HUMAN	171.08	19	6	1 HSP90AA2
P40429 RL13A_HUMAN	170.67	25	8	8 RPL13A
Q01085 TIAR_HUMAN	169.08	12	3	1 TIAL1
Q01085-2 TIAR_HUMAN	169.08	12	3	1 TIAL1
P06309 KV205_HUMAN	168.93	46	5	3 4 SV
Q5XKE5 K2C79_HUMAN	168.75	11	8	1 KRT79
P62244 RS15A_HUMAN	168.33	38	5	5 RPS15A
P42677 RS27_HUMAN	165.98	40	4	4 RPS27
Q92499 DDX1_HUMAN	165.77	9	4	4 DDX1
O75964 ATP5L_HUMAN	165.57	27	2	2 ATP5L
P62888 RL30_HUMAN	164.1	41	3	3 RPL30
P62979 RS27A_HUMAN	163.75	27	4	4 RPS27A
P47914 RL29_HUMAN	163.36	21	4	4 RPL29
Q00325-2 MPCP_HUMAN	163.13	19	6	6 SLC25A3
P08727 K1C19_HUMAN	162.81	14	7	1 KRT19
Q14103-4 HNRPD_HUMAN	162.44	9	3	1 HNRNPD
Q14103-2 HNRPD_HUMAN	162.44	7	3	1 HNRNPD
P83731 RL24_HUMAN	162.02	23	4	4 RPL24
P55884 EIF3B_HUMAN	161.79	7	5	5 EIF3B
P55884-2 EIF3B_HUMAN	161.79	6	5	5 EIF3B
P14625 ENPL_HUMAN	161.27	8	4	3 HSP90B1
P46783 RS10_HUMAN	161.12	31	5	5 RPS10
Q9Y3U8 RL36_HUMAN	161	34	5	5 RPL36
Q9Y520-6 PRC2C_HUMAN	160.94	5	8	8 PRRC2C
Q9Y520-4 PRC2C_HUMAN	160.94	5	8	8 PRRC2C
P08708 RS17_HUMAN	160.86	40	4	4 RPS17
P0CW22 RS17L_HUMAN	160.86	40	4	4 RPS17L
P35030-5 TRY3_HUMAN	160.8	34	5	5 PRSS3
P17812 PYRG1_HUMAN	160	9	5	5 CTPS1
P43358 MAGA4_HUMAN	159.72	12	3	3 MAGEA4
P04843 RPN1_HUMAN	158.39	13	5	5 RPN1
Q9Y6M1-1 IF2B2_HUMAN	157.98	16	6	1 IGF2BP2
Q9Y6M1 IF2B2_HUMAN	157.98	15	6	1 IGF2BP2
P06748-2 NPM_HUMAN	157.35	13	4	4 NPM1
P06748 NPM_HUMAN	157.35	12	4	4 NPM1
Q9UPQ9-1 TNR6B_HUMAN	156.44	4	5	5 TNRC6B
Q9UPQ9 TNR6B_HUMAN	156.44	4	5	5 TNRC6B
O75531 BAF_HUMAN	156.34	51	4	4 BANF1
Q9NZI8 IF2B1_HUMAN	154.85	12	5	4 IGF2BP1
Q14566 MCM6_HUMAN	154.05	6	4	4 MCM6
P05387 RLA2_HUMAN	152.41	63	5	5 RPLP2
Q9Y285 SYFA_HUMAN	152.02	7	3	3 FARSA
P26599 PTBP1_HUMAN	151.52	15	5	5 PTBP1
P26599-2 PTBP1_HUMAN	151.52	15	5	5 PTBP1
P26599-3 PTBP1_HUMAN	151.52	14	5	5 PTBP1

Q9C002 NMES1_HUMAN	151.43	52	3	3 NMES1
P01857 IGHG1_HUMAN	150.5	18	5	2 IGHG1
P25398 RS12_HUMAN	150.4	50	6	6 RPS12
P16401 H15_HUMAN	149.43	10	3	3 HIST1H1B
Q3ZCQ8-2 TIM50_HUMAN	149.21	9	3	3 TIMM50
Q9Y310 RTCB_HUMAN	148.73	16	4	4 RTCB
Q6NZI2-2 PTRF_HUMAN	147.78	10	2	2 PTRF
Q6NZI2 PTRF_HUMAN	147.78	7	2	2 PTRF
P09429 HMGB1_HUMAN	147.53	24	4	1 HMGB1
Q58FG1 HS904_HUMAN	147.43	11	4	1 HSP90AA4P
P61006 RAB8A_HUMAN	147.27	17	3	1 RAB8A
P18085 ARF4_HUMAN	147.09	23	3	1 ARF4
P46109 CRKL_HUMAN	146.51	18	5	5 CRKL
P48735 IDHP_HUMAN	146.27	11	4	4 IDH2
Q15233 NONO_HUMAN	144.28	13	5	5 NONO
P50990-3 TCPQ_HUMAN	144.27	13	5	5 CCT8
P50990-2 TCPQ_HUMAN	144.27	12	5	5 CCT8
P50990 TCPQ_HUMAN	144.27	11	5	5 CCT8
Q15436 SC23A_HUMAN	143.91	5	3	2 SEC23A
Q13148 TADBP_HUMAN	142.36	10	3	3 TARDBP
Q96PK6 RBM14_HUMAN	141.77	10	4	4 RBM14
P01871 IGHM_HUMAN	141.23	10	4	1 IGHM
P55084-2 ECHB_HUMAN	141.02	11	5	5 HADHB
P55084 ECHB_HUMAN	141.02	11	5	5 HADHB
P07195 LDHB_HUMAN	140.96	16	4	3 LDHB
P07910-2 HNRPC_HUMAN	140.57	15	4	4 HNRNPC
P07910 HNRPC_HUMAN	140.57	14	4	4 HNRNPC
P11216 PYGB_HUMAN	140.39	6	4	1 PYGB
Q9BXB4 OSB11_HUMAN	140.15	8	4	4 OSBPL11
A0M8Q6 LAC7_HUMAN	139.33	46	3	1 IGLC7
P33992 MCM5_HUMAN	137.42	6	3	3 MCM5
O00567 NOP56_HUMAN	137.31	6	3	3 NOP56
Q9Y4L1 HYOU1_HUMAN	136.68	8	4	4 HYOU1
Q8IUE6 H2A2B_HUMAN	136.2	35	6	1 HIST2H2AB
Q9Y2X3 NOP58_HUMAN	136.05	10	4	4 NOP58
Q71DI3 H32_HUMAN	135.95	33	8	8 HIST2H3A
P68431 H31_HUMAN	135.95	33	8	8 HIST1H3A
Q16695 H31T_HUMAN	135.95	33	8	8 HIST3H3
P06737-2 PYGL_HUMAN	135.41	5	4	1 PYGL
P06737 PYGL_HUMAN	135.41	5	4	1 PYGL
P06493 CDK1_HUMAN	134.97	14	4	4 CDK1
P61026 RAB10_HUMAN	134.24	16	3	1 RAB10
P05388 RLA0_HUMAN	133.78	18	5	5 RPLP0
Q15084-3 PDIA6_HUMAN	133.63	7	2	2 PDIA6
Q15084 PDIA6_HUMAN	133.63	7	2	2 PDIA6
Q15084-2 PDIA6_HUMAN	133.63	7	2	2 PDIA6
P21333-2 FLNA_HUMAN	133.12	3	5	4 FLNA
P21333 FLNA_HUMAN	133.12	3	5	4 FLNA
P51148 RAB5C_HUMAN	132.33	16	3	1 RAB5C
P51148-2 RAB5C_HUMAN	132.33	14	3	1 RAB5C
P67809 YBOX1_HUMAN	130.4	14	2	2 YBX1
P06310 KV206_HUMAN	130.18	20	4	2 4 SV
Q9H9B4 SFXN1_HUMAN	130.14	14	4	4 SFXN1
P69905 HBA_HUMAN	129.68	28	3	3 HBA1
P11388 TOP2A_HUMAN	129.55	3	4	2 TOP2A
P11388-2 TOP2A_HUMAN	129.55	3	4	2 TOP2A
P11388-3 TOP2A_HUMAN	129.55	3	4	2 TOP2A
P11388-4 TOP2A_HUMAN	129.55	2	4	2 TOP2A
Q92928 RAB1C_HUMAN	128.9	22	4	2 RAB1C
Q9H0U4 RAB1B_HUMAN	128.9	22	4	2 RAB1B
P62820 RAB1A_HUMAN	128.9	21	4	2 RAB1A
Q5T9A4 ATD3B_HUMAN	128.25	10	5	2 ATAD3B
Q04637-3 EIF4G1_HUMAN	127.83	3	3	3 EIF4G1
P40763-2 STAT3_HUMAN	127.61	9	4	4 STAT3
P40763 STAT3_HUMAN	127.61	9	4	4 STAT3
Q15366-7 PCBP2_HUMAN	127.53	11	3	1 PCBP2
Q15366-4 PCBP2_HUMAN	127.53	11	3	1 PCBP2
Q15366-5 PCBP2_HUMAN	127.53	11	3	1 PCBP2
Q15366-6 PCBP2_HUMAN	127.53	10	3	1 PCBP2
Q15366-3 PCBP2_HUMAN	127.53	10	3	1 PCBP2
Q15366 PCBP2_HUMAN	127.53	10	3	1 PCBP2

Q15366-2 PCBP2_HUMA	127.53	10	3	1 PCBP2
Q08J23-2 NSUN2_HUMA	127.47	4	2	2 NSUN2
Q08J23 NSUN2_HUMAN	127.47	4	2	2 NSUN2
P46087-3 NOP2_HUMAN	127.4	5	3	3 NOP2
P46087-2 NOP2_HUMAN	127.4	4	3	3 NOP2
P46087 NOP2_HUMAN	127.4	4	3	3 NOP2
P46087-4 NOP2_HUMAN	127.4	4	3	3 NOP2
P50914 RL14_HUMAN	126.97	11	2	2 RPL14
P36507 MP2K2_HUMAN	125.32	12	5	5 MAP2K2
P11217 PYGM_HUMAN	124.95	6	4	1 PYGM
Q14019 COTL1_HUMAN	124.64	18	3	3 COTL1
Q14978 NOLC1_HUMAN	124.46	6	4	4 NOLC1
Q14978-3 NOLC1_HUMA	124.46	6	4	4 NOLC1
Q14978-2 NOLC1_HUMA	124.46	6	4	4 NOLC1
Q9Y678 COPG1_HUMAN	123.41	4	3	2 COPG1
Q6PKG0 LARP1_HUMAN	123.29	5	4	4 LARP1
P42224 STAT1_HUMAN	122.69	7	4	4 STAT1
P09874 PARP1_HUMAN	122.09	3	2	2 PARP1
P62273 RS29_HUMAN	121.82	34	2	2 RPS29
Q71U19 H2AV_HUMAN	121.27	23	3	1 H2AFV
P0C055 H2AZ_HUMAN	121.27	23	3	1 H2AFZ
P46782 RS5_HUMAN	120.75	12	2	2 RPS5
Q9NVI7-3 ATD3A_HUMA	120.61	10	4	1 ATAD3A
Q9NVI7-2 ATD3A_HUMA	120.61	9	4	1 ATAD3A
E9PAV3 NACAM_HUMA	120.58	1	2	2 NACA
O60260-6 PRKN2_HUMA	120.34	14	2	2 PARK2
O60260-7 PRKN2_HUMA	120.34	12	2	2 PARK2
O60260-8 PRKN2_HUMA	120.34	11	2	2 PARK2
O60260-2 PRKN2_HUMA	120.34	10	2	2 PARK2
O60260 PRKN2_HUMAN	120.34	10	2	2 PARK2
Q9Y224 CN166_HUMAN	120.33	12	2	2 C14orf166
P39019 RS19_HUMAN	120.08	30	5	5 RPS19
P02100 HBE_HUMAN	119.65	26	3	2 HBE1
Q9UH17-3 ABC3B_HUMA	118.83	16	4	1 APOBEC3B
Q9UH17 ABC3B_HUMAN	118.83	15	4	1 APOBEC3B
Q96I24 FUBP3_HUMAN	118.33	12	4	4 FUBP3
P04181 OAT_HUMAN	118.01	8	3	2 OAT
P62851 RS25_HUMAN	117.97	26	4	4 RPS25
Q92945 FUBP2_HUMAN	117.8	8	3	3 KHSRP
Q8IVF2-3 AHNK2_HUMA	117.62	1	3	3 AHNAK2
Q8IVF2 AHNK2_HUMAN	117.62	1	3	3 AHNAK2
P68871 HBB_HUMAN	116.19	16	2	1 HBB
P53992 SC24C_HUMAN	115.67	6	5	5 SEC24C
P20340 RAB6A_HUMAN	115.6	18	3	2 RAB6A
P20340-2 RAB6A_HUMA	115.6	18	3	2 RAB6A
P61513 RL37A_HUMAN	115.13	22	2	2 RPL37A
O00425 IF2B3_HUMAN	114.36	7	3	1 IGF2BP3
Q14498-3 RBM39_HUMA	113.9	4	2	2 RBM39
Q14498-2 RBM39_HUMA	113.9	4	2	2 RBM39
Q14498 RBM39_HUMAN	113.9	4	2	2 RBM39
P30101 PDIA3_HUMAN	113.89	9	3	3 PDIA3
Q9Y305 ACOT9_HUMAN	113.66	11	5	5 ACOT9
P25205 MCM3_HUMAN	113.59	4	3	3 MCM3
P53007 TXTP_HUMAN	113.3	14	4	4 SLC25A1
Q02880-2 TOP2B_HUMA	112.82	2	3	1 TOP2B
Q02880 TOP2B_HUMAN	112.82	2	3	1 TOP2B
P62857 RS28_HUMAN	112.57	23	2	2 RPS28
Q9H223 EHD4_HUMAN	112.17	4	2	2 EHD4
P31483 TIA1_HUMAN	111.46	11	3	1 TIA1
P29692-2 EF1D_HUMAN	108.93	6	3	3 EEF1D
P56537 IF6_HUMAN	108.74	13	2	2 EIF6
Q99439-2 CNN2_HUMA	108.65	17	3	3 CNN2
Q99439 CNN2_HUMAN	108.65	15	3	3 CNN2
Q7L0Y3 MRRP1_HUMAN	108.38	9	3	3 TRMT10C
P16615-5 AT2A2_HUMA	108.16	4	3	2 ATP2A2
P16615-2 AT2A2_HUMA	108.16	4	3	2 ATP2A2
P16615-3 AT2A2_HUMA	108.16	4	3	2 ATP2A2
P16615 AT2A2_HUMAN	108.16	4	3	2 ATP2A2
P62829 RL23_HUMAN	107.78	18	4	4 RPL23
Q9BQ39 DDX50_HUMAN	107.32	3	2	1 DDX50
P31689-2 DNJA1_HUMA	107	10	2	2 DNAJA1
P31689 DNJA1_HUMAN	107	8	2	2 DNAJA1

P14868 SYDC_HUMAN	106.89	8	4	4 DARS
Q15365 PCBP1_HUMAN	106.24	12	3	2 PCBP1
P33991 MCM4_HUMAN	105.83	4	3	3 MCM4
P15924 DESP_HUMAN	104.99	1	3	3 DSP
Q13283 G3BP1_HUMAN	104.23	8	3	3 G3BP1
P62861 RS30_HUMAN	103.56	19	2	2 FAU
Q14444-2 CAPR1_HUMAN	103.35	3	2	2 CAPRIN1
Q14444 CAPR1_HUMAN	103.35	3	2	2 CAPRIN1
O43707 ACTN4_HUMAN	103	3	2	1 ACTN4
P01023 A2MG_HUMAN	102.81	3	4	3 A2M
Q96HJ9-2 CGO55_HUMAN	102.52	6	2	2 C7orf55
O14980 XPO1_HUMAN	102.35	4	3	3 XPO1
P27708 PYR1_HUMAN	101.71	1	2	2 CAD
Q9Y5X1 SNX9_HUMAN	101.52	7	3	3 SNX9
Q59GN2 R39L5_HUMAN	101.05	24	2	2 RPL39P5
P62891 RL39_HUMAN	101.05	24	2	2 RPL39
O00148 DX39A_HUMAN	100.98	7	2	2 DDX39A
P39656 OST48_HUMAN	100.44	4	2	2 DDOST
O60716-16 CTND1_HUMAN	100.04	4	2	2 CTNND1
O60716-15 CTND1_HUMAN	100.04	4	2	2 CTNND1
O60716-13 CTND1_HUMAN	100.04	4	2	2 CTNND1
O60716-11 CTND1_HUMAN	100.04	4	2	2 CTNND1
O60716-8 CTND1_HUMAN	100.04	4	2	2 CTNND1
O60716-7 CTND1_HUMAN	100.04	4	2	2 CTNND1
O60716-5 CTND1_HUMAN	100.04	4	2	2 CTNND1
O60716-3 CTND1_HUMAN	100.04	4	2	2 CTNND1
Q8WWM7-8 ATX2L_HUMAN	99.9	3	2	2 ATXN2L
P12883 MYH7_HUMAN	98.97	1	2	1 MYH7
P62841 RS15_HUMAN	98.42	27	2	2 RPS15
P62906 RL10A_HUMAN	98.38	25	5	5 RPL10A
P21796 VDAC1_HUMAN	98.32	8	2	1 VDAC1
O95299 NDUFA10_HUMAN	97.96	9	2	2 NDUFA10
Q96EP5-2 DAZP1_HUMAN	97.93	8	2	2 DAZAP1
Q96EP5 DAZP1_HUMAN	97.93	8	2	2 DAZAP1
P18583-2 SON_HUMAN	97.93	1	2	2 SON
P18583-5 SON_HUMAN	97.93	1	2	2 SON
P05386 RLA1_HUMAN	97.86	14	1	1 RPLP1
P00558 PGK1_HUMAN	97.54	4	1	1 PGK1
P12956 XRCC6_HUMAN	96.25	5	2	2 XRCC6
P60903 S100A_HUMAN	96.12	40	2	2 S100A10
Q15907 RB11B_HUMAN	92.51	14	3	3 RAB11B
Q9BZE4 NOG1_HUMAN	91.39	6	4	4 GTPBP4
Q8N1N4 K2C78_HUMAN	90.69	4	3	1 KRT78
P46013-2 KI67_HUMAN	90.55	1	2	2 MKI67
P46013 KI67_HUMAN	90.55	1	2	2 MKI67
P62195-2 PRS8_HUMAN	90.45	5	1	1 PSMC5
P62195 PRS8_HUMAN	90.45	5	1	1 PSMC5
P49207 RL34_HUMAN	90.04	13	2	2 RPL34
A7E2Y1 MYH7B_HUMAN	89.28	1	2	1 MYH7B
P50238 CRIP1_HUMAN	88.13	36	1	1 CRIP1
P48047 ATPO_HUMAN	87.7	6	1	1 ATP5O
P06753-3 TPM3_HUMAN	86.49	15	3	1 TPM3
P06753-4 TPM3_HUMAN	86.49	15	3	1 TPM3
P06753-5 TPM3_HUMAN	86.49	15	3	1 TPM3
P06753-2 TPM3_HUMAN	86.49	15	3	1 TPM3
P06753-6 TPM3_HUMAN	86.49	15	3	1 TPM3
O75821 EIF3G_HUMAN	86.49	8	1	1 EIF3G
Q9Y277 VDAC3_HUMAN	85.67	11	2	1 VDAC3
Q9Y277-2 VDAC3_HUMAN	85.67	11	2	1 VDAC3
P54577 SYYC_HUMAN	85.27	4	2	2 YARS
P35606 COPB2_HUMAN	84.59	3	2	2 COPB2
O00154-5 BACH_HUMAN	84.45	3	1	1 ACOT7
P07814 SYEP_HUMAN	82.58	2	2	2 EPRS
Q07666-2 KHDR1_HUMAN	82.38	5	2	2 KHDRBS1
Q07666 KHDR1_HUMAN	82.38	5	2	2 KHDRBS1
O14828-2 SCAM3_HUMAN	82.32	5	1	1 SCAMP3
O14828 SCAM3_HUMAN	82.32	5	1	1 SCAMP3
P62495 ERF1_HUMAN	81.75	3	1	1 ETF1
Q9GVZ4 IFSA2_HUMAN	81.13	8	1	1 EIFSA2
P63241 IFSA1_HUMAN	81.13	8	1	1 EIFSA
Q6IS14 IFSA1_HUMAN	81.13	8	1	1 EIFSAL1
P63241-2 IFSA1_HUMAN	81.13	7	1	1 EIFSA
Q9UNL2 SSRG_HUMAN	80.99	8	1	1 SSR3

P20742 PZP_HUMAN	80.3	2	2	1 PZP
O14497-3 ARI1A_HUMAN	79.82	3	4	4 ARID1A
O14497 ARI1A_HUMAN	79.82	3	4	4 ARID1A
P61586 RHOA_HUMAN	79.36	6	1	1 RHOA
P37802 TAGL2_HUMAN	79.34	6	1	1 TAGLN2
P63167 DYL1_HUMAN	79.07	37	2	2 DYNLL1
P51149 RAB7A_HUMAN	79.03	5	1	1 RAB7A
P04004 VTNC_HUMAN	78.71	5	2	2 VTN
P99999 CYC_HUMAN	77.69	18	2	2 CYCS
Q06210-2 GFPT1_HUMAN	77.46	3	2	2 GFPT1
Q06210 GFPT1_HUMAN	77.46	3	2	2 GFPT1
P56134-4 ATPK_HUMAN	76.73	27	1	1 ATP5J2
P56134-3 ATPK_HUMAN	76.73	24	1	1 ATP5J2
P56134-2 ATPK_HUMAN	76.73	15	1	1 ATP5J2
P56134 ATPK_HUMAN	76.73	14	1	1 ATP5J2
O00299 CLIC1_HUMAN	76.56	7	1	1 CLIC1
P49327 FAS_HUMAN	76.49	1	2	2 FASN
P47897 SYQ_HUMAN	75.91	4	2	2 QARS
Q9NZ01 TECR_HUMAN	75.37	7	2	2 TECR
P37108 SRP14_HUMAN	75.32	10	1	1 SRP14
Q86UP2-2 KTN1_HUMAN	75.3	3	3	3 KTN1
P40616 ARL1_HUMAN	75.08	8	1	1 ARL1
Q14137 BOP1_HUMAN	74.65	2	2	2 BOP1
P02787 TRFE_HUMAN	74.12	1	1	1 TF
P50570-3 DYN2_HUMAN	73.55	2	1	1 DNM2
P50570-2 DYN2_HUMAN	73.55	2	1	1 DNM2
P50570-5 DYN2_HUMAN	73.55	2	1	1 DNM2
P50570-4 DYN2_HUMAN	73.55	2	1	1 DNM2
P50570 DYN2_HUMAN	73.55	2	1	1 DNM2
P55769 NH2L1_HUMAN	73.34	9	1	1 NHP2L1
Q9BXW7-2 CECR5_HUMAN	73.13	2	1	1 CECR5
Q9BXW7 CECR5_HUMAN	73.13	2	1	1 CECR5
P48507 GSHO_HUMAN	72.67	11	2	2 GCLM
P55060-4 XPO2_HUMAN	70.74	2	2	2 CSE1L
P55060-3 XPO2_HUMAN	70.74	2	2	2 CSE1L
P55060 XPO2_HUMAN	70.74	2	2	2 CSE1L
O43390-3 HNRPR_HUMAN	70.69	3	2	2 HNRNPR
O43390 HNRPR_HUMAN	70.69	3	2	2 HNRNPR
O75190-4 DNJB6_HUMAN	70.48	5	1	1 DNAJB6
O75190 DNJB6_HUMAN	70.48	5	1	1 DNAJB6
P01624 KV306_HUMAN	70.33	13	4	3 1 SV
P04207 KV308_HUMAN	70.33	11	4	3 1 SV
Q13418 ILK_HUMAN	69.88	2	1	1 ILK
P19823 ITH2_HUMAN	69.7	2	1	1 ITH2
P05204 HMG2_HUMAN	68.98	17	1	1 HMG2
P51116 FXR2_HUMAN	66.59	2	2	1 FXR2
P62333 PRS10_HUMAN	66.54	4	1	1 PSMC6
B5ME19 EIFCL_HUMAN	66.42	3	2	2 EIF3CL
P02775 CXCL7_HUMAN	65.42	11	1	1 PPBP
P63173 RL38_HUMAN	65.41	19	1	1 RPL38
P23381-2 SYWC_HUMAN	65.21	4	1	1 WARS
P23381 SYWC_HUMAN	65.21	3	1	1 WARS
P08243-3 ASNS_HUMAN	64.86	2	1	1 ASNS
P08243-2 ASNS_HUMAN	64.86	2	1	1 ASNS
P08243 ASNS_HUMAN	64.86	2	1	1 ASNS
O76021 RL1D1_HUMAN	64.58	4	3	3 RSL1D1
Q13428-2 TCOF_HUMAN	64.29	2	2	2 TCOF1
Q13428-8 TCOF_HUMAN	64.29	2	2	2 TCOF1
Q13428-6 TCOF_HUMAN	64.29	2	2	2 TCOF1
Q13428-7 TCOF_HUMAN	64.29	2	2	2 TCOF1
Q13428 TCOF_HUMAN	64.29	2	2	2 TCOF1
Q13428-3 TCOF_HUMAN	64.29	2	2	2 TCOF1
Q13428-4 TCOF_HUMAN	64.29	2	2	2 TCOF1
P20929-3 NEBU_HUMAN	64.28	0	1	1 NEB
O94905 ERL12_HUMAN	63.67	4	1	1 ERLIN2
O75477 ERL11_HUMAN	63.67	3	1	1 ERLIN1
Q6YN16-2 HSDL2_HUMAN	63.01	4	1	1 HSDL2
Q6YN16 HSDL2_HUMAN	63.01	3	1	1 HSDL2
Q09028-4 RBBP4_HUMAN	62.94	2	1	1 RBBP4
Q09028-3 RBBP4_HUMAN	62.94	2	1	1 RBBP4
Q09028-2 RBBP4_HUMAN	62.94	2	1	1 RBBP4

Q09028 RBBP4_HUMAN	62.94	2	1	1 RBBP4
Q14694 UBP10_HUMAN	62	3	2	2 USP10
P28331-5 NDUS1_HUMAN	61.67	5	2	2 NDUFS1
Q9P2J5 SYLC_HUMAN	61.61	1	1	1 LARS
P13073 COX41_HUMAN	61.59	6	1	1 COX41
Q9NS69 TOM22_HUMAN	61.43	8	1	1 TOMM22
Q96JB1-2 DYH8_HUMAN	60.8	0	1	1 DNAH8
Q96JB1 DYH8_HUMAN	60.8	0	1	1 DNAH8
Q08554-2 DSC1_HUMAN	60.25	2	1	1 DSC1
Q08554 DSC1_HUMAN	60.25	2	1	1 DSC1
P63220 RS21_HUMAN	60.23	11	1	1 RPS21
Q9NWT8 AKIP_HUMAN	59.83	7	1	1 AURKAIP1
O14773-2 TPP1_HUMAN	59.7	4	1	1 TPP1
O14773 TPP1_HUMAN	59.7	2	1	1 TPP1
Q9BQGO-2 MBB1A_HUMAN	58.76	1	1	1 MYBBP1A
P61160 ARP2_HUMAN	58.37	5	1	1 ACTR2
P62316-2 SMD2_HUMAN	58.22	9	1	1 SNRPD2
P62316 SMD2_HUMAN	58.22	8	1	1 SNRPD2
P46060 RAGP1_HUMAN	57.91	4	2	2 RANGAP1
Q96SU4-3 OSBL9_HUMAN	57.63	2	1	1 OSBPL9
Q96SU4-7 OSBL9_HUMAN	57.63	2	1	1 OSBPL9
Q96SU4-2 OSBL9_HUMAN	57.63	2	1	1 OSBPL9
Q96SU4 OSBL9_HUMAN	57.63	2	1	1 OSBPL9
Q96SU4-6 OSBL9_HUMAN	57.63	2	1	1 OSBPL9
Q86VP6-2 CAND1_HUMAN	56.89	1	1	1 CAND1
O75369-5 FLNB_HUMAN	56.48	1	2	1 FLNB
O75369-4 FLNB_HUMAN	56.48	1	2	1 FLNB
O75369-6 FLNB_HUMAN	56.48	1	2	1 FLNB
O75369-3 FLNB_HUMAN	56.48	1	2	1 FLNB
O75369-2 FLNB_HUMAN	56.48	1	2	1 FLNB
O75369-9 FLNB_HUMAN	56.48	1	2	1 FLNB
O75369 FLNB_HUMAN	56.48	1	2	1 FLNB
O75369-8 FLNB_HUMAN	56.48	1	2	1 FLNB
Q9Y266 NUDC_HUMAN	56.3	3	1	1 NUDC
P17096-2 HMGA1_HUMAN	55.96	15	1	1 HMGA1
Q15061 WDR43_HUMAN	55.4	2	1	1 WDR43
Q05707-3 COEA1_HUMAN	55.29	1	1	1 COL14A1
Q05707-2 COEA1_HUMAN	55.29	1	1	1 COL14A1
Q05707 COEA1_HUMAN	55.29	1	1	1 COL14A1
Q86UA1 PRPF39_HUMAN	54.92	2	1	1 PRPF39
O75746-2 CMC1_HUMAN	54.52	1	1	1 SLC25A12
Q9UJS0 CMC2_HUMAN	54.52	1	1	1 SLC25A13
Q9UJS0-2 CMC2_HUMAN	54.52	1	1	1 SLC25A13
O75746 CMC1_HUMAN	54.52	1	1	1 SLC25A12
P17980 PRSGA_HUMAN	54.5	2	1	1 PSMC3
Q01518-2 CAP1_HUMAN	54.44	2	1	1 CAP1
Q01518 CAP1_HUMAN	54.44	2	1	1 CAP1
O15145 ARPC3_HUMAN	53.82	6	1	1 ARPC3
Q9Y5A9-2 YTHDF2_HUMAN	53.81	2	1	1 YTHDF2
Q9Y5A9 YTHDF2_HUMAN	53.81	2	1	1 YTHDF2
P01621 KV303_HUMAN	53.65	9	5	5 1 SV
P13010 XRCC5_HUMAN	53.6	1	1	1 XRCC5
P60900-2 PSA6_HUMAN	53.55	6	1	1 PSMA6
P60900 PSA6_HUMAN	53.55	5	1	1 PSMA6
P08133-2 ANXA6_HUMAN	53.04	2	1	1 ANXA6
P08133 ANXA6_HUMAN	53.04	2	1	1 ANXA6
O94776 MTA2_HUMAN	52.25	1	1	1 MTA2
Q16891-4 IMMT_HUMAN	52	2	1	1 IMMT
Q15532-2 SSXT_HUMAN	51.67	3	1	1 SS18
Q15532 SSXT_HUMAN	51.67	3	1	1 SS18
Q6UB35 C1TM_HUMAN	51.23	1	1	1 MTHFD1L
Q9UBT2 SAE2_HUMAN	50.74	1	1	1 UBA2
P80748 LV302_HUMAN	50.01	7	1	1 1 SV
Q8N1F7-2 NUP93_HUMAN	49.96	1	1	1 NUP93
Q8N1F7 NUP93_HUMAN	49.96	1	1	1 NUP93
Q5T4S7-3 UBR4_HUMAN	49.5	0	1	1 UBR4
Q5T4S7 UBR4_HUMAN	49.5	0	1	1 UBR4
Q15208 STK38_HUMAN	49.42	4	1	1 STK38
O96013-2 PAK4_HUMAN	49.06	4	1	1 PAK4
O96013-3 PAK4_HUMAN	49.06	3	1	1 PAK4
O96013-4 PAK4_HUMAN	49.06	3	1	1 PAK4

O96013 PAK4_HUMAN	49.06	3	1	1 PAK4
Q8TD84-2 DSC11_HUMAN	48.86	0	1	1 DSCAML1
Q8TD84 DSC11_HUMAN	48.86	0	1	1 DSCAML1
P12004 PCNA_HUMAN	48.85	3	1	1 PCNA
P35610-3 SOAT1_HUMAN	48.77	3	1	1 SOAT1
O00116 ADAS_HUMAN	48.47	2	1	1 AGPS
P62318 SMD3_HUMAN	48.37	7	1	1 SNRPD3
Q96HS1-2 PGAM5_HUMAN	48.29	4	1	1 PGAM5
Q96HS1 PGAM5_HUMAN	48.29	3	1	1 PGAM5
Q16625-5 OCLN_HUMAN	47.51	8	1	1 OCLN
Q16625-4 OCLN_HUMAN	47.51	6	1	1 OCLN
Q16625-2 OCLN_HUMAN	47.51	3	1	1 OCLN
Q16625 OCLN_HUMAN	47.51	3	1	1 OCLN
Q8N752 KC1A1_HUMAN	47.06	3	1	1 CSNK1A1L
P48729 KC1A_HUMAN	47.06	3	1	1 CSNK1A1
Q9H4M9 EHD1_HUMAN	46.59	2	1	1 EHD1
P19525-2 E2AK2_HUMAN	46.49	2	1	1 EIF2AK2
P19525 E2AK2_HUMAN	46.49	2	1	1 EIF2AK2
Q14667-2 K0100_HUMAN	46.36	0	1	1 KIAA0100
Q14667 K0100_HUMAN	46.36	0	1	1 KIAA0100
O75367-2 H2AFY_HUMAN	46.34	5	1	1 H2AFY
O75367-3 H2AFY_HUMAN	46.34	5	1	1 H2AFY
O75367 H2AFY_HUMAN	46.34	5	1	1 H2AFY
P84103 SRSF3_HUMAN	46.07	5	1	1 SRSF3
Q13151 ROAO_HUMAN	45.78	5	1	1 HNRNPA0
Q96KG9-6 NTKL_HUMAN	45.71	2	1	1 SCYL1
Q9NTJ3-2 SMC4_HUMAN	45.67	1	1	1 SMC4
Q9NTJ3 SMC4_HUMAN	45.67	1	1	1 SMC4
P57088 TMM33_HUMAN	44.87	5	1	1 TMEM33
P10599 THIO_HUMAN	44.78	19	1	1 TXN
Q7Z2K6 ERMP1_HUMAN	44.54	1	1	1 ERMP1
P35573-2 GDE_HUMAN	44.04	1	1	1 AGL
P35573-3 GDE_HUMAN	44.04	1	1	1 AGL
P35573 GDE_HUMAN	44.04	1	1	1 AGL
O75694-2 NUP155_HUMAN	43.55	1	1	1 NUP155
P35568 IRS1_HUMAN	43.33	1	1	1 IRS1
Q08211 DHX9_HUMAN	42.7	1	1	1 DHX9
O14647-2 CHD2_HUMAN	41.86	1	1	1 CHD2
O14647 CHD2_HUMAN	41.86	1	1	1 CHD2
Q06945 SOX4_HUMAN	41.14	3	1	1 SOX4
P56192 SYMC_HUMAN	40.95	2	1	1 MARS
Q86UP3-5 ZFXH4_HUMAN	40.75	1	1	1 ZFXH4
P62330 ARF6_HUMAN	40.38	6	1	1 ARF6
P31947 1433S_HUMAN	40.32	3	1	1 SFN
O60762 DPM1_HUMAN	39.66	4	1	1 DPM1
Q9NVP1 DDX18_HUMAN	39.6	1	1	1 DDX18
P15259 PGAM2_HUMAN	39.37	4	1	1 PGAM2
P18669 PGAM1_HUMAN	39.37	4	1	1 PGAM1
Q13838 DX39B_HUMAN	39.35	3	1	1 DDX39B
Q13838-2 DX39B_HUMAN	39.35	3	1	1 DDX39B
Q81WI9-3 MGAP_HUMAN	39.3	0	1	1 MGA
Q81WI9 MGAP_HUMAN	39.3	0	1	1 MGA
Q81WI9-4 MGAP_HUMAN	39.3	0	1	1 MGA
P17096 HMGA1_HUMAN	39.28	23	1	1 HMGA1
Q7Z2K8 GRIN1_HUMAN	38.91	2	1	1 GPRIN1
Q9C0G6 DYH6_HUMAN	38.84	0	1	1 DNAH6
Q15008 PSMD6_HUMAN	38.83	4	1	1 PSMD6
P02765 FETUA_HUMAN	38.79	2	1	1 AHSG
Q5VZ46-2 K1614_HUMAN	38.75	1	1	1 KIAA1614
Q5VZ46 K1614_HUMAN	38.75	1	1	1 KIAA1614
P40818-2 UBP8_HUMAN	38.71	1	1	1 USP8
P40818 UBP8_HUMAN	38.71	1	1	1 USP8
O60229-2 KALRN_HUMAN	38.61	1	1	1 KALRN
O60229 KALRN_HUMAN	38.61	0	1	1 KALRN
O60242 BAI3_HUMAN	38.55	0	1	1 BAI3
Q55T30 SYVM_HUMAN	38.27	1	1	1 VARS2
P49755 TMEDA_HUMAN	38.22	5	1	1 TMED10
Q8N568-2 DCLK2_HUMAN	38.06	1	1	1 DCLK2
Q8N568 DCLK2_HUMAN	38.06	1	1	1 DCLK2
P24539 AT5F1_HUMAN	37.97	6	1	1 ATP5F1

Q9P0W8-3 SPAT7_HUMAN	37.69	2	1	1 SPATA7
Q9P0W8-2 SPAT7_HUMAN	37.69	1	1	1 SPATA7
Q9P0W8 SPAT7_HUMAN	37.69	1	1	1 SPATA7
P54886-2 P5CS_HUMAN	37.37	1	1	1 ALDH18A1
P16070-18 CD44_HUMAN	37.23	3	1	1 CD44
P16070-12 CD44_HUMAN	37.23	3	1	1 CD44
P16070-14 CD44_HUMAN	37.23	3	1	1 CD44
P16070-13 CD44_HUMAN	37.23	2	1	1 CD44
P16070-11 CD44_HUMAN	37.23	2	1	1 CD44
P16070-10 CD44_HUMAN	37.23	2	1	1 CD44
P16070-16 CD44_HUMAN	37.23	1	1	1 CD44
P16070-8 CD44_HUMAN	37.23	1	1	1 CD44
P16070-17 CD44_HUMAN	37.23	1	1	1 CD44
P16070-4 CD44_HUMAN	37.23	1	1	1 CD44
P16070-6 CD44_HUMAN	37.23	1	1	1 CD44
P16070-3 CD44_HUMAN	37.23	1	1	1 CD44
P16070-7 CD44_HUMAN	37.23	1	1	1 CD44
P16070-5 CD44_HUMAN	37.23	1	1	1 CD44
P16070 CD44_HUMAN	37.23	1	1	1 CD44
Q9Y6X6 MYO16_HUMAN	37.15	1	1	1 MYO16
Q86XE3 MICU3_HUMAN	35.92	3	1	1 MICU3
Q13425 SNTB2_HUMAN	35.91	2	1	1 SNTB2
Q8NEZ4-2 KMT2C_HUMAN	35.59	0	1	1 KMT2C
Q8NEZ4 KMT2C_HUMAN	35.59	0	1	1 KMT2C
Q8NEZ4-3 KMT2C_HUMAN	35.59	0	1	1 KMT2C
Q9H6R4-3 NOL6_HUMAN	35.29	1	1	1 NOL6
Q9H6R4-2 NOL6_HUMAN	35.29	1	1	1 NOL6
Q9H6R4-4 NOL6_HUMAN	35.29	1	1	1 NOL6
Q9H6R4 NOL6_HUMAN	35.29	1	1	1 NOL6
Q00535-2 CDK5_HUMAN	34.65	4	1	1 CDK5
Q00535 CDK5_HUMAN	34.65	4	1	1 CDK5
Q6P2H3 CEP85_HUMAN	34.41	2	1	1 CEP85
O95389 WISP3_HUMAN	33.87	2	1	1 WISP3
Q14571 ITPR2_HUMAN	33.71	0	1	1 ITPR2
Q15382 RHEB_HUMAN	32.98	7	1	1 RHEB
O75914-2 PAK3_HUMAN	32.34	2	1	1 PAK3
O75914 PAK3_HUMAN	32.34	2	1	1 PAK3
O75914-4 PAK3_HUMAN	32.34	2	1	1 PAK3
O75914-3 PAK3_HUMAN	32.34	2	1	1 PAK3
Q9H8M2-6 BRD9_HUMAN	32.1	1	1	1 BRD9
Q92502-2 STAR8_HUMAN	31.95	1	1	1 STAR8
P28749-2 RBL1_HUMAN	31.82	2	1	1 RBL1
P28749 RBL1_HUMAN	31.82	2	1	1 RBL1
P51172 SCNN1D_HUMAN	31.69	1	1	1 SCNN1D
P51172-2 SCNN1D_HUMAN	31.69	1	1	1 SCNN1D
Q7Z494 NPHP3_HUMAN	31.45	1	1	1 NPHP3
O60423 ATP8B3_HUMAN	30.68	2	1	1 ATP8B3
Q12792 TWF1_HUMAN	30.59	2	1	1 TWF1
Q12792-3 TWF1_HUMAN	30.59	2	1	1 TWF1
Q5THJ4-2 VPS13D_HUMAN	30.39	0	1	1 VPS13D
Q5THJ4 VPS13D_HUMAN	30.39	0	1	1 VPS13D
Q9NRZ9 HELLS_HUMAN	30.17	1	1	1 HELLS
P42285 SKIV2L2_HUMAN	30.17	1	1	1 SKIV2L2
Q8IYA2 C144C_HUMAN	30.08	1	1	1 CCDC144CP

Appendix 3: Table 3: Lists from Venn diagram (Chapter 6, figure 6.2)

Control List_ONLY	Both_Lists	IP List_ONLY
TUBB2B	HSPA9	TUBB4A
ACTG2	ACTB	HDLBP
ACTA2	VIM	TUBB8
POTEE	HSPA5	KRT6A
PRSS1	HSPA8	KRT16
HIST1H2BL	ACTG1	KRT6B
HIST3H2BB	TUBB	HNRNPH2
H3F3A	TUBB4B	POTEF
PFKP	MYH9	LARP4
RPL36A	TUBB2A	EEF1A2
ENO2	TUBB3	ARF1
PDCD5	PKM	PSMD3
PLEC	EEF1A1	KRT17
MAP4	TUBA1C	MYH10
EIF4B	ENO1	FBL
TRIP13	HSPA1A	MTHFD1
SHROOM3	ALB	EIF3D
ERCC6	KRT1	PFKL
DPYSL2	HSPA2	TRAP1
CROCC	HSP90AB1	RANBP1
CALM1	ACTA1	PABPC1
MT-CO2	KRT8	COPA
RPL35A	ATPSA1	HNRNPA1
HNRNPL	GAPDH	NAMPT
DRG1	ATPSB	DES
MYO18A	RAN	RPS18
LUC7L2	TCP1	HSP90AA2
HSPF1	FUS	TIAL1
GPI	HSPA1L	KRT79
RAB5B	HNRNPH1	RPS27
RAB5A	HIST1H4A	DDX1
NME2	KRT2	ATP5L
NME1	HSPB1	RPS27A
DDN	KRT9	KRT19
FKBP15	KRT10	EIF3B
SRSF5	HSP90AA1	PRRC2C
SRSF6	CFL1	CTPS1
SRSF4	EEF2	IGF2BP2
CACNA1C	KRT18	BANF1
SPN51	EIF4A1	IGF2BP1
ARL6IP4	ANXA2	MCM6
CAMSAP3	HNRNPA2B1	NMES1
ARHGEF17	RPS3	RPS12
FAM179B	PRDX1	TIMMSO
COL4A1	MVP	RTCB
CYP1B1	RPS3A	PTRF
GLYR1	RPL13	HMG81
ZNF620	RPS8	HSP90AA4P
RAP1B	RPS6	RAB8A
2 SV	RPL9	CRKL
RAP1A	TUBB6	IDH2
ANO8	AHNAK	NONO
FUBP1	LGALS1	SEC23A
BCHE	DDX3X	TARDBP
PTPN21	RPL4	RBM14
SEZ6L2	RPS7	HADHB
ST6GAL2	CCT3	OSBPL11
EFR3B	EWSR1	IGLC7
EBF2	RPL6	MCM5
YWHAZ	TKT	NOP56
FERMT1	HNRNPH3	HYOU1
ATP11A	RPS11	HIST2H2AB
TRAK1	HNRNPK	NOP58
PLEKHA5	TAF15	PYGL
	RPS4X	RAB10
	GNB2L1	FLNA
	GART	YBX1
	HNRNPD	TOP2A
	HNRNPF	RAB1C
	RPL7A	RAB1B
	RPL10	RAB1A
	RPL35	ATAD3B
	RPL18	PCBP2
	HIST1H1E	NSUN2

Control List_ONLY	Both_Lists	IP List_ONLY
	TUFM	NOP2
	CCT6A	MAP2K2
	RPL27A	PYGM
	RPS20	COTL1
	RPL7	NOLC1
	HIST1H1C	LARP1
	RPL8	STAT1
	DDX5	PARP1
	SERPINH1	H2AFV
	RPL19	H2AFZ
	RPL32	ATAD3A
	HNRNPU	NACA
	RPS16	PARK2
	SLC25A6	C14orf166
	RPSA	RPS19
	CCT4	APOBEC3B
	HIST2H2AC	FUBP3
	HIST2H2AA3	OAT
	RPS14	AHNAK2
	RPL3	RAB6A
	RPS9	RPL37A
	BOLA2	IGF2BP3
	PDCD6IP	PDIA3
	MCM7	ACOT9
	KRT5	MCM3
	SLC25A5	SLC25A1
	RPS2	TOP2B
	HIST3H2A	TIA1
	HIST1H2AB	EIF6
	HIST1H2AC	CNN2
	DDX17	TRMT10C
	RPL17	ATP2A2
	LDHA	RPL23
	RPL12	DDX50
	HNRNPD	DNAJA1
	HSPD1	DARS
	RPL24	PCBP1
	RPLP0	MCM4
	TFG	DSP
	MYL6	ACTN4
	MAGEA4	A2M
	RPL23A	C7orf55
	RPL21	CAD
	HIST2H3A	SNX9
	HIST1H3A	RPL39P5
	HIST3H3	RPL39
	CCT2	DDOST
	VDAC2	CTNND1
	EIF4A3	ATXN2L
	HNRNPAB	MYH7
	RPS23	RPS15
	GARS	NDUFA10
	IGKC	DAZAP1
	UQCRC2	SON
	TRIM21	XRCC6
	RPL26	S100A10
	CLTC	RAB11B
	RPL11	GTPBP4
	EEF1G	KRT78
	NCL	MKI67
	PDIA6	PSMC5
	PHB2	CRIP1
	HBA1	ATP5O
	RPL28	TPM3
	IGLL5	EIF3G
	KRT14	YARS
	CARM1	COPB2
	RPL27	ACOT7
	VAR5	EPRS
	FARSA	KHDRBS1
	EIF3A	SCAMP3
	RPL14	ETF1
	RPL36	SSR3
	RPS24	PZP

Control List_ONLY	Both_Lists	IP List_ONLY
	RPL22	ARID1A
	EIF2S3	RHOA
	RPL18A	TAGLN2
	HSP90B1	DYNLL1
	RPS15A	VTN
	RPL13A	CYCS
	HNRNPUL1	GFPT1
	4SV	ATP5J2
	HNRNPA3	CLIC1
	RPL30	FASN
	RPL29	QARS
	RPS26	TECR
	IGHM	KTN1
	CCT8	ARL1
	HIST1H1B	BOP1
	PCMT1	TF
	KHSRP	DNM2
	VDAC1	NHP2L1
	RPL31	CECR5
	PPIA	GCLM
	AHSA1	CSE1L
	GRN	HNRNPR
	RPL36AL	DNAJB6
	CDK1	ILK
	PRPF39	ITIH2
	MYO1C	HMGN2
	ATP5C1	FXR2
	RBM39	PSMC6
	DYNC1H1	EIF3CL
	RPL5	ASNS
	RPS10	RS�1D1
	RPS13	TCOF1
	RPLP2	NEB
	SEC24C	ERLIN2
	PTBP1	ERLIN1
	LDHB	HSDL2
	TFRC	RBBP4
	PRSS3	USP10
	SLC25A3	NDUFS1
	IGHG1	LARS
	HBE1	COX4I1
	RPS25	TOMM22
	RAB2A	DSC1
	HBB	AURKAIP1
	XPO1	TPP1
	EIF3I	MYBBP1A
	CCT7	ACTR2
	RPN1	SNRPD2
	RPS17	RANGAP1
	RPS17L	OSBPL9
	RPSS	CAND1
	SFXN1	FLNB
	LMNA	NUDC
	VDAC3	HMGA1
	RPL10A	WDR43
	RAB7A	COL14A1
	PYGB	SLC25A12
	CCT5	SLC25A13
	PHB	PSMC3
	DDX39A	CAP1
	COPG1	ARPC3
	RPS28	YTHDF2
	PRKDC	XRCC5
	PFN1	PSMA6
	DDX21	ANXA6
	IQGAP1	MTA2
	HNRNPM	IMMT
	HNRNPC	SS18
	RPL15	MTHFD1L
	RPS21	UBA2
	EEF1D	NUP93
	KPNA2	UBR4
	RPN2	STK38
	PPBP	PAK4

Control List_ONLY	Both_Lists	IP List_ONLY
	SRP14	PCNA
	EIF4G1	SOAT1
	CAPRIN1	AGPS
	FAU	SNRPD3
	DNAH8	PGAM5
	RPL34	OCLN
	STAT3	CSNK1A1L
	RPLP1	CSNK1A1
	MYOF	EHD1
	MYH7B	EIF2AK2
	G3BP1	KIAA0100
	RPL38	H2AFY
	WARS	SRSF3
	NPM1	HNRNPA0
	DSCAML1	SCYL1
	EHD4	SMC4
	TNRC6B	TMEM33
	RPS29	TXN
	ARF4	ERMP1
	RAB5C	AGL
	CSTB	NUP155
	ARF3	IRS1
	EIF5A2	DHX9
	EIF5AL1	CHD2
	EIF5A	SOX4
	1 SV	MARS
	PGK1	ZFHX4
	AHSG	ARF6
		SFN
		DPM1
		DDX18
		PGAM2
		PGAM1
		DDX39B
		MGA
		GPRIN1
		DNAH6
		PSMD6
		KIAA1614
		USP8
		KALRN
		BAI3
		VAR52
		TMED10
		DCLK2
		ATP5F1
		SPATA7
		ALDH18A1
		CD44
		MYO16
		MICU3
		SNTB2
		KMT2C
		NOL6
		CDK5
		CEP85
		WISP3
		ITPR2
		RHEB
		PAK3
		BRD9
		STARD8
		RBL1
		SCNN1D
		NPHP3
		ATP8B3
		TWF1
		VPS13D
		HELLS
		SKIV2L2
		CCDC144CP

Appendix 3: Table 4: MaxQuant/Perseus analysis of data generated by mass spectrometry analysis of RACK1 IPs.

At p=0.01, 66 proteins were deemed to co-immunoprecipitate with RACK1 (GNB2L1) and these proteins are listed in the table below.

List from Perseus	Edited list	
VARS	VARS	
CCT8	CCT8	
ATP5C1	ATP5C1	
RPL18A	RPL18A	
PHB2	PHB2	
HNRNPC	HNRNPC	
CCT5	CCT5	
HNRNPUL1	HNRNPUL1	
RPL31	RPL31	
MYOF	MYOF	
RANBP1	RANBP1	
PHB	PHB	
RPL30	RPL30	
PABPC1;PABPC3	PABPC1	Example of edit made
RPL15	RPL15	
PRKDC	PRKDC	
RPL28	RPL28	
LARP4	LARP4	
ATP5L	ATP5L	
NONO	NONO	
MTHFD1	MTHFD1	
MYO1C	MYO1C	
DDX17	DDX17	
UQCRC2	UQCRC2	
NOP56	NOP56	
PSMD3	PSMD3	
BANF1	BANF1	
IGHG1;IGHG3;IGHG4	IGHG1	
LMNA	LMNA	
TFRC	TFRC	
CSTB	CSTB	
NPM1	NPM1	
PARP1	PARP1	
DARS	DARS	
HSPA6;HSPA7	HSPA6	
FBL	FBL	
RPS12	RPS12	
RPL12	RPL12	
DNAJA1	DNAJA1	
RPL13A;RPL13AP3	RPL13A	
GARS	GARS	
RPS27;RPS27L	RPS27	
NAMPT	NAMPT	
VDAC2	VDAC2	
RPL29	RPL29	
HNRNPM	HNRNPM	
KPNA2	KPNA2	
ARF1;ARF3;ARF5	ARF1	
RPL27	RPL27	
RPS23	RPS23	
RPS18	RPS18	
RPS13	RPS13	
PPIA	PPIA	
GNB2L1	GNB2L1	
YBX1;YBX3	YBX1	
SLC25A3	SLC25A3	
HDLBP	HDLBP	
CLTC	CLTC	
PFKP	PFKP	
TUBB3	TUBB3	
EIF3A	EIF3A	
DYNC1H1	DYNC1H1	
PLEC	PLEC	
AHNAK2	AHNAK2	
FUBP3	FUBP3	
OSBPL11	OSBPL11	
DDX21	DDX21	

Appendix 3: Table 5: MaxQuant/Perseus analysis of data generated by mass spectrometry analysis of RACK1 IPs.

At p=0.05, 116 proteins were deemed to co-immunoprecipitate with RACK1 (GNB2L1) and these proteins are listed in the table below.

List from Perseus	Edited list
RPL23A	RPL23A
EWSR1	EWSR1
VAR5	VAR5
MCM5	MCM5
CCT8	CCT8
IDH2	IDH2
ATP5C1	ATP5C1
RPL18A	RPL18A
EIF3D	EIF3D
PHB2	PHB2
HNRNPC	HNRNPC
CCT5	CCT5
TKT	TKT
HADHB	HADHB
HNRNPUL1	HNRNPUL1
RPL31	RPL31
CCT7	CCT7
RPL37A	RPL37A
MYOF	MYOF
RANBP1	RANBP1
RPL24	RPL24
PHB	PHB
EIF4G1	EIF4G1
RBM39;RNPC2	RBM39
LARP1	LARP1
RPL30	RPL30
RPS24	RPS24
PABPC1;PABPC3	PABPC1
RPL15	RPL15
PRKDC	PRKDC
RPL28	RPL28
LARP4	LARP4
RPL8	RPL8
ATP5L	ATP5L
NONO	NONO
TRIM21	TRIM21
MTHFD1	MTHFD1
SEC23A;SEC23B	SEC23A
MYO1C	MYO1C
RPL6	RPL6
C14orf166	C14orf166
RPL36A-HNRNPH2;RPL36A;RPL36AL	RPL36A
TNRC6B	TNRC6B
DDX17	DDX17
UQCRC2	UQCRC2
PCMT1	PCMT1
RPL17	RPL17
RPS15	RPS15
NOP56	NOP56
DDX3X;DDX3Y	DDX3X
PSMD3	PSMD3
BANF1	BANF1
IGKC	IGKC
IGHG1;IGHG3;IGHG4	IGHG1
LMNA	LMNA
TFRC	TFRC
CSTB	CSTB

List from Perseus	Edited list
RPN1	RPN1
HIST1H2AC;HIST3H2A;HIST1H2AB	HIST1H2AC
CDK1	CDK1
NPM1	NPM1
PFN1	PFN1
PARP1	PARP1
HSPD1	HSPD1
DARS	DARS
HSPA6;HSPA7	HSPA6
PFKL	PFKL
NCL	NCL
FBL	FBL
GART	GART
RPS12	RPS12
PTBP1	PTBP1
GRN	GRN
RPL12	RPL12
DNAJA1	DNAJA1
MCM7	MCM7
MAP2K2	MAP2K2
RPL13A;RPL13AP3	RPL13A
EIF2S3;EIF2S3L	EIF2S3
GARS	GARS
RPS27;RPS27L	RPS27
NAMPT	NAMPT
VDAC2	VDAC2
RPS5	RPS5
RPS10;RPS10P5	RPS10
RPL29	RPL29
CCT3	CCT3
HNRNPA3	HNRNPA3
HNRNPM	HNRNPM
KPNA2	KPNA2
HNRNPH2	HNRNPH2
RAB2A	RAB2A
ARF1;ARF3;ARF5	ARF1
RPL27	RPL27
RPS7	RPS7
RPS23	RPS23
RPS18	RPS18
RPS13	RPS13
RPL10A	RPL10A
PPIA	PPIA
GNB2L1	GNB2L1
YBX1;YBX3	YBX1
SLC25A3	SLC25A3
HDLBP	HDLBP
CLTC	CLTC
PFKP	PFKP
TUBB3	TUBB3
EIF3A	EIF3A
DYNC1H1	DYNC1H1
PLEC	PLEC
CARM1	CARM1
AHNAK2	AHNAK2
DDX1	DDX1
FUBP3	FUBP3
OSBPL11	OSBPL11
NMES1;C15orf48	NMES1
DDX21	DDX21
NOP58	NOP58