RACK1 facilitates the activity and substrate specificity of PP2A in breast cancer cells

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Doctor of Philosophy

By

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2014
You, the people have the power...
the power to create happiness...
to make this life free and beautiful...
to make this life a wonderful adventure...
let us use that power...let us all unite
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DECLARATION
I hereby declare that I am the sole author of this thesis and that it has not been submitted for any other University or higher institution, or for any other academic award in this University. References and acknowledgements have been made, where necessary, to the work of others.

Signature:        Date:

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ABSTRACT

Breast cancer is a leading cause of cancer death worldwide but targeted therapies have proven to be very successful in the treatment of this disease. The aim of this study was to develop models and parameters that will help identify potential therapeutic targets and strategies for breast cancer treatment. We utilized the RTCA xCELLigence platform to develop protocols and determine the effect of novel compounds on breast cancer cells. We found that the vitamin K (VK) derivative, VK2 reduced the viability of Triple Negative Breast Cancer (TNBC) cells. Culturing the cells in media with a combination of VK2 and low glucose shows a further reduction in cell viability. This contributes to the body of knowledge about the benefits of calorie restriction (CR) in cancer patients. Using the platforms and parameters we developed, we expanded our study to investigate the effects of PRIMA1Met on cancer cell behaviour. PRIMA1Met is a small molecule that can revert mutant p53 back to Wild Type. Treatment of a panel of breast cancer cell lines with PRIMA1Met showed cell lines with mutant p53 were sensitive to the molecule. These results correlated very well with more traditional cell based assays. This is the first known analysis of breast cancer cells treated with either VK2 or PRIMA1Met conducted in real time.

We next characterised the interaction between RACK1 and PP2A in order to identify a possible role for the complex in breast cancer cells. It is known that RACK1 and PP2A bind directly in an IGF-1 dependant manner and play a role in cell migration. We confirmed that RACK1 was in a complex with both the PP2A C and A subunits. Mutation of the interaction sites between RACK1 and the PP2A C subunit allowed us to determine the consequences for disruption of the complex. Using stable cell lines expressing the mutant subtype, we showed that the RACK1/PP2A complex plays a role
in the progression of many important cellular processes including adhesion, proliferation, migration and invasion. It also plays a role in maintenance of the cancer phenotype.

We hypothesized that RACK1 was scaffolding PP2A to a novel set of substrates through its role as a signalling hub. To identify novel interacting proteins of the RACK1/PP2A complex we isolated the complex from breast cancer cells and used mass spectrometry to identify 66 novel binding partners of the complex. Our aim was to characterise some of these proteins with a particular focus on those involved in cancer. We chose Tau Tubulin Kinase-1 (TTBK-1) and Metadherin for further study and we suggest that TTBK-1 has the potential to be a prognostic indicator of disease. Gene expression analysis identified Metadherin to be upregulated in a cohort of breast cancer patient samples. We confirmed the interaction between Metadherin and the RACK1/PP2A complex and mapped potential binding sites for RACK1 on Metadherin.

These findings confirm the direct interaction between RACK1 and PP2A and are the first to characterise the interaction between these proteins in cancer cells. Our results show that the RACK1/PP2A complex is interacting with proteins known to be involved in the advancement of breast cancer disease and disturbance of the RACK1/PP2A complex shows anti-tumourigenic potential. We have determined that disruption of the RACK1/PP2A complex has implications for a wide range of cellular processes involved in maintenance and progression of cancer including migration and invasion and suggests that targeting the RACK1/PP2A complex may provide novel therapeutic approaches in cancer.
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>°C</td>
<td>Degrees Celsius</td>
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<td>µl</td>
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<td>A</td>
<td>Alanine</td>
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<td>AD</td>
<td>Alzheimers Disease</td>
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<td>ASBD</td>
<td>A subunit binding</td>
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<td></td>
<td>domains</td>
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<td>Cell Index</td>
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<td>CR</td>
<td>Calorie Restriction</td>
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<td>DMEM</td>
<td>Dulbeccos Modified</td>
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<td></td>
<td>Eagles Medium</td>
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<td>ER</td>
<td>Estrogen Receptor</td>
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<td>Human influenza</td>
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<td>Haemagglutinin</td>
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<td>Hepatocellular</td>
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<td>F</td>
<td>Phenylalanine</td>
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<td>FAK</td>
<td>Focal Adhesion Kinase</td>
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<td>FBS</td>
<td>Foetal Bovine Serum</td>
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<td>GNB2L1</td>
<td>Guanine Nucleotide</td>
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<td>Binding Protein, Beta</td>
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<td>Polypeptide 2-Like 1</td>
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<td>GST</td>
<td>Glutathione-S-Transfease</td>
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<td>GTP</td>
<td>Guanosine triphosphate</td>
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<td>GTPase activating protein</td>
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<td>HER2</td>
<td>Human Epidermal</td>
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<td>Growth Factor 2</td>
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<td>TBS</td>
<td>Tris Buffered Saline</td>
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<td>TBS-T</td>
<td>Tris Buffered Saline containing Tween-20</td>
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<tr>
<td>Thr</td>
<td>Threonine</td>
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<tr>
<td>TNBC</td>
<td>Triple Negative Breast Cancer</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
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<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
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<tr>
<td>TTBK-1</td>
<td>Tau Tubulin Kinase-1</td>
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Chapter 1:

General Introduction.
1.1 Breast Cancer

1.1.1 Breast Cancer Incidence

A comprehensive study of cancer prevalence between 2004 and 2008 records 28.8 million cases of cancer diagnosed worldwide. Cancer of the breast is the most commonly diagnosed cancer at 5.2 million cases worldwide (18% of all cases) (Figure 1.1) (Bray et al. 2013).

In America, breast cancer is estimated to be the most commonly diagnosed malignancy in women with a predicted 235,030 new cases in 2014 (Siegel et al. 2014). These numbers mirror those reported in Europe as breast cancer was estimated to be the most commonly diagnosed invasive malignancy in 2012 when taking both males and females into account at 13.48% (Figure 1.2) closely followed by cancers of the large bowel and lung at 12.99% and 11.92% respectively (Ferlay et al. 2013). Breast cancer is the leading cause of cancer mortality for females in Europe (Figure 1.3) and is third for overall cancer mortality when both sexes are taken into account (Ferlay et al. 2013) (Figure 1.4).

In Ireland, breast cancer is the most commonly diagnosed invasive cancer type in females accounting for 31% of all cancers (Comber 2014). It is responsible for 16.4% of cancer deaths making it the second leading cause of cancer death for women in Ireland. However, it is important to note that 5 year relative survival rates have increased year on year from 1994 to present and in 2010, they were estimated to be at 85% (Comber 2014). This is an indication that early screening methods and improved therapies are proving successful in the fight against breast cancer.
Figure 1.1: Worldwide Prevalence of cancer by site of disease.

Figure 1.2: Estimated European Cancer Incidence for both sexes in 2012. Breast cancer is the most commonly diagnosed malignancy at 13.48%.
Figure 1.3: Estimated Cancer Mortality in Europe (female), 2012. Breast cancer is the leading cause of cancer death in females at 16.85%.

Table 1.4: Estimated Cancer Mortality for both sexes in Europe, 2012. Breast cancer is the third most common cause of cancer mortality in Europe for both sexes; cancer of the lung is the leading cause of cancer death at 20.15% followed by cancer of the large bowel at 12.24%.
1.1.2 Breast Cancer Classification

For over a decade, breast cancer has been classified based on gene expression profiles of breast cancer patients (Perou et al. 2000, Sørlie et al. 2003, Van De Vijver et al. 2002). This classification has been pivotal in recognising that breast cancer is comprised of a number of distinctive and separate disease entities which show a large amount of heterogeneity (Sørlie et al. 2003, Norum et al. 2014). It has also led to more informed decisions on how to diagnose and treat breast cancer patients as well as allowing studies into prognosis based on gene expression profiles (van't Veer et al. 2002).

The four main intrinsic breast cancer subtypes are Luminal A, Luminal B, HER2+ and Basal-like (Goldhirsch et al. 2013). A normal breast like group has also been characterised and studied (Perou et al. 2000). Luminal A breast cancer accounts for 50-60% of breast cancer, making it the most common subtype (Eroles et al. 2012). Its characteristics include positive expression of the estrogen receptor (ER+), progesterone receptor (PGR+) and an absence of HER2 expression (HER2-) (Sørlie et al. 2001). The proliferation rate of this subtype has been determined to be low (<14%) based on Ki-67 immunohistological staining (Cheang et al. 2009). Patients with Luminal A breast cancer have better prognosis compared to other subtypes (Feeley et al. 2013) with a lower relapse rate (27.8%) and longer survival from time of first distant metastasis (median 2.2 years) (Kennecke et al. 2010). Ten year survival rates show that patients diagnosed with Luminal A tumours have the highest rate of survival of all the subtypes at 70% (Kennecke et al. 2010).

Luminal B breast cancer accounts for around 10-20% of total breast cancer cases. The vast majority of Luminal B tumours are ER+ and can be either HER2+ or HER2-. Just 6% of Luminal B tumours are reported to be ER-/HER2- (Eroles et al. 2012, Cheang et al. 2009). ER+/HER2- tumours are differentiated from Luminal A by a high
rate of proliferation with Ki-67 levels of >14% and a negative or low level of PGR (Goldhirsch et al. 2013). Patients with Luminal B breast cancer have a poorer prognosis when compared to the Luminal A subtype (Sørlie et al. 2001, Sørlie et al. 2003, Parker et al. 2009a). The tumours have more aggressive phenotype with a relapse rate of 42.9% for Luminal B HER2 수도 tumours and 47.9% for Luminal B HER2 수도 tumours (Kennecke et al. 2010). Median survival from first distant metastasis after relapse is less than Luminal A cases at 1.6 years (Kennecke et al. 2010). It has been reported that the HER2 수도 status of the Luminal B subtype drives the aggressive phenotype in ER 수도 tumours resulting in poorer prognosis (Jerjees et al. 2014). The ten year survival rate for patients with a Luminal B tumour is 54.4%.

The HER2 수도 subtype accounts for 15-20% of breast cancer cases (Sørlie et al. 2001). These tumours show overexpression of the HER2 gene which can be accompanied by overexpression of other genes (such as GRB7 and MED24) located in the same 17q12 chromosomal location (Perou et al. 2000). The majority of tumours in this subtype are ER 수도/PGR 수도 (Norum et al. 2014). These HER2 수도/ER 수도/PGR 수도 tumours are associated with aggressive disease and have been reported to have a recurrence rate of 51.4% with median survival from first metastasis after relapse at 0.7 years (Kennecke et al. 2010). Ten year survival for this subtype is 48.1% (Kennecke et al. 2010).

Basal-like breast cancer tumours are negative for ER, PGR and HER2 expression and represent 10-20% of breast cancer patients (Sørlie et al. 2001). The terms Basal-like and Triple Negative are sometimes used interchangeably however this is not entirely accurate as there can be up to a 20% difference between tumours classified as Basal-like and those classified as Triple Negative (Goldhirsch et al. 2013). Basal-like tumours are defined by the absence of ER, PGR, HER2 and presence of Epidermal Growth Factor Receptor (EGFR) and/or Cytokeratin(CK) 수도/6 (Nielsen et al.
Some tumours can be Triple Negative but may not be positive for EGFR or CK5/6. Basal-like tumours tend to have a younger demographic with a prominence in women of African origin and are characterised by an aggressive phenotype (Eroles et al. 2012). There is a 43.1% relapse rate for patients with a basal-like tumour after first distant metastasis and median survival after relapse is just 0.5 years. Ten year survival rate for this subtype is 52.6% with 62.6% of patients with Triple Negative (non-basal) tumours still alive after ten years. (Kennecke et al. 2010).

Another classification of note is Claudin-low breast cancer which is associated with poor prognosis (Prat et al. 2010). This subtype was identified much later than the main intrinsic subtypes described above and is characterized by low expression of genes known to play a role in cell-cell adhesion including claudin 3, 4 and 7 (Herschkowitz et al. 2007). This subtype share similarities with the Basal-like subtype in that they are mostly ER-/PGR-/HER2- but unlike the Basal-like tumours, they display high expression of a number of immune-response genes (Prat et al. 2010). To date, the Claudin-low subtype has not been fully recognised as an intrinsic breast cancer subtype in Europe (Goldhirsch et al. 2013).

### 1.1.3 Current Therapeutic Strategies

Increased survival rates for patients with breast cancer is a very welcome trend and can be directly attributed to screening programs and improved therapy (Berry et al. 2005, DeSantis et al. 2014). However, disease recurrence and relapse are still a huge problem as for the most part, breast cancer metastasis is incurable and can occur in up to 40% of patients (Guarneri and Conte 2009). Current therapeutic strategies for treatment of breast cancer are based on disease subtype and it has been shown that these subtypes act as both prognostic indicators and as indicators of response to therapy.
Luminal A tumour subtypes are suitable for endocrine therapy as they have a positive hormone status. Chemotherapy may also be recommended for this breast cancer subtype if certain criteria are met. For example, in America, adjuvant chemotherapy is advised if there is nodal positivity and similarly in Europe where there are 4 or more positive nodes (NCCN 2014, Goldhirsch et al. 2013). Other criteria for administration of adjuvant chemotherapy include advanced disease (grade 3) and/or a high recurrence score which is based on a 21-gene assay (NCCN 2014, Goldhirsch et al. 2013, Paik et al. 2004).

Luminal B tumours are recommended to be treated with endocrine therapy in combination with chemotherapy in the majority of cases (Goldhirsch et al. 2013). Luminal B tumours that are HER2$^+$ can also be treated with anti-HER2 therapy along with both endocrine therapy and chemotherapy (Goldhirsch et al. 2013). Even though, Luminal B tumours have a poorer prognosis compared to Luminal A, they can respond better to chemotherapy (Paik et al. 2004, Parker et al. 2009b). A number of clinical trials, both completed and on-going have examined the use of inhibitors targeting the PI3K/AKT/mTOR pathways for this cancer subtype (clinicaltrials.gov).

Patients diagnosed with HER2$^+$ tumours have benefitted greatly in recent years from the development of direct HER2 therapies. Trastuzumab is a monoclonal antibody that targets HER2$^+$ tumours (Vogel et al. 2002). It is approved by both America and Europe to be used as a treatment for HER2+ breast cancer in conjugation with chemotherapy (NCCN 2014, Goldhirsch et al. 2013). This availability of a targeted therapy has reduced the recurrence rates and reduced the mortality rates that were associated with this subtype (Ross et al. 2009, Arteaga et al. 2011). Laptinib is another drug which has been approved specifically for the treatment of HER2$^+$ breast cancer patients with advanced disease. This drug alone has been shown to inhibit HER2 and
has shown promise when used in combination with trastuzumab (Konecny et al. 2006). Resistance to trastuzumab has become an increasing issue and it has been suggested that lapatinib may have an effect on patients that have acquired resistance to trastuzumab (Arteaga et al. 2011).

There is no targeted therapy currently available for patients with Basal-like breast tumours, making it difficult to treat. Chemotherapy is the recommended treatment for this subtype and a subset of patients with this tumour type do respond (NCCN 2014, Goldhirsch et al. 2013, Conte and Guarneri 2009). The limited treatment options for this subtype make it an essential and pertinent area of research as this subtype is associated with particularly aggressive disease (Conte and Guarneri 2009). Strategies currently being explored are hoped to be successful in targeting Basal-like breast cancer. For example, poly-ADP ribosepolymerase (PARP-1) inhibitors have been the subject of clinical trials where they have shown encouraging effectiveness against triple negative breast cancer cells in combination with chemotherapy (O’Shaughnessy et al. 2009) and alone (Tutt et al. 2009, Tutt et al. 2010).
1.2 Receptor for Activated C Kinase 1 (RACK1)

Receptor for Activated C Kinase 1 (RACK1) is an adaptor protein that plays a fundamental role in many important physiological processes including development, central nervous system function, circadian rhythm, immune response, cell adhesion, cell proliferation, cell migration and protein synthesis (reviewed in (Adams et al. 2011, Ron et al. 2013, Gandin et al. 2013, McCahill et al. 2002)). RACK1 binds to a diverse array of proteins with up to 90 identified to date (Adams et al. 2011). It has four main influences on its binding partners; recruiting proteins and shuttling them to substrates thus allowing for activation of downstream signalling pathways, modifying the activity of the partners, changing intermolecular interaction and modulating the stability of binding proteins (Li and Xie 2014). RACK1 plays a role in so many essential physiological processes, it is no surprise that changes in RACK1 expression have been associated with many diseases and disorders including Alzheimer’s Disease (BATTAINI and PASCALE 2005), bi-polar disorder (Wang and Friedman 2001), addiction (He et al. 2002, Escribá and García-Sevilla 1999, Wan et al. 2009) and cancer (reviewed in (Li and Xie 2014)).

1.2.1 RACK1: Gene and expression

Human RACK1 is encoded by the gene guanine nucleotide binding protein, beta polypeptide 2-like 1 (GNB2L1) which is located on chromosome 5q35.3 (Del Vecchio et al. 2009, Wang et al. 2003). GNB2L1 codes for a protein with 317 amino acids which registers as a 36 kDa protein on an SDS-PAGE gel (Adams et al. 2011). The GNB2L1 gene is evolutionarily conserved across such diverse species as *Tetrahymena thermophila*, *Saccharomyces cerevisiae* and *Drosophila melanogaster* (Adams et al.
2011). RACK1 is highly expressed in many tissue types including the brain, kidney, bone marrow, thymus and liver (Ashique et al. 2006, Chou et al. 1999).

1.2.2 RACK1: Protein structure

RACK1 is a member of the tryptophan, aspartic acid repeat (WD-repeat) family of proteins. This family of proteins are ancient and well-conserved and are found in both prokaryotes and eukaryotes (McCahill et al. 2002). WD-repeats are sequences of 44-60 amino acids typically ending with a WD dipeptide at the C-terminus (Adams et al. 2011). Genes encoding WD-repeats have been found on almost all chromosomes except for chromosome 20 and 22. This is reflective of their importance as a family (Adams et al. 2011). RACK1 is a protein with seven WD-repeats which form a β-propeller structure. Four-stranded antiparallel β-sheets make up each propeller structure. RACK1 is homologous to G-protein β subunit which is the most extensively studied member of the WD-repeat family of proteins. The crystal structure of RACK1 was published recently at a resolution of 2.45 Å (Ruiz Carrillo et al. 2012).

1.2.3 RACK1: Role as a scaffolding protein

It is well established that RACK1 functions as a scaffold and a shuttle for proteins within distinct and diverse signalling pathways to regulate fundamental cellular processes including cell proliferation and protein synthesis (reviewed in (Adams et al. 2011, Ron et al. 2013)). RACK1 was initially identified as a binding partner of PKCβII (Ron et al. 1994). RACK1 binds to activated PKCβII and it shuttles PKCβII to locations in the cell where it is in closer proximity to its substrates, allowing the kinase to phosphorylate its targets (Stebbins and Mochly-Rosen 2001, Ron et al. 1999, Ron et al. 1994), Ron et al., 1999, Ron et al., 1994). In contrast to this, RACK1 binds to the tyrosine kinases Src and Fyn to maintain these kinases in an inactive state until the
Figure 1.5: Amino acid sequence of human Receptor for Activated C Kinase (RACK1). (a) The GNB2L1 gene codes for RACK1, a protein with 317 amino acids. The seven tryptophan-aspartic acid (WD) repeats are shown in red (WD1), orange (WD2), yellow (WD3), green (WD4), blue (WD5), purple (WD6), pink (WD7). (b) Model of RACK1 structure depicting the seven WD repeat domains coloured as in (a). Modelling was completed using the resolved human RACK1 crystal structure (PDB code 4AOW).
appropriate signal occurs to trigger dissociation of the complex. For example, IGF-1 stimulation activates Src by mediating the release of RACK1 from Src (Kiely et al. 2005). The scaffolding function of RACK1 is mediated by the presence of seven WD-repeats. These repeats can bind to numerous protein domains including SH2 domains (e.g. Src and Fyn) (Chang et al. 2002, Yaka et al. 2002), pleckstrin homology (PH) domains (e.g. dynamin and p120GAP) (Koehler and Moran 2001, Rodriguez et al. 1999) and C2 domains such as Protein Kinase C (PKC) (Ron et al. 1995).

1.2.4 RACK1, the IGF-IR and PP2A

RACK1 interacts with several receptors including the Insulin-like Growth Factor Receptor I (IGF-IR) (Kiely et al. 2002), β-integrin receptor and the common beta-chain of the IL-5/IL-3/GM-CSF receptor (Geijsen et al. 1999). The interaction between RACK1 and the IGF-IR is particularly well studied (Hermanto et al. 2002, Kiely et al. 2002, Kiely et al. 2005). RACK1 interacts with the IGF-IR in a wide variety of cells and the interaction site has been mapped to the C-terminus of the IGF-IR. At the IGF-IR, RACK1 recruits a series of proteins including the adaptor proteins, Shc and IRS-1/2, the phosphatase, Shp2, the transcription factor, STAT3 (Kiely et al. 2002, Kiely et al. 2005, Zhang et al. 2006) and Focal Adhesion Kinase (FAK) (Kiely et al. 2009). This results in construction of protein complexes built by RACK1 to facilitate signalling downstream of the IGF-IR. RACK1 also binds the β subunits of integrin (Liliental and Chang 1998) integrating and facilitating signalling between the IGF-IR and integrins during tumour cell migration (Hermanto et al. 2002, Kiely et al. 2002, Kiely et al. 2009). This is mediated in part by the serine/threonine phosphatase PP2A as RACK1 binds PP2A and β1 integrin directly in a mutually exclusive manner (Kiely et al. 2008, Kiely et al. 2006). PP2A and β1 integrin compete to bind with RACK1 on Tyr302. In serum-starved
cells, RACK1 is bound to PP2A, however upon stimulation with IGF-1, PP2A disassociates with RACK1 and β1 integrin then binds to RACK1 at the same site. This interaction between RACK1 and PP2A stabilizes the activity of PP2A (Kiely et al. 2006). This is an example of the role RACK1 plays in stabilizing its binding partners. By bridging the IGF-IR and β1 integrin into one complex, RACK1 is facilitating the IGF-I and adhesion-mediated recruitment of other proteins to the complex to promote cell migration. Binding of RACK1 to integrins also promotes the engagement of survival signalling pathways (De Toni-Costes et al. 2010), the MAPK pathway (Vomastek et al. 2007) and the recruitment of several PKC isoforms (Besson et al. 2002, Buensuceso et al. 2001, Lee et al. 2002).

1.2.5 RACK1: Role in cancer

RACK1 has been identified as a factor in the progression of many cancer types including hepatocellular carcinoma, metastatic melanoma, ovarian cancer and cancers of the oesophagus, prostate, lung and breast (Ruan et al. 2012, Bourd-Boittin et al. 2008, Egidy et al. 2008, Williams et al. 2004, Li et al. 2012, Hu et al. 2013, Hellberg et al. 2002, Shi et al. 2012, Xi-Xi Cao et al. 2010, Al-Reefy and Mokbel 2010). There are several mechanisms by which RACK1 contributes to cancer progression. For example, the interaction between RACK1 and FAK plays a role in cancer where this interaction is required at the leading edge of polarized cancer cells to sense direction (Serrels et al. 2010). This RACK1/FAK complex binds to PDE4D5 to control adhesion signalling (Serrels et al. 2011, Serrels et al. 2010). RACK1 also regulates paxillin, integrins and Src to regulate cell adhesion and spreading (Cox et al. 2003, Kiely et al. 2005, Liliental and Chang 1998, Kiely et al. 2009). RACK1 contributes to the progression of
Figure 1.6: Interaction between RACK1 and PP2A. PP2A and β1 integrin compete to bind with RACK1 on Tyr302 downstream of the IGF-IR. (a) In serum-starved cells, RACK1 is bound to PP2A. (b) Upon stimulation with IGF-1, PP2A disassociates with RACK1 and β1 integrin then binds to RACK1 at the same site.
cancer through angiogenesis where it is up-regulated and promotes activation of the PI3K/Akt-Rac1 pathway (Berns et al. 2000, Wang et al. 2011).

RACK1 interacts with around 80 proteins either directly or indirectly in large complexes. Many of these proteins are phosphatases and kinases whose activity is altered in cancer. RACK1 also scaffolds many central components of the MAPK pathway (Vomastek et al. 2007) and the PI3K pathway (Hermanto et al. 2002, Kiely et al. 2002) which can also be dysregulated in cancer. RACK1 is involved in so many fundamental processes which require tight regulation and so, even subtle changes in the protein expression of RACK1 can be expected to have consequences on the regulation of its binding partners within key signalling pathways.
1.3 Protein Phosphatase 2A (PP2A)

1.3.1 Role of phosphatases in cellular signalling

Reversible phosphorylation is the most common mechanism of signal transduction in eukaryotic cells and is mediated by a series of phosphatases (Hunter 1995, Pawson and Scott 1997). These phosphatases allow proteins to react rapidly to external environmental cues by tightly regulating the activity, location and substrate specificity of the proteins. Disruption of the regulation of protein phosphorylation can lead to aberrant cellular behaviour and result in many diseases including cancer (Eichhorn et al. 2009, Tonks 2006, Östman et al. 2006, Gallego and Virshup 2005, Stebbing et al. 2013). Protein Phosphatase 2A (PP2A) is a major serine/threonine phosphatase that is expressed ubiquitously in animals. It is one of the most conserved eukaryotic proteins and together with PP1, is responsible for up to 90% of all serine/threonine activity in a cell (Eichhorn et al. 2009, Arroyo and Hahn 2005, Westermarck and Hahn 2008). As a phosphatase, PP2A is essential to promote the post translational modifications that reverse kinase activity in all of the major cell signalling pathways such as those that regulate the cell cycle, metabolism, cell migration and survival (Janssens and Goris 2001, Schönthal 2001, Seshacharyulu et al. 2013, Perrotti and Neviani 2013).

1.3.2 PP2A: Structure and assembly

PP2A is, in fact, a complex of 3 specific and individual subunit proteins that function as a holoenzyme. The core dimer consists of a 65kDa scaffolding subunit (A) and a 36kDa catalytic subunit (C). In mammals, the A subunit exists in two isoforms (Aα and Aβ) that share almost 87% sequence identity (Hemmings et al. 1990). Despite the similarity, approximately 90% of PP2A holoenzymes contain the Aα isoform leaving
Figure 1.7. PP2A Structure and assembly. (a) Structural representation of the PP2A holoenzyme. (b) Isoforms of the different subunits of PP2A, their nomenclature and chromosomal location (reviewed in Seshacharyulu et al. 2013).
just 10% of holoenzymes containing the Aβ isoform (Yang and Phiel 2010). This suggests that there is divergent function of the A subunits and is illustrated by the fact that Aβ is unable to substitute for Aα in mice (Eichhorn et al. 2009). Aα and Aβ are detected primarily in the cytoplasm but differ in their ability to bind to the B regulatory subunits (Zhou et al. 2003). Aβ is detected at high levels in embryogenesis but is rare in adult tissues (Bosch et al. 1995) and Aα increases gradually as Aβ decreases after late embryogenesis (Hendrix et al. 1993). Two isoforms of the catalytic subunit also exist (Cα and Cβ). Cα localises primarily in the plasma membrane with Cβ localising primarily to the cytoplasm and nucleus (Stone et al. 1987, Seshacharyulu et al. 2013, Chen et al. 2013). Both isoforms have been detected in many rat and porcine tissue types including brain, heart, liver, kidney and ovaries but they are most abundant in the brain and heart (Yeesim Khew-Goodall 1991).

Full activity, specific subcellular location and substrate specification is conferred upon PP2A only when the core dimer interacts with the B regulatory subunit to form a heterotrimeric holoenzyme (Xu et al. 2006). In humans, at least 26 different B subtypes have been identified to date (Seshacharyulu et al. 2013). These are alternate transcripts and splice forms encoded from 15 genes (Eichhorn et al. 2009, Seshacharyulu et al. 2013). This allows up to 96 possible combinations of the assembled holoenzyme (Janssens et al. 2008). The expression levels of these subunits is highly variable and is dependent on both cell and tissue type (Xu et al. 2008). The diversity in regulatory subunit availability is primarily responsible for the widespread substrate specificity and function of the PP2A holoenzyme. As well as this, the PP2A holoenzyme has the ability to interchange B subunits rapidly allowing PP2A to respond quickly to specific environmental cues (Janssens et al. 2008). The variable regulatory B subunits are categorized into 4 recognised subfamilies; B/B55/PR55, B’/B56/PR61,
B′′/B72/PR72 and B′′′/PR93(SG2NA)/PR110(Striatin). In contrast to the A and C subunits, the sequence and structural similarity amongst the 4 regulatory subfamilies is very low and is a major contributor to the diversity of PP2A holoenzymes location and function (Sents et al. 2013, Eichhorn et al. 2009, Chen et al. 2013).

A large body of work has been done to determine specific functions of known PP2A regulatory subunits and is reviewed extensively (Seshacharyulu et al. 2013, Eichhorn et al. 2009, Yang and Phiel 2010, Janssens et al. 2005, Kurimchak and Graña 2012b). PP2A-B56 is the best studied regulatory subunit subfamily. This subfamily has at least eight isoforms which are represented by five different genes (PPP2R5 α-ε) (Eichhorn et al. 2009, Sents et al. 2013). PP2A-B56α, PP2A-B56β and PP2A-B56ε are expressed in the cytoplasm of cells. The PP2A-B56y1-4 isoforms are expressed in the nucleus while PP2A-B56δ is expressed in both the cytoplasm and nucleus (Eichhorn et al. 2009). Substrates of the PP2A holoenzyme containing B56 include the basic proteins, shugoshins (Sgo1) (Kitajima 2006, Tanno et al. 2010). Together, PP2A and Sgo1 function to protect centromeres from premature chromosome segregation in both mitosis and meiosis.

There are at least 6 members of the PP2A-B55 family which are the products of 4 different genes (PPP2R2 α-δ) (Eichhorn et al. 2009, Sents et al. 2013). PP2A-B55α and PP2A-B55δ are, for the most part, expressed ubiquitously. PP2A-B55β and PP2A-B55γ are highly expressed in the brain and are tightly controlled during development (Strack et al. 1999). Some of the proteins that have been shown to directly interact with the PP2A-B55 include AKT, p53, SRC, RAF1, pRB, p107, p130, CDK1 and KSR1 (Eichhorn et al. 2009, Adams et al. 2005, Ory et al. 2003). In humans, there are three genes coding for members of the PP2A-B72 subfamily (PPP2R3 A-C)
Figure 1.8: Amino acid sequences of the PP2A Catalytic isoforms α and β. Transcription from the PPP2CA gene results in an isoform of the PP2A Catalytic subunit that is 309 amino acids in length. Transcription from the PPP2CAB gene results in an isoform of the PP2A Catalytic subunit that is also 309 amino acids in length. The sequences were aligned using Clustal Omega 1.2.1. The sequences share 98% homology. Differences between the sequences are highlighted in red.
(Sents et al. 2013). The best known members of this subfamily include PR72, PR130, PR70 and PR48 (Eichhorn et al. 2009). Most known functions of PP2A-B72 involve the regulation of the G1/S part of the cell cycle and the regulation of DNA replication during S phase (Eichhorn et al. 2009).

Three genes, STRN, STRN3 and STRN4 code for the striatin proteins that make up the B”’ subtype (Sents et al. 2013). S/G2 Nuclear Autoantigen (SG2NA) and striatin are two proteins in the B”’ subfamily (Moreno 2000). Striatin is found in high numbers in post synaptic membranes. S/G2 Nuclear Autoantigen (SG2NA), targeted to the nucleus, is expressed mostly during the S/G2 phases of the cycle (Janssens et al. 2008).

1.3.3 PP2A: A key regulator in Growth Factor signalling

PP2A has also been shown to play a considerable role in the regulation of growth factor signalling (Ugi et al. 2004, Kiely et al. 2006, Kiely et al. 2008, Ugi et al. 2002, Yoshizaki et al. 2004). Stimulation of cells with EGF, Insulin and IGF-1 inhibits PP2A activity and promotes sustained activation of the MAP kinase pathway (Ugi et al. 2002). The growth factor stimulation causes disassociation of PP2A from the adaptor protein Shc. This leads to increased and sustained Shc phosphorylation and subsequent activation of the MAP kinase pathway to promote cell proliferation (Ugi et al. 2002). In gastric cancer cells, the EGFR positively regulates the expression of the PP2A inhibitor CIP2A (Khanna et al. 2011) to drive metastasis.

Downstream of the Insulin receptor, PP2A directly dephosphorylates Akt on Ser473 and Thr308 to negatively regulate metabolic signalling (Ugi et al. 2004, Yoshizaki et al. 2004). The PP2A-B56 regulatory subunit has been implicated in the negative regulation of insulin signalling through Akt dephosphorylation at Thr308 (Padmanabhan et al. 2009). However, the PP2A holoenzyme containing PP2A-B55α
has also been identified as a regulator of Akt phosphorylation at Thr308 acting to negatively regulate Akt mediated cell proliferation and survival (Kuo et al. 2008).

In breast cancer cells, PP2A associates with the scaffolding protein RACK1 in an IGF-1 dependant manner and regulates cell migration and proliferation by controlling the formation of a complex between the IGF-IR, RACK1 and β1 integrin (Kiely et al. 2006, Kiely et al. 2008). In this context, it is not PP2A activity that is the key regulator; rather, it is the ability of PP2A to compete with β1 Integrin for binding to Tyr302 of RACK1.

1.3.4 PP2A: Role in cancer

Mutations in PP2A subunits have been found in a variety of human cancers and include deletions, point mutations and mutations that generate alternate transcripts (Perrotti and Neviani 2013, Kurimchak and Graña 2012a). Some of these mutations prevent the A subunit from binding to the B and C subunits. This can cause disruption of the core complex which has implications for PP2A activity (Ruediger et al. 2001a, Ruediger et al. 2001b, Chen et al. 2013, Sablina et al. 2007). The gene encoding the Aα subunit, PPP2R1A has been shown to be mutated in just over 40% of high grade endometrial tumours (McConkey et al. 2011). PPP2R1A has also been shown to be mutated in 9.1% of Type I ovarian tumours and 6.8% of Type I uterine carcinomas (Shih et al. 2011). As well as this, specific mutations of PPP2R1A have been identified in lung carcinoma, breast carcinoma and melanoma. These mutations result in a decreased binding to other PP2A subunits (Ruediger et al. 2001b, Calin et al. 2000). In mice expressing two Aα mutations (E64D and E64G), the presence of either mutation resulted in defective binding of the Aα subunit to B subunits (Ruediger et al. 2011). Mice expressing the E64D mutation demonstrated increased incidence of p53 dependant
l lung cancer in a p53 dependant manner (Ruediger et al. 2011). This study highlights the role of PP2A as a tumour suppressor and suggests that disruption of the PP2A holoenzyme may contribute to the development of carcinogenesis.

PPP2R1B, the gene encoding the Aβ subunit of PP2A is located at 11q23 which is a chromosomal region that is frequently deleted in cancer cells (Baysal et al. 1998). PPP2R1B is mutated in 15% of both primary lung tumours and colorectal carcinomas (Wang et al. 1998). One PPP2R1B mutation seen in 6% of lung cancer cell lines was defective in binding to the PP2A C subunit (Wang et al. 1998). Five missense mutations of PPP2R1B have been identified in colorectal tumours (Takagi et al. 2000). Other PPP2R1B mutations have been detected in human hepatocellular carcinomas (Chou et al. 2007) and are also found in melanoma and cancers of the breast and lung (Calin et al. 2000).

PP2A B subunit mutations are less frequent, but some deletion mutations have been identified in breast, prostate and ovarian cancers (Curtis et al. 2012, Cheng et al. 2011, Network 2011, Kurimchak and Graña 2012a). Reduced expression of PP2A-B55α has been reported in leukaemia (Ruvolo et al. 2011). PP2A-B55β is epigenetically inactivated by DNA hypermethylation in colorectal cancer which has consequences for myc signalling (Tan et al. 2010). PP2A-B55β has also been shown to be mutated by hypermethylation in breast cancer (Muggerud et al. 2009).

1.3.4.1 PP2A: Role in cancer. Viral targeting of PP2A.

There is very strong evidence to suggest that certain PP2A holoenzymes are genuine tumour suppressors. This tumour suppressor function for PP2A was realised when it was shown that okadaic acid, a tumour promoter, inhibits PP2A (Bialojan 1988). PP2A has a regulatory function in many signalling pathways that control cell growth, cell
migration and apoptosis. This makes it an obvious target for oncogenes targeting deregulation of the cell cycle. In transformed cells, several mechanisms have evolved that inhibit PP2A and target carcinogenesis (Marc 2007). For example, some DNA tumour viruses produce viral proteins that displace B subunits from PP2A and can function as pseudo B subunits themselves (Guergnon et al. 2011, Eichhorn et al. 2009). This results in altered activity and location of PP2A, leading to dysregulation of the signalling pathways where PP2A normally functions (Eichhorn et al. 2009). Retroviruses such as HIV1 VPR also interfere with PP2A to induce cell cycle arrest (ZHAO and T ELDER 2005, Guergnon et al. 2011).

Polyoma small t (pyst), polyoma middle T (pyMT) and simian virus small t (ST) are viral proteins that can form complexes with the PP2A core dimer (Walter et al. 1990, Pallas et al. 1990, Campbell et al. 1995, Cristobal et al. 2014). When pyMT displaces the B subunit of the PP2A holoenzyme, the MAP kinase pathway is activated and interaction of pyMT with PP2A is required for pyMT induced transformation (Campbell et al. 1995). A number of PP2A B subunits inhibit and activate proteins in the MAP kinase signalling pathways including RAF and ERK (Eichhorn et al. 2009). PP2A-B56\textsuperscript{y} dephosphorylates ERK which inhibits ERK activity (Letourneux et al. 2006). PP2A-B55 has been shown in a variety of capacities within this pathway. PP2A-B55\textsubscript{α} or PP2A-B56 positively regulate RAF1 MAP kinase signalling by dephosphorylating an inhibition site on RAF1. PP2A-B55\textsubscript{α} dephosphorylates key 14-3-3 binding sites on Kinase Suppressor of RAS (KSR1) which also positively regulates the MAP kinase pathway (Ory et al. 2003). These specific holoenzymes can also negatively regulate MAP kinase signalling by dephosphorylating and deactivating ERK further downstream of RAF (Silverstein et al. 2002) (Figure 1.9). The ability of pyMT to specifically activate the MAP kinase pathway suggests that deregulation of PP2A
assembly and disruption of PP2A-B55α or PP2A-B56 binding to the holoenzyme promotes deregulated growth control (Rodriguez-Viciana et al. 2006).

Small t antigen (ST) is a simian virus 40 ER viral onco-protein which when co-expressed with large T antigen, the telomerase catalytic subunit and the H-RAS oncogene, is able to transform human cells. ST requires interaction with PP2A in order for this transformation to occur (Hahn 2002, Yu et al. 2001, Marc 1995). ST binds to the Aα subunit to regulate the Akt pathway (Eichhorn et al. 2009). ST also displaces PP2A-B55α, PP2A-B56y and PP2A-B56y3 regulatory subunits and when bound to the core subunit, ST will suppress PP2A activity (Chen et al. 2004). Displacement of a subset of these PP2A complexes (PP2A-B56γ and PP2A-B56γ3) increases cell proliferation and confers an ability to grow in an anchorage independent manner leading to tumour development in animal hosts (Chen et al. 2004). Induction of increased cell proliferation in cells where PP2A has been displaced by ST has previously been shown to be mediated by stimulation of the MAP kinase pathway (Sontag et al. 1993, Ugi et al. 2002). ST, when interacting with PP2A, directly regulates the dephosphorylation of Akt. In cells where growth factors are absent, ST interaction with PP2A enhances the anti-apoptotic activity of Akt. Conversely, in the presence of growth factors, ST interaction with PP2A enhances the pro-apoptotic activity of Akt. (Andrabi. S. 2007).

1.3.4.2 PP2A: Role in cancer. Inhibition of PP2A

Okadaic acid (OA) is a well-known PP2A inhibitor and carcinogen (Bialojan 1988, Suganuma et al. 1988, Fujiki and Suganuma 1993). There are other mechanisms of PP2A inhibition that contribute to carcinogenesis. Cancerous Inhibitor of PP2A (CIP2A) is a PP2A inhibitor which when over expressed, is associated with poor outcome (Lin et al. 2012, Junttila et al. 2007). Specifically, CIP2A inhibits the activity
Figure 1.9: PP2A as a tumour suppressor within signalling pathways. The PP2A holoenzyme containing B55γ negatively regulates the MAP kinase pathway through inhibition of c-SRC (Eichhorn et al. 2009, Eichhorn et al. 2007, Stokoe and McCormick 1997). PP2A holoenzymes containing B56β and B56γ directly dephosphorylate ERK to negatively regulate the MAP kinase pathway (Letourneux et al. 2006). PP2A negatively regulates Akt activity through dephosphorylation of Akt at Thr 308 to regulate cell survival (Kuo et al. 2008). As well as functioning as a phosphatase, PP2A also controls cell migration by acting as a regulator of growth factor and adhesion receptor assembly through its interaction with RACK1 during cell migration (Kiely et al. 2006).
of PP2A which promotes stabilisation of the known oncogenic transcription factor c-Myc through phosphorylation on Ser62. CIP2A also promotes anchorage independent growth and tumour formation (Junttila et al. 2007). Transcription of CIP2A is decreased when the checkpoint kinase and DNA damage response protein, CHK1 is inhibited. This induces PP2A activity allowing dephosphorylation of c-MYC on Ser62 which impairs cancer cell survival (Khanna et al. 2013) Overexpression of SET, a potent PP2A inhibitor is associated with poor prognosis in Acute Myeloid Leukaemia (AML) (Cristóbal et al. 2012). Other PP2A inhibitors known to progress carcinogenesis include Cytostatin (Kawada et al. 1999) and Rubratoxin A which inhibit PP2A and suppresses metastasis (Wada et al. 2010).

1.3.4.3 PP2A: Role in cancer. Key regulator of apoptosis

The predominant perception of PP2A functioning solely as a tumour suppressor and a regulator of pathways that promote apoptosis is being challenged. A growing body of evidence suggests an anti-apoptotic role for PP2A. This was first noted in Drosophila (Van Hoof and Goris 2003) and in mammalian models where inactivation of PP2A induces apoptosis in a number of cancer cell types including cancers of the pancreas, testicles, liver and leukaemia (Wei Li et al. 2010, Schweyer et al. 2007, Duong et al. 2014, Lu et al. 2009, Boudreau et al. 2007). PP2A plays a dual regulatory role in apoptosis, facilitating both pro and anti-apoptotic signalling depending on the holoenzyme assembled and pathways targeted (Santoro et al. 1998, Li et al. 2002, MacKeigan et al. 2005, Ruvolo et al. 2001). Particularly well studied is the relationship between Bcl-2 and PP2A. PP2A dephosphorylates Bcl-2, however, depending on the cellular location, this can manifest as either a pro or anti-apoptotic signal (Ruvolo et al. 2001, Ruvolo et al. 2002, Simizu et al. 2004). For example, PP2A-B56α promotes apoptosis through dephosphorylation of Bcl-2 on Ser70 in the mitochondria (Ruvolo et
al. 2001, Ruvolo et al. 2002). However, PP2A inhibits apoptosis by dephosphorylating Bcl-2 on Ser87 in tumour cell lines and this inhibition is not seen in normal human blood cells (Simizu et al. 2004). This presents a good example of the ‘molecular tightrope’ that PP2A walks, and highlights the importance of holoenzyme assembly and the central role being played by the regulatory subunit which regulates the sub-cellular location of PP2A.

P53 is a major and extensively studied tumour suppressor protein that is mutated or deleted in many cancers (Brown et al. 2009). Under stress conditions such as DNA damage, p53 is stabilised and accumulated by phosphorylation events conferred by a number of stress activated kinases that induce cell cycle arrest or apoptosis (Ajay et al. 2010). Ser15 has been identified as an important phosphorylation site in the regulation of p53. A number of phosphatases, including PP2A, are known to play a regulatory role by targeting p53 phosphorylation on Ser15. For example, PP1 dephosphorylates p53 at this site as a negative regulator to promote cell survival (Li et al. 2006) and Wip1 which is involved in cell cycle arrest in tumour cells (Crescenzi et al. 2013). PP2A has been described as both a negative and positive regulator of p53 function through mediation of p53 phosphorylation on Ser15. PP2A regulates p53 by dephosphorylating and deactivating MDM2 (Okamoto et al. 2002). This results in the degradation of p53 by proteosomes which, in resting cells, keeps p53 levels in check. However, in cells that have accumulated DNA damage and cellular stress, the pro-apoptotic role of p53 can be dysfunctional. Inhibition of PP2A in this case, keeps p53 phosphorylated and therefore ‘active’ as a tumour suppressor (Ajay et al. 2010). In osteosarcoma cells, PP2A-B56γ3 interacts with p53 through an Ataxia Telangetasia Mutated (ATM) dependant mechanism. Phosphorylation of p53 by ATM at Ser15 promotes PP2A mediated dephosphorylation of p53 on Thr55 to inhibit cell proliferation. This, conversely, is
Figure 1.10: PP2A as a promoter of carcinogenesis. The PP2A holoenzymes containing B55α and B55δ play a positive regulatory role in the MAP kinase pathway through direct dephosphorylation of RAF1 on Ser259. The PP2A holoenzyme containing B55α also dephosphorylates KSR1 on Ser392 and RAF on Ser295 to activate ERK (Adams et al. 2005, Ory et al. 2003). Inhibition of PP2A by Cantharidin promotes apoptosis in cancer cells by mediating the prolonged phosphorylation of IκB kinase α (IKK) and activation of NFκB (Wei Li et al. 2011). Cantharidin also induces apoptosis by blocking PP2A mediated activation of the MAP kinase pathway (Wei Li et al. 2011, Schweyer et al. 2007). Inhibition of PP2A using okadaic acid increases the sensitivity of breast cancer cells to the drug lapatinib which has an anti-proliferative effect on cells (McDermott et al. 2014).
strong evidence in support of a tumour suppressor role for the PP2A holoenzyme containing B56γ (Shouse et al. 2008, Li et al. 2007). Mutations that disrupt the binding of B56γ to the A and C subunits in their own right have been shown to disrupt the p53 dependant tumour suppressor functions (Nobumori and Liu 2014).

1.3.5 PP2A: Therapeutic strategies

Even subtle changes in expression or activity of phosphatases will lead to a diseased state. In cancer, phosphatases present as a major focus of therapeutic targets as they are deregulated in almost all cancer types (Stebbing et al. 2013), and strategies that restore activity of PP2A merit serious consideration as novel treatments for cancer (Yang et al. 2012). Compounds are available that reverse the action of PP2A inhibition, to restore PP2A activity levels. Forskolin, (a PP2A activator) is used to reduce growth of AML cells and induce apoptosis through caspase dependant mechanisms (Cristobal et al. 2011). Ceramide is a bioactive lipid which activates PP2A through targeting of the interaction between PP2A and SET and has been shown to induce apoptosis in cancer cells (Dent 2013).

FTY720 is a sphingosine analog drug and a potent immunosuppressant that has been approved by the FDA as a treatment for multiple sclerosis (ms) after a series of successful clinical trials (Kappos et al. 2010, Khatri et al. 2011, O'Connor et al. 2009, Cohen et al. 2010, Brinkmann et al. 2010). FTY720 is an activator of PP2A (Matsuoka et al. 2003) and this mechanism of action has shown promise as an anti-cancer therapy in many pre-clinical studies. These studies have investigated the use of FTY720 as a potential therapy for a number of cancer cell types including but not limited to, neuroblastoma, bladder, renal, colorectal, breast, ovarian and lung cancers (Li et al. 2013, Azuma et al. 2003, Ubai et al. 2007, Cristobal et al. 2014, Marvaso et al. 2014,
Ning Zhang et al. 2013, Saddoughi et al. 2013) and an *in vivo* study of renal cancer using mouse models (Ubai et al. 2007). For example, in colorectal cancer, PP2A inactivation is a common occurrence and is accompanied by up-regulation of a number of well-known PP2A inhibitors including CIP2A (Cristobal et al. 2014). Treatment of colorectal cancer cells with FTY720 shows reduced proliferation and an increase in the pro-apoptotic factors caspase-3 and caspase-7 and appears to be accompanied by an increase in PP2A expression (Cristobal et al. 2014). As well as this, treatment of colorectal cells with FTY720 enhances the effect of a number of well-established chemotherapy drugs such as 5-fluorouracil and oxalipatin (Cristobal et al. 2014).

One pre-clinical study with breast cancer cell lines showed that cell lines associated with oestrogen receptor loss (ER-) were more sensitive to FTY720 than cells that express the oestrogen receptor (ER+) (Baldacchino et al. 2014). This is attributed to ER- breast cancer cell lines having suppressed levels of PP2A activity in comparison to ER+ breast cancer cell lines thus having a higher sensitivity to a PP2A activator such as FTY720. This predictive study suggests a potential assessment for patients where identification of a subset of patients with reduced PP2A activity could indicate their suitability for treatment with a PP2A activator such as FTY720.

Many pre-clinical studies have focused on the effect of FTY720 on leukaemia cell lines. A number of myeloid leukaemia (AML) cell lines are sensitive to FTY720, particularly those with a specific D816V mutation in the tyrosine kinase domain of C-KIT (Yang et al. 2012, Roberts et al. 2010). Cell lines with this mutation show inhibition of PP2A activity with decreased expression of the A subunit of PP2A, PP2A-B55α and PP2A-B56α, γ and δ (Roberts et al. 2010). The toxic effect of FTY720 in these cells is mediated by reactivation of PP2A. This reactivation occurs via downregulation of the PP2A inhibitor SET, upregulation of PP2A A subunit and PP2A-
B55α and dephosphorylation of the PP2A C subunit (Yang et al. 2012). Restoration of PP2A activity then promotes the induction of apoptosis and inhibition of proliferation (Yang et al. 2012, Roberts et al. 2010). Over expression of PP2A Aα in cells harbouring the D816V mutation also induces apoptosis and inhibits proliferation (Roberts et al. 2010).

However, reports also describe the merits of PP2A inhibition in certain circumstances. Overexpression of the catalytic subunit of PP2A in hepatocellular cancer models and virus infected cells disrupts p53 phosphorylation and inhibits p53 mediated apoptosis (Duong et al. 2014). Mouse models with the catalytic subunit of PP2A overexpressed, have more and larger hepatocellular tumours suggesting that PP2A has a role to play in tumour progression (Duong et al. 2014, Duong et al. 2004). Studies like these suggest that PP2A should be considered as a therapeutic target in some cancer types.

Cantharidan is a toxin isolated from *Mylabris*. It is a known anti-cancer agent (Wei Li et al. 2010) and a potent PP2A inhibitor (Honkanen 1993). Its anti-cancer effect, as seen in pancreatic cell models, is mediated through induction of pro apoptotic proteins including caspase-8 and caspase-9 as well as tumour necrosis factor- alpha leading to a subsequent, dose dependant increase in apoptosis. This is accompanied by a simultaneous decrease in the expression of the anti- apoptotic factor Bcl-2 (Wei Li et al. 2010). Inhibition of malignant testicular germ cell tumours using okadaic acid and cantharidan induces an anti-apoptotic effect through phosphorylation and subsequent activation of both MEK and Erk which, in turn, activate one of the most prominent inducers of apoptosis, caspase-3 (Schweyer et al. 2007). In leukaemia cells, PP2A inhibition again leads to activation of caspase-3 as well as activation of caspases-8 and caspase-9 leading to caspase dependant apoptosis, DNA fragmentation and
mitochondrial permeabilization (Boudreau et al. 2007, Riordan et al. 1998). However, cantharidin has very toxic side effects at concentrations > 10µmol/L and has been associated with poisoning, coma and death so even though it effectively induces apoptosis at lower concentrations (2-5µM), the associated side effects will more than likely prevent it from ever becoming a mainstream treatment for cancer (Bonness et al. 2006). A less toxic demethylated analog of cantharidin, norcatharidin, shows a promising ability to induce apoptosis in a number of cancer cell types including melanoma, breast, oral and gallbladder cancers with some effectiveness (Huang et al. 2009, Liu et al. 2011, Kok et al. 2003, Fan et al. 2007).

Work is continuing to develop small molecules that will inhibit PP2A with reduced side effects. LB-102 inhibits PP2A to increase the efficacy of some well-known chemotherapy drugs including doxorubicin in xenograft animal models of glioblastoma. In this situation, inhibition of PP2A blocks the DNA damage defence mechanisms in the cell that is being targeted by the chemotherapeutic agent (Lu et al. 2009). Inactivation of PP2A, in particular the holoenzymes involving the regulatory subunits B56γ and B56δ, induces phosphorylation of the apoptosis inducing protein Apoptin. Apoptin is a protein derived from an avian virus which, when phosphorylated, can induce apoptosis specifically in transformed human cells and not in normal cells (Zimmerman et al. 2012). Apoptin has been shown to work in a broad spectrum of transformed cells and is considered to be a potentially safe and effective anti-cancer treatment.

Increased PP2A activity has recently been shown to contribute to the mechanism of drug resistance in the HER2 positive subtype of breast cancer (McDermott et al. 2014). Lapatinib is a drug that is approved to treat patients with HER2 positive, metastatic, trastuzumab refractory breast cancer when used in combination with
capecitabine (Geyer et al. 2006). The drug prolongs median survival time (Geyer et al. 2006). In one study, increased PP2A activity has been identified as a contributory factor in the development of resistance to lapatinib (McDermott et al. 2014). PP2A activity was found elevated in two lapatinib resistant breast cancer cell lines. Both cell lines, as expected, demonstrated increased sensitivity to OA and OA induced inhibition of PP2A in these cells resulted in increased sensitivity to lapatinib. It was also demonstrated in this study that treatment of a lapatinib sensitive breast cancer cell line with the PP2A activator FTY720, decreased its sensitivity to lapatinib. Many other mechanisms of lapatinib resistance have undergone investigation (Liu et al. 2009, Aird et al. 2010, Rexer et al. 2011, Xia et al. 2006, Xia et al. 2010, Jegg et al. 2012), however the authors of this study suggest that PP2A has potential as a novel biomarker of lapatinib resistance and PP2A inhibition in combination with HER2 inhibition merits further investigation as a potential therapy for this type of breast cancer (McDermott et al. 2014).

PP2A is an indispensable enzyme in cells which criss-crosses cellular signalling pathways to regulate a huge number of proteins. In this review, our objective is to highlight that although best known as a tumour suppressor, PP2A has a dark side. Accumulating evidence points to inactivation of PP2A being an appropriate course of treatment in particular sets of cancer and PP2A has emerged as an attractive therapeutic target in malignancy.

1.4 Aims and objectives
The body of work contained in this thesis is focused on identifying new therapeutic strategies and targets in breast cancer. The first part of this thesis describes the design and execution of Real Time Cell Analysis protocols that were developed to investigate
how two novel compounds, VK2 and PRIMA-1\textsuperscript{Met}, affect the growth and transformed phenotype of triple negative breast cancer cells.

The second part of this thesis builds on previous studies and is aimed at getting a better understanding of how RACK1 regulates the activity and substrate specificity of PP2A. It has been postulated that signalling hubs like RACK1 can stabilize the activity of both kinases and phosphatases and that they direct enzymes around the cell in response to specific environmental cues. Using a series of synergistic and complementary cellular and molecular approaches, we set out to characterise the interaction between RACK1 and PP2A and to determine exactly how RACK1 scaffolds the catalytic subunit of PP2A. In doing so, we gain further insight into this dynamic relationship and establish the role of the RACK1/PP2A complex in the development and/or progression of breast cancer.

RACK1 does not have enzymatic activity but there are close to 80 known interacting proteins. Due to this prominent role as a scaffolding protein, we hypothesized that RACK1 serves to shuttle PP2A to specific subcellular locations where it can be in closer proximity to its substrates. The final aim of this research is to identify novel interacting binding partners of the RACK1/PP2A complex and to characterize a subset of these proteins to determine possible roles for the proteins in the progression of cancer.
Chapter 2:

Materials and Methods
2.1 Materials

2.1.1 General Chemicals and Reagents

Vitamin K2 (VK2) was purchased from Sigma-Aldrich Ltd (Wicklow, IE). PRIMA-1Met was obtained from Prof Michael. J. Duffy, Conway Institute, UCD. E-plates for the RTCA xCELLigence platform were purchased from ACEA Biosciences, (Cambridge, U.K). Prestained protein marker and 1kb DNA ladder were purchased from Fermentas (York, UK). Nitrocellulose membrane was purchased from Perkin Elmer (Dublin, IE). Protein G beads and Glutathione beads were purchased from Expedeon (Cambridgeshire, UK). Plasmid purification kits were purchased from Sigma-Aldrich Ltd. (Wicklow, IE). Culture inserts were purchased from Ibidi® (Munich, DE). Matrigel™ was purchased from Sparks Ltd (Dublin, IE.) The RNeasy Plus Mini Kit and MaXtract tubes were purchased from Qiagen (Manchester, UK). The Vilo cDNA synthesis kit was purchased from Invitrogen (Dublin, IE). Taqman® assays were purchased from Life Technologies (Dublin, IE). Endo-ribonuclease prepared siRNA (esiRNA) and siRNA were purchased from Sigma-Aldrich Ltd (Wicklow, IE). Lipofectamine 2000 and Oligofectamine™ were purchased from Life Technologies, (Dublin, IE). All other chemicals and salts were purchased from Sigma-Aldrich Ltd (Wicklow, IE).

2.1.2 Plasmid stocks and source

PP2A wild type was a gift from Professor David Pallas (Emory University School of Medicine, Atlanta) and was cloned together with PP2A C subunit mutant plasmids HA-FR/AA and HA-R214A. RACK1 constructs and empty vector plasmids (HA and GST) by Dr Patrick Kiely in the Laboratory of Cellular and Molecular Biology, UL. PP2A C subunit mutant plasmid HA-Y218F was made in the Laboratory of Cellular and
Molecular Biology, UL using G-block technology from Integrated DNA Technologies (Glasgow, UK). HA-Metadherin was a gift from Prof Paul Fisher (Virginia Commonwealth University, Richmond, VA, Canada).

2.1.3 Antibodies

The mouse anti-RACK1 antibody was purchased from BD Transduction Laboratories (Heidelberg, Germany). The mouse anti-PP2A C subunit antibody, anti-PP2A A subunit antibody and rabbit anti-Metadherin antibody were purchased from Cell Signalling Technology (Beverly, MA). The mouse anti-HA antibody was purchased from Roche (Dublin, IE). The mouse anti-Actin monoclonal antibody, anti-GST antibody and anti-Metadherin antibody were from Sigma-Aldrich (Wicklow, IE). Secondary antibodies were purchased from LICOR (Cambridge, UK) (for Western blotting), and Jackson Immunoresearch (Suffolk, UK) (for immunofluorescence).

2.1.4 Cell Culture Reagents

Dulbeccos Modified Eagles Medium (DMEM), RPMI 1640, foetal bovine serum (FBS), horse serum (HS), Penicillin/Streptomycin antibiotic mix, L-glutamine and Trypsin-EDTA were purchased from Sigma-Aldrich Ltd (Wicklow, IE). Optimem, Oligofectamine, Lipofectamine 2000 transfection reagents were purchased from Life Technologies (Dublin, IE).

2.1.5 Cell lines

Hs578T(i8)2 cells (human breast cancer cell line), MDA-MB-468 cells (human breast cancer cell line) and MDA-MB-453 (human breast cancer cell line) were all obtained from Prof Michael. J. Duffy, Conway Institute, UCD. MCF-7 cells (human breast cancer cell line) and MDA-MB-231 cells (human breast cancer cell line) were obtained from the Laboratory of Cellular and Molecular Biology, UL. MCF-7 cells and MDA-
MB-231 cells were maintained in DMEM supplemented with L-glutamine, penicillin/streptomycin and 10% FBS. Hs578T(i8)2, MDA-MB-468 cells and MDA-MB-453 cells were maintained in RPMI 1640 supplemented with L-glutamine, penicillin/streptomycin and 10% FBS.

2.2 Methods

2.2.1 Cell adhesion, spreading and proliferation using the RTCA xCELLigence system.

For experiments with VK2, MDA-MB-231 cells were harvested with trypsin/EDTA, washed with DMEM, and re-suspended in the DMEM with 10% FBS. The cells were counted using a haemocytometer. Cells were seeded in each well of the E-plate. The impedance values of each well were automatically monitored by the xCELLigence system and expressed as a cell index value (CI). The baseline impedance is recorded using control wells containing DMEM only with no cells (See Figure 2.1). VK2 was dissolved in ethanol and diluted to the required concentrations. The maximum amount of ethanol was added to cells and found to have no effect (data not shown). The appropriate concentration of VK2 was added to the wells of the E-plate. Unless otherwise stated, cells were seeded onto the E-plate at a density of 20,000 per well. The E-plate was then placed into the xCELLigence system. Scans were run with sweeps every minute for the first eight hours to detect early stages of cell adhesion and spreading. Subsequent sweeps were taken every 15 minutes for the duration of the experiment.

For experiments with PRIMA-1\textsuperscript{Met}, cells were harvested with trypsin/EDTA, washed with the appropriate culture medium (cell line dependant), and re-suspended in
Figure 2: Cell adhesion, spreading and proliferation using the xCELLigence. Cells are seeded into wells of an E-Plate. The electrodes at the base of wells detect the presence of the cells based on impedance which is measured by a Cell Index (CI) value. The CI increases as impedance increases due to cell adhesion, spreading and proliferation.
the appropriate culture medium with 10% FBS. The cells were counted using a haemocytometer. Cells were seeded in each well of the E-plate. The impedance values of each well were automatically monitored by the xCELLigence system and are expressed as a cell index value (CI). The baseline impedance is recorded using control wells containing DMEM only with no cells. PRIMA-1<sup>Met</sup> was dissolved in water and diluted to the required concentrations. The appropriate concentration of PRIMA-1<sup>Met</sup> was added to the wells of the E-plate. For this PRIMA-1<sup>Met</sup> study, unless otherwise stated, all cell lines were seeded onto the E-plate at a density of 10,000 per well. The E-plate was then placed into the xCELLigence system. Scans were run with sweeps every minute for the first eight hours to detect early stages of cell adhesion. Subsequent sweeps were taken every 15 minutes for the duration of the experiment.

2.2.2 Generation of stable cell lines.

To generate stable transfectants of PP2A mutants, MCF-7 cells were transfected with pcDNA3/HA-Empty Vector, pcDNA3/HA-PP2A(Wild Type), pcDNA3/HA-FR/AA, pcDNA3/HA-R214A and pcDNA3/HA-Y218F using Lipofectamine 2000 transfection reagent. Then, 24 hours post transfection the cells were split into DMEM medium containing 10% FBS, 10mM l-glu and G418 (1mg/ml) and maintained for 14 days, with regular replenishment of medium and drug. At this time the pool was expanded, and screened for expression of the HA tagged plasmids by Western blotting. Clones of MCF-7 cells stably overexpressing the plasmids were maintained in DMEM supplemented with 1 mg/ml G418.

2.2.3 Preparation of cellular protein extracts

Cellular protein extracts were prepared by placing cells on ice, removing media and washing three times in ice cold PBS. Cells were scraped into ice cold lysis buffer
(10mM Tris HCl pH 7.4, 150mM NaCl, NaF, 1% NP40) plus the tyrosine phosphatase inhibitor Na$_3$VO$_4$ (1mM), protease inhibitors PMSF (1mM), pepstatin (1µM) and aprotinin (1.5µg/ml). Lysates were incubated on ice for 20 minutes before centrifugation at 14,000 rpm for 15 minutes at 4°C to remove nuclei and cellular debris. Lysates were analysed for protein concentration using the Bradford assay and boiled in sample buffer for SDS-PAGE or used in immunoprecipitation experiments.

2.2.4 Immnoprecipitation of proteins from cellular extracts

Protein extracts were precleared with 20µl Protein G beads by incubation at 4°C for 1 hour rotating. The lysates were recovered from the beads by centrifugation at 3,000 rpm for 3 minutes and transferred to a new tube containing primary antibody (2µg), 40µl Protein G beads, 500µl lysis buffer and made up to 1ml with dH$_2$O. Samples were incubated at 4°C rotating overnight. Immune complexes were pelleted with the beads by centrifugation at 3,000 rpm for 3 minutes at 4 °C. The beads were washed three times with ice cold lysis buffer and removed from beads by boiling for 5 minutes in 25µl of 2x SDS PAGE sample buffer for electrophoresis and western blot analysis.

For a RACK1 IP, 5µl of linker antibody is also added to the sample when incubated overnight to ensure binding between the RACK1 antibody (IgM) and the protein G beads (IgG). Samples were incubated with an IP buffer (150 mM Tris-HCL, 1mM EDTA, 1mM EGTA, 1% deoxycholate) overnight instead of lysis buffer.

2.2.5 SDS-PAGE and Western Blotting

Protein samples for western blot analysis were separated by 12% SDS-PAGE gels. Following separation on the gel, proteins were transferred using electrophoresis onto a nitrocellulose membrane and blocked for 1 hour at room temperature shaking in 5%
milk (w/v) in TBS containing 0.5% Tween-20 (TBS-T). Membranes were incubated overnight at 4°C with primary antibody. Primary antibody dilutions were as follows:

- **Anti-PP2A C subunit**, 1:1000 TBS-T/5% milk
- **Anti-PP2A A subunit**, 1:1000 TBS-T/5% milk
- **Anti-RACK1**, 1:1000 TBS-T/5% milk
- **Anti-HA**, 1:1000 TBS-T/5% milk
- **Anti-GST**, 1:1000 TBS-T/5% BSA
- **Anti-Actin**, 1:5000 TBS-T/5% milk
- **Anti-Metadherin**, 1:1000 TBS-T/5% milk

Appropriate secondary antibodies (IRDye® 680LT and 800CW- Infrared Dye coupled anti-rabbit or anti-mouse (LI-COR Biosciences)) were diluted 1:10000 in TBS-T/5% milk for 1 hour. Antibody reactive bands were detected with the Odyssey® infrared imaging system (LI-COR Biosciences).

### 2.2.6 Immunofluorescence to detect protein co-localisation in cells

MCF-7 cells and the HA tagged PP2A mutant stable cell lines were seeded at a density of 5000 cells on 10 mm glass coverslips. The cells were fixed with 4% paraformaldehyde for 1 h and permeabilized by incubation in PHEM (60 mM PIPES, 25mM HEPES, 10mM EGTA, 2mM MgCl₂, made up with dH₂O, pH=6.9), 0.1% Triton X-100 for 15 min. The cells were rinsed three times in PHEM and blocked with 5% of normal goat serum (NGS) for 30 min and finally incubated for 2 h at room temperature with primary antibodies. The cells were then incubated for 1 h with the appropriate dye-labelled secondary antibodies: Cy3 (red), DyLight 488 (green) and Hoechst 33342 (stains nuclei). Coverslips were mounted using Vinol (Sigma) and cells were examined with a Zeiss LSM 710 META confocal laser scanning microscope equipped with an
argon/krypton laser. All experiments were analysed in sequential scanning mode. Images (1024 × 1024 pixels) were obtained with a x63 magnification oil-immersion objective. Images were merged and Pearson’s coefficients were calculated with MacBiophotonics Image J software.

2.2.7 PP2A Phosphatase Activity Assay

Cellular PP2A activity was measured using threonine phosphopeptide as the substrate with the PP2A immunoprecipitation phosphatase assay kit (Millipore). Cells were washed 3 times with PBS at 4°C before being lysed using a lysis buffer containing 20mM imidazole-HCL, 2mM EDTA, 2mM EGTA, pH 7.0 with 10µg/ml aprotinin and 1mM PMSF. Cells were sonicated for 10 seconds and centrifuged at 2000 x g for 5 min. Clarified supernatants were incubated with anti- HA antibody (4µg) with protein A agarose slurry for 18 h at 4º with gentle rocking. Beads were washed 3 times with 700µl TBS, followed by one wash with 500µl Ser/Thr assay buffer. The beads were then incubated with 60µl diluted phosphopeptide and 20µl Ser/Thr assay buffer at 30°C for 10 min in a shaking incubator. The beads were centrifuged briefly and the samples were analysed in a colorimetric assay using malachite green at an absorbance of 650nm.

2.2.8 Adhesion Assay

Collagen plates were prepared by coating wells of a 96 well plate with 100µl of 10µg/ml collagen (Type IV). The plates were incubated at 4°C overnight or alternatively incubated at 37°C for 2-3 hours. The wells were washed twice with PBS and blocked with 50µl PBS/2.5% BSA and incubated at 37° for 1-2 hours. Cells were washed 3 times with PBS. 2000 cells were plated into the pre-prepared wells and incubated at 37°C in 5% CO₂ for 1 hour. Then, cells were washed 3 times with PBS and fixed in 100µl methanol at -20°C for 5 minutes. The methanol was removed and cells
were stained with 0.1% crystal violet for 15 minutes at room temperature. Cells were carefully washed with water and left overnight to dry. The plates were then read at 590nm on a spectrophotometer.

2.2.9 Migration Assay

Plastic culture inserts with an adhesive bottom layer were placed into wells of a 24 well plate. 20,000 cells were plated into the wells containing the inserts. The cells were left overnight at 37 °C to adhere fully. The insert was removed which leaves a cell free gap of 500µm ± 50µm. The cells were photographed using a Nikon microscope with a x63 lens at 0 hours and 24 hours. The migration of cells was analysed using Ibidi® Quantitative Image Analysis.

2.2.10 Invasion Assay

Cell invasion was monitored in real-time with the xCELLigence system CIM-plates. 4 hours prior to conducting the experiment the PP2A stable mutant cells were serum starved. The upper chamber of the CIM plates was coated with 1 µg/µl of fibronectin and a 1:40 solution of Matrigel™. A total of 20,000 cells were seeded in each well of the upper chamber in serum-free media. DMEM media containing 10% FBS was added to each well of the lower chamber (See Figure 2.2). The CIM-plate was left in an 5% CO₂ incubator at 37°C for 1 hour to allow cell attachment. The impedance value of each well was automatically monitored by the xCELLigence system for duration of the experiment and expressed as a CI value.

2.2.11 Plating Efficiency Assay

The stable PP2A mutant cell lines were harvested with trypsin/EDTA, washed with DMEM and counted using a haemocytometer. 500 cells of each were plated per well of
Figure 2.2: Invasion using the RTCA xCELLigence system. (a) Using a Cell/Invasion Migration (CIM) plate, the base of the upper chamber is coated with a layer of Matrigel™ and the underside of the upper chamber is coated with fibronectin. Cells are seeded in the upper chamber in serum free media. Media with a chemoattractant (e.g. 10% FBS) is put in the lower chamber. (b) If the cells have the ability to invade, they will invade through the Matrigel, towards the chemoattractant and cause an electrical signal due to impedance on the electrode which is recorded as a CI value.
a 6 well plate. A full 6 well plate was used for each cell line i.e. 6 wells with 500 cells each. The plates were incubated at 37° in 5% CO$_2$ at 37°C for 10 days. After 10 days, the cells were fixed in 96% ethanol for 10 min and subsequently stained with 0.05% crystal violet (made in 20% ethanol) for 20 min. The wells were washed carefully in trays of water and allowed to dry. Colonies were counted and recorded. A colony was deemed to be of 50 cells or more in size. This was done in triplicate.

2.2.12 Soft Agar Assay

Wells were coated with a 0.6% agarose layer which was made in DMEM with 10% FBS. This was left for 20 minutes to allow the agarose to solidify. The stable PP2A mutant cell lines were harvested with trypsin/EDTA, washed with DMEM, and re-suspended in the DMEM with 10% FBS containing 0.3% agarose. The cells were counted using a haemocytometer and plated in quadruplicate. The cells were overlaid very carefully with DMEM with 10% FBS. Cells were left to incubate for 14 days. Colonies were then stained with 0.01% crystal violet overnight and counted using a light microscope which aids in creating contrast.

2.2.13 Spot Synthesis of peptides and Overlay analysis

Peptides arrays of PP2A A subunit in nitrocellulose were generated as previously described (Frank 2002, Frank and Overwin 1996, Kramer and Schneider-Mergener 1998). Scanning libraries of overlapping 18-mer peptides covering the entire sequence of a protein, e.g. PP2A A subunit were produced by automatic SPOT synthesis and synthesized on Whatman 50 cellulose using Fmoc (9-fluorenylmethyloxycarbonyl) chemistry with the AutoSpot-Rosbot ASS 222 (Intavis Bioanalytical Instruments). The interaction of GST and GST-tagged proteins, e.g. GST-RACK1 with the protein array was investigated by overlaying the cellulose membranes with 10 µg/ml concentrations
of each recombinant protein. Bound protein was detected with specific mouse antisera for each protein and a secondary anti-mouse antibody coupled with LICOR dye 680 and scanned on the Odyssey Infrared Scanner. Once the binding site of RACK1 on the full-length PP2A A subunit array was determined, specific alanine scanning substitution arrays were generated for selected peptides using the same synthesis procedure.

2.2.14 Alanine substitution array analysis

Specific alanine scanning substitution arrays were generated for selected peptides using the same synthesis procedure as described above. The specific interaction sites of two proteins was determined by overlaying the cellulose membranes with 10μg/ml concentration of the recombinant protein, e.g. GST-RACK1. Bound protein was detected with specific mouse antisera for each protein and a secondary anti-mouse antibody coupled with LICOR dye 680 and scanned on the Odyssey Infrared Scanner. The signal intensity of each spot was measured and compared to the control. Spots with a signal intensity of 40% or less of the control were considered important.

2.2.15 3D Cell Culture

Unless otherwise stated, the protocol for the on-top assay was followed as described in (Genee Y Lee et al. 2007). The Matrigel™ was thawed overnight at 4°. A pre-chilled culture dish was coated with a thin layer of Matrigel™ and spread evenly over the surface. The dish was incubated at 37° for 15-20 minutes to allow the Matrigel™ to solidify. Cells were trypsinized from a monolayer to a single cell suspension. Cells were counted using a haemocytometer, pelleted by centrifugation and resuspended in half the amount of required media. Cells were plated on to the coated surface and allowed to settle and attach to the Matrigel™ layer for 10-30 minutes at 37°. The remaining media
was chilled on ice and Matrigel™ was added to 10% volume. This 10% Matrigel™ solution was gently added to the plated cells. Cells were incubated at 37° for ten days.

For extraction of the 3D cultures, media was aspirated carefully off the cells and cells were washed twice with ice-cold PBS. 2-3 volumes of ice-cold PBS/0.5M EDTA was used to scrape the cultures from the bottom of the well using a pipette tip. The solution was placed in a tube and shook gently on ice for at least 30 minutes. When the Matrigel™ had fully dissolved, the cultures were centrifuged and the supernatant was removed and cells were lysed as described in 2.2.3.

2.2.16 Mass Spectrometry Analysis to identify proteins interacting with the RACK1/PP2A complex.

RACK1 was immunoprecipitated from MCF-7 cells as described in 2.2.4. The agarose resin was washed with lysis buffer and reincubated with anti-PP2A C subunit for 3 hours at 4°C. Immunoprecipitated proteins were eluted in loading buffer (2% SDS, 23% beta-mercaptoethanol, 50 mM Tris-HCl, pH 6.8, 10% glycerol, 1 mM EDTA, and 0.1% bromophenol blue) and resolved by 12% SDS-PAGE. Gel was stained with Gel Code Blue (Thermo Scientific) according to manufacturer’s instructions and scanned. The gel was preserved in Gel Preserving Solution (Glycerol and dH2O) and stored @ 4°C until it was sent for Mass Spectrometry analysis.

The whole lanes (Control versus RACK1) of interest were manually excised from the gel and cut into small pieces. Mass spectrometry analysis was performed by Dr Catherine Botting, BSRC Mass Spectrometry and Proteomics Facility, University of St Andrews, Fife. Proteins were subjected to trypsin digestion, and proteolytic peptide mixtures were separated by nano-LC utilizing an Eksigent two-dimensional LC NanoLC system (Eksigent/Applied Biosystems Sciex) interfaced with a QStar XL mass
spectrometer (Applied Biosystems Sciex). MASCOT (Matrix Science) was used for peak detection, mass peak list generation, and database searches.

2.2.17 Gene expression

RNA was extracted from cell lines using the RNeasy Plus mini kit (Qiagen) as per manufacturer’s protocol. Cells were lysed and physically broken up by vortexing and passing through a needle a number of times. Breast tissue was obtained from patients undergoing surgery in University Hospital Limerick. RNA extraction from tissue was completed using an RNeasy Lipid Tissue mini kit (Qiagen). To extract the RNA, tissue was homogenised by snap freezing in liquid nitrogen. 1ml of QIAzol was added to the sample which was ground in a pestle and mortar. The sample was transferred to a MaXtract tube and 200µl chloroform was added. The tube was centrifuged at 4°C for 15 minutes at 12,000g. The upper aqueous layer was removed and placed in a sterile container. For both cells and tissue, RNA was precipitated out by adding an equal volume of 70% ethanol to the sample. The sample and ethanol were then centrifuged in an RNeasy spin column with the sample remaining in the column and the waste being pushed through the column as flow through which is discarded (RNeasy Plus Mini Kit). The column was washed 3 times with 700µl RW1 buffer (RNeasy Plus Mini Kit) which was centrifuged for 15 seconds at 8000g. The flow throw was discarded after each wash. The column was washed once with 500µl RPE buffer (RNeasy Plus Mini Kit) for 15 seconds at 800g and then washed again with 500µl RPE buffer for 2 minutes at 8000g. Again, the flow through was discarded. After this, 500µl 80% ethanol was added to the column and centrifuged for 2 minutes at 8000g. The RNeasy spin column was placed in a new collection tube and centrifuged again for 2 minutes at 8000g to remove any residual ethanol. After centrifugation the spin column was removed from the
collection tube so that the column did not make contact with the flow-through. The column was placed in a new 1.5ml collection tube and allowed to air dry for at least 6 minutes with the cap open. After this, 30µl of RNase-free water was added directly to the spin column membrane. The lid was closed gently and allowed to sit for 2-3 minutes. The column was centrifuged for 1 minute at 8000g to elute the RNA. The concentration and quality of the RNA was determined by reading a sample of the RNA on the Nanodrop. Integrity of the RNA was determined by running a sample on an agarose gel.

RNA was synthesised into cDNA using the Invitrogen Vilo cDNA synthesis kit. Real time PCR was carried out on the cDNA using Taqman® assays in a thermocycler. All data was normalized using reference genes and analysed using REST© software.

2.2.18 Gene knockdown

The MTDH gene was knocked down using esiRNA (endo-ribonuclease prepared siRNA) (Sigma-Aldrich Ltd) with Oligofectamine™ (Life Technologies, Dublin, IE). EsiRNA are a heterogeneous mixture of siRNA’s that all target the same mRNA sequence. These multiple silencing triggers lead to highly specific and effecyove gene silencing. The oligofectamine protocol was followed as per manufacturer’s instructions. Knockdown efficiency was optimised over different time points and at different concentrations. Knockdown efficiency was again optimised over different time points and at different concentrations.

2.2.19 Statistical Analysis

IC 50 values were calculated by obtaining the slope of the line using Excel. All statistical analysis was performed using SPSS version 20 statistical package. Student T test was used to calculate statistical significance. Differences between groups were
determined using Welsh Anova and multiple groups were compared using Bonferroni correction. A p value of less than 0.05 was considered statistically significant.
Chapter 3:

Utilization of a real time cell analysis tool to analyse the anti-cancer effects of compounds on breast cancer cells.
3.1 Abstract

The Real Time Cell Analysis (RTCA) xCELLigence platform, ACEA Biosciences, (Cambridge, U.K) is a tool which facilitates capturing real-time data on cell behaviour in response to novel compounds. The continuous monitoring of the xCELLigence platform makes it advantageous over traditional end-point assays. The objective of our study was to utilize the xCELLigence platform to determine the effect of two novel compounds (Vitamin K (VK) 2 and PRIMA-1\textsuperscript{Met}) on Triple Negative Breast Cancer (TNBC) cells. VK2 is known primarily as an anti-haemorrhagic agent but has also shown anti-carcinogenic properties. We determined that VK2 has an inhibitory effect on the adhesion and viability of TNBC cells. Culturing the cells in a low glucose media in combination with VK2 decreases cell viability even further. We also utilized the system to determine the effect of the small molecule PRIMA-1\textsuperscript{Met} on a panel of breast cancer cell lines. PRIMA-1\textsuperscript{Met} restores wild type conformation and function to mutant p53. Sensitivity of cell lines to PRIMA-1\textsuperscript{Met} was found to correlate with mutant p53 status. MDA-MD-468 and MDA-MB-453 cells lines were found to be most sensitive to the compound and both are known to harbour p53 mutants. Hs578T(i8)2 cells showed lower sensitivity to PRIMA-1\textsuperscript{Met} even though these cells have a recorded p53 mutation. MCF-7 cells do not have mutant p53 and showed complete resistance to PRIMA-1\textsuperscript{Met} at the concentrations tested. In conclusion, our results are the first to demonstrate the anti-cancer effect of VK2 in real time both alone and in combination with a reduced glucose culture media on TNBC cells. We have also shown, for the first time, the effect of PRIMA-1\textsuperscript{Met} on the growth and viability of breast cancer cells in real time.
3.2 Introduction

Breast cancer is the most prevalent cancer type in the vast majority of countries worldwide (Bray et al. 2013). Although there is an on-going debate into the overall value of breast cancer screening due to possible over diagnosis (Screening 2012), the fact remains that breast cancer is the second leading cause of cancer deaths in women over the age of 50 (Ekwueme et al. 2014). It is clear that current treatments are not sufficient against breast cancer in every case and both improving current treatments and developing novel strategies should be a priority in dealing with this disease.

There are a number of very well established end point assays widely utilised in the field of cancer cell biology to determine essential cell characteristics such as cell viability, cell adhesion, cell migration and cell proliferation in response to cytotoxic agents. These include the MTT assay and WST test (Mosmann 1983, Ishiyama et al. 1996) for cell viability and the scratch wound test for cell migration (Todaro et al. 1965, Liang et al. 2007). However, there are many disadvantages associated with assays like these and others of a similar nature. For example, as an end point assay, only the end physiological state of the cells can be determined and no information can be gained as to the effect of a compound on cells during the course of the treatment (Moniri et al. 2014). The Real Time Cell Analysis (RTCA) xCELLigence platform, ACEA Biosciences, (Cambridge, U.K) is a tool which facilitates label free and operator independent cell analysis in real time (Ke et al. 2011). It has been shown to correlate well with the more traditional end-point assay with the added advantage of continuous monitoring (Ke et al. 2011). Here, we will describe the use of the xCELLigence platform to capture real-time data on breast cancer cells behaviour in response to novel compounds.
Vitamin K (VK) is a fat soluble vitamin that is historically known for its role in blood coagulation where it acts as a co-factor in gamma carboxylation of clotting factors II, VII, IX and X (Nimptsch et al. 2010). It exists in three forms, vitamin K1 (phylloquinone), vitamin K2 (menaquinone) and vitamin K3 (menadione). Vitamin K1 (VK1) is mainly sourced from the diet, found in green, leafy vegetables and certain oils of vegetable origin; Vitamin K2 (VK2) is synthesized by the gut microbiota and is also obtained in smaller amounts from fermented dietary products. Vitamin K3 (VK3) is a synthetic compound which does not occur naturally but which is used widely in animal feed (Nimptsch et al. 2010).

In addition to its established function as an anti-haemorrhagic agent, studies have illustrated the anti-carcinogenic properties of VK. In particular, VK2 has showed promise as an anti-carcinogenic agent in studies involving many cancer cell types including leukaemia and cancers of the liver, stomach, lung, ovary, glioblastoma and prostate (YOSHIJI et al. 2005, Tokita et al. 2006, Yokoyama et al. 2005, Sibayama-Imazu et al. 2008, Oztopcu et al. 2004, Samykutty et al. 2013, Karasawa et al. 2013). The anti-cancer activity of VK2 has also been demonstrated in *in vivo* studies of colon and prostate cancer (Ogawa et al. 2007, Samykutty et al. 2013). VK1 has been shown to potentiate the anti-proliferative effects of calcitriol (the hormonally active form of vitamin D) on calcitriol resistant MCF-7 breast cancer cells in addition to having an anti-cancer effect on MCF-7 cells when used alone (Marchionatti et al. 2009). However, any anti-cancer activity of VK2 in breast cancer is largely unexplored. The exact mechanism underlying this anti-cancer property of VK2 has yet to be fully elucidated however a number of processes have been suggested including apoptosis and cell cycle arrest (Lu Li et al. 2010, Matsumoto et al. 2006, Yao et al. 2012). It has also been suggested that VK2 may also play a role in the prevention of cancer, as a randomized
trial of 43 women with viral hepatitis treated with high dose VK2 showed an 80% decreased risk of developing hepatocellular carcinoma (Habu et al. 2004). VK2 is not used as an anti-cancer treatment in current clinical practise, however it has been suggested that that it does have potential as an alternative to some currently used cancer drugs due to the lack of adverse side effects associated with VK2 (even at high doses) (Karasawa et al. 2013).

Calorie restriction (CR) without malnutrition can increase life span and protect against cancer (Longo and Fontana 2010, Longo and Mattson 2014, Meynet and Ricci 2014, Hursting et al. 2010). Even when commenced late in life, CR can delay tumourigenesis and increase survival (Dhahbi et al. 2004). Reduced consumption of food has been shown to decrease levels of growth factors, reduce oxidative stress, increase cell repair mechanisms and possibly inhibit glycolysis (Hursting et al. 2010, Fontana and Klein 2007, Sebastián et al. 2012). CR also plays a role in response to chemotherapy as fasting has been shown to protect normal cells from the toxicity associated with some chemotherapy drugs (Meynet and Ricci 2014). This may occur because normal cells can adapt to conditions of deprivation but cancer cells cannot (Meynet and Ricci 2014).

In particular, the circulating serum growth factor insulin-like growth factor-1 (IGF-1) plays a prominent role with regard to the effect CR can have on cancer. Reduction of circulating serum IGF-1 as a result of CR has been shown to mediate the anti-apoptotic and anti-proliferative effects usually observed with CR (Hursting et al. 2010, Longo and Fontana 2010). Reduction of circulating serum IGF-1 levels by CR has also been shown to confer protection from the adverse effects of chemotherapy (Lee and Longo 2011, Lee et al. 2012). Furthermore, the addition of IGF-1 is enough to
reverse the sensitisation of cancer cells to chemotherapy which is CR mediated (Safdie et al. 2012).

Other factors thought to be involved in the anti-cancer effect attributed to CR include changes in the expression levels of hormones including leptin, androgens and estrogen. It is also thought that CR can cause a reduction of the chronic systemic inflammation which is associated with obesity and has been linked to cancer (Hursting et al. 2010, Hursting et al. 2007). Conversely, increased food consumption and increased levels of adipose tissue have been shown to alter expression of genes controlling cell proliferation, apoptosis and DNA repair which can lead to DNA damage, increasing the chance of a malignant transformation (Calle and Kaaks 2004).

Due to the accumulating evidence in support of CR as an anti-cancer strategy, a wide number of clinical trials have been completed and are currently on-going where CR and CR mimetics such as metformin are being administered either alone or in combination with conventional chemotherapy (reviewed extensively in (Meynet and Ricci 2014)). These trials hope to establish whether CR or CR mimetics can not only enhance the efficiency of chemotherapy but also reduce the toxic side effects of chemotherapy by protecting healthy cells.

The small molecule p53-dependant Reactivation and Induction of Massive Apoptosis (PRIMA)-1 is a small molecule first described in 2002 that induces apoptosis in human cancer cells and inhibits tumour growth in mice by restoring wild-type function to mutant p53 (Bykov et al. 2002). p53 is an important and extensively studied tumour suppressor protein that is mutated or deleted in many cancers (Brown et al. 2009). PRIMA-1\textsuperscript{Met} is a methylated analog of PRIMA-1 which is more active and specific to mutant p53 cells (Bykov et al. 2005). The mechanism of action of PRIMA-1\textsuperscript{Met} involves conversion of the small molecule to methylene quinuclidinone which has
the ability to covalently bind to cysteine residues on p53 (Peng et al. 2013, Wassman et al. 2013). This covalent modification has shown to be enough to reactivate mutant p53 as it restores sequence specific DNA binding and active conformation to p53 (Bykov et al. 2002). PRIMA-1\textsuperscript{Met} also activates a number of p53 target genes and pro-apoptotic genes. Through the ability of PRIMA-1\textsuperscript{Met} (also called APR-246) to re-establish wild type p53 function, PRIMA-1\textsuperscript{Met} has shown apoptotic, anti-cancer effects on lung and leukaemia cancer cell lines (Zandi et al. 2011, Ali et al. 2011) as well as in animal models of multiple myeloma and lung cancer (Saha et al. 2013, Zandi et al. 2011). Also, combination treatment of PRIMA-1\textsuperscript{Met} with established chemotherapy drugs such as doxorubicin and cisplatin has a synergistic anti-cancer effect in cancer types such multiple myeloma and acute myeloid leukaemia both \textit{in vitro} and \textit{in vivo} (Saha et al. 2013, Ali et al. 2011, Bykov et al. 2005). It should also be noted that PRIMA-1\textsuperscript{Met} was shown to have anti-cancer effects irrespective of p53 mutant status as it can reactivate wild-type p53 to increase expression of p53 pro-apoptotic targets (Saha et al. 2013, Bao et al. 2011).

PRIMA-1\textsuperscript{Met} has been the subject of a recent Phase I/II clinical trial to treat patients with prostate cancer and haematological malignancies (Lehmann et al. 2012). The drug was well tolerated and concluded to be safe at the concentrations tested. The most common adverse effects reported included headaches and dizziness. Molecular results were favourable also with tumour cells having increased levels of apoptosis, cell cycle arrest and up-regulation of p53 target genes (Lehmann et al. 2012). Results of this trial have found that PRIMA-1\textsuperscript{Met} could potentially be used as a treatment in clinical practise. However, to date, the effect of PRIMA-1\textsuperscript{Met} on breast cancer is a largely unexplored area of study.
The aim of this investigation was to utilize the RTCA xCELLigence platform to analyse the anti-cancer effects of compounds on breast cancer cell lines. The specific objectives were (i) to investigate if VK2 has an inhibitory effect on TNBC cells, (ii) to investigate if a combination of low glucose media and VK2 has an added inhibitory effect on TNBC cells and (iii) to determine the effect of PRIMA-1\textsuperscript{Met} on a panel of breast cancer cell lines both with and without p53 mutations (See Table 3.1). To the best of our knowledge, this is the first study of its kind documenting the effects of these 2 compounds on breast cancer cells using the RTCA xCELLigence platform.

**Table 3.1 p53 mutation status of a panel of breast cancer cell lines.** Information obtained from the UMD database (Edlund et al. 2012).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Subtype</th>
<th>p53 mutant status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hs578T(i8)2</td>
<td>TNBC</td>
<td>p.V157F</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Luminal</td>
<td>Wild Type</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>TNBC</td>
<td>p.R249S</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>Her2+</td>
<td>p.H368 del/ins Q</td>
</tr>
</tbody>
</table>
3.3 Results

3.3.1 Utilization of a real time cell analysis tool to analyse the anti-cancer effects of compounds on breast cancer cells.

Statistical analysis in this section was performed using SPSS v20 statistical package as described in Materials and Methods. IC50 values were calculated from the slope of the line using excel.

3.3.1.1 Optimisation cell number to facilitate monitoring cell behaviour in real time.

The first objective of this study was to use the RTCA xCELLigence system to monitor the effect that the V K derivative, VK2 has on the adhesion and viability of TNBC cells. To begin with, it was necessary to determine a suitable seeding concentration to allow analysis of the cells over the time course of experiments. MDA-MB-231 cells were seeded in wells of an E-plate at numbers ranging from 5,000 to 20,000 cells and were monitored every minute for the first 8 hours and every 30 minutes up to 48 hours. Readouts from RTCA systems are expressed as cell index (CI) values. As shown in Figure 3.1, two distinct patterns can be seen on the representative xCELLigence graph, which can be attributed to cell adhesion and spreading (0–8 h) and cell proliferation (8–24 h). During the first hour, very little impedance is measured during initial adhesion and this is reflected in the lack of readings during this time. The rate of proliferation was calculated for each seeding number by analysing the slope of the line between 8 and 24 hours (Figure 3.1.1) and based on the patterns observed, we determined that the optimum cell seeding density to monitor cell behaviour of MDA-MB-231 cells is 20,000 cells/well.
Figure 3.1.1: Optimizing cell number to monitor cell behaviour in real time. MDA-MB-231 cells were seeded at numbers 5,000, 10,000 and 20,000 in each well of an E-plate. Cell behaviour was monitored in real time using the xCELLigence system. Readings were taken every minute for the first 8 hours and every 15 minutes for the subsequent 40 hours with readings expressed as Cell Index (CI) values. (a) The xCELLigence graph is representative of the average of duplicate wells comparing the growth curve of MDA-MB-231 cells at 5,000 cells (red line), 10,000 cells (green line) and 20,000 cells (blue line). (b) The rate of proliferation at the various cell concentrations as determined by analysing the slope of the line over 8-24 hours. N=3.
3.3.1.2 **VK2 has a significant inhibitory effect on the adhesion of breast cancer cells.**

Having optimised conditions to monitor MDA-MB-231 cell behaviour, we then wanted to investigate whether VK2 had any effect on the cancer cells ability to adhere and grow. To do this, the cells were seeded in E-plate wells with DMEM containing VK2 concentrations of 35, 50 and 75µM. As seen in **Figure 3.1.2 (a)**, modest effects were seen after 16 hours of monitoring. Our next objective was to determine concentrations of VK2 that had a significant effect on the behaviour of the MDA-MB-231 cells. To do this, the cells were seeded in E-plate wells with DMEM containing a range of VK2 concentrations (0-150µM). Cell behaviour was monitored using the RTCA platform over a period of 48 hours. To investigate any effect on cell adhesion and spreading, data was extracted from the first 8 hours of cell monitoring (**Figure 3.1.2 (b)**). **Figure 3.1.2 (c)** shows that adherence of the cells is inhibited at each VK2 concentration tested in comparison to the untreated control. Comparison of the percentage difference in mean cell index between untreated cells and cells treated with different concentrations of VK2 showed that CI values are reduced from 20% to over 95% as the VK2 concentration was increased (**Figure 3.1.2(c)**). This shows that the TNBC cells are sensitive to VK2 at a concentration of 100µM.

3.3.1.3 **VK2 has a significant inhibitory effect on the proliferation of breast cancer cells.**

Having shown that VK2 had an effect on the adhesion of TNBC cells, we extended the study to investigate the effect of VK2 on TNBC cell proliferation. MDA-MB-231 cells were seeded in E-plate wells with DMEM containing a range of VK2 concentrations (0-150µM). Cell behaviour was monitored using the RTCA platform over a period of 48
Figure 3.1.2: VK2 has a significant inhibitory effect on the adhesion of breast cancer cells. MDA-MB-231 cells were plated at a seeding density of 20,000 cells in wells of an E-plate in DMEM containing a range of VK2 concentrations (0µM, 35µM, 50µM, 75µM). The cells were monitored in real time using the xCELLigence system. Readings were taken every minute over the 8 hours with readings expressed as Cell Index (CI) values. (a) Initial concentrations of VK2 tested. (b) The xCELLigence graph is representative of the average of duplicate wells comparing the effect of different VK2 concentrations on cell adhesion. (c) Comparison of the percentage difference in mean cell index between untreated cells and cells treated with different concentrations of VK2. Data is represented as mean ± SEM. A statistical analysis was performed using SPSS 20 statistical package. Differences between groups were determined using Welsh Anova and multiple groups were compared using Bonferroni correction. A p value of less than 0.05 was considered statistically significant. ***p<0.001. N=3.
Figure 3.1.3: VK2 has an inhibitory effect on the proliferation of breast cancer cells. MDA-MB-231 cells were plated at a seeding density of 20,000 cells in an E-plate in DMEM containing a range of VK2 concentrations (0µM-150µM). Proliferation was monitored in real time using the xCELLigence system. Readings were taken every minute over 8 hours and then every 30 minutes up to 48 hours with readings expressed as CI values. (a) The xCELLigence graph is representative of duplicate wells comparing the effect of different VK2 concentrations on cell proliferation over 48 hours. (b) Comparison of the percentage difference in mean cell index between cells treated with different concentrations of VK2. CI values were reduced by 30% to almost 70% with increasing concentration of VK2. IC50 = 124.37µM. Data is represented as mean ± SEM. Differences between groups were determined using Welsh Anova and multiple groups were compared using Bonferroni correction. A p value of less than 0.05 was considered statistically significant. ***p<0.001. N=3.
hours and CI values were analysed. Results show that VK2 significantly inhibited the proliferation of the cells at each VK2 concentration tested in comparison to the untreated control (Figure 3.1.3). Comparison of the percentage difference in mean cell index between untreated cells and cells treated with different concentrations of VK2 showed that CI values were reduced by 30% to almost 70% depending on the concentration of VK2 used (Figure 3.1.3 (b)). The IC50 is calculated to be 124.37µM.

3.3.1.4 A combination of low glucose media and VK2 has an added inhibitory effect on breast cancer cell adhesion.

Following this, it was investigated if lowering the glucose concentration in the cell culture media would have an effect on breast cancer cell growth both alone and in combination with VK2 as calorie restriction has been shown to improve patient response to chemotherapy (Meynet and Ricci 2014). The RTCA platform was utilised again for this experiment. MDA-MB-231 cells were seeded in an E-plate with either a high or low glucose media alone and also in the presence of VK2 at a concentration of 100µM. Adhesion and spreading were monitored over an 8 hour period. As seen in Figure 3.1.4, culturing the cells in low glucose media had an inhibitory effect on breast cancer cell adhesion when compared to cells cultured in high glucose media. Over the first 8 hours of cell adhesion and spreading, cells cultured in low glucose media had a 20% reduction in adhesion when compared to the CI values of the cells cultured in high glucose (Figure 3.1.4 (b)). This inhibition is shown in real time and represented by analysing the data in 2 hour blocks (Figure 3.1.4 (c)). Culturing of cells with VK2 (100µM) in combination with the low glucose media is shown to have had a greater, statistically significant, inhibitory effect on cell adhesion than when culturing cells with low glucose alone (Figure 3.4 (c)).
Figure 3.1.4: A combination of low glucose media and VK2 has an added inhibitory effect on breast cancer cell adhesion. MDA-MB-231 cells were plated at a seeding density of 20,000 cells in wells of an E-plate in DMEM containing either high glucose or low glucose. Cells were treated with 100µM VK2 where indicated. Adhesion was monitored in real time using the xCELLigence system. Readings were taken every minute over 8 hours with readings given as CI values. (a) The xCELLigence graph is representative of the average of duplicate wells comparing the combinatory effect of different glucose conditions and VK2 treatment on cell adhesion. (b) Comparison of the CI values of cells treated with different glucose conditions and treatment with VK2 (100µM) where indicated. (c) Comparison of the percentage difference in mean CI values between cells treated with different glucose conditions and treatment with VK2 (100µM) where indicated. Data is shown in 2 hour blocks between 0 and 8 hours. Data is represented as mean ± SEM. Differences between groups were determined using Welsh Anova and multiple groups were compared using Bonferroni correction. A p value of less than 0.05 was considered statistically significant. ***p<0.001, +++p<0.001, N=3.
Figure 3.1.5: A combination of low glucose media and VK2 has an added inhibitory effect on cell proliferation. MDA-MB-231 cells were plated at a seeding density of 20,000 cells in wells of an E-plate in DMEM containing either high glucose or low glucose. Cells were treated with 100µM VK2 where indicated. Proliferation was monitored in real time using the xCELLigence system. Readings were automatically taken every minute over the 24 hours with readings given as CI values (a) The xCELLigence graph is representative of duplicate wells comparing the combinatory effect of different glucose conditions and VK2 treatment on cell adhesion. (b) Comparison of the percentage difference in mean CI values between cells treated with different glucose conditions and treatment with VK2 (100µM) where indicated. Data is represented as mean ± SEM. Differences between groups were determined using Welsh Anova and multiple groups were compared using Bonferroni correction. A p value of less than 0.05 was considered statistically significant. ***p<0.001. +++p<0.001. N=3.
3.3.1.5 A combination of low glucose media and VK2 has an added inhibitory effect on cell proliferation.

Following from looking at adhesion, the next objective was to test if the combination of low glucose media and VK2 had an added effect on breast cancer cell proliferation. MDA-MB-231 cells were seeded in an E-plate with either a high or low glucose media alone and also in the presence of VK2 at a concentration of 100µM. Data was analysed over a 24 hour period. From this it was determined that cells cultured in low glucose media have lower CI values when compared to the CI index values of cells cultured in high glucose media which indicates reduced proliferation (Figure 3.1.5(a)). Results show that proliferation is inhibited to the greatest extent (74%) when cells are cultured with a combination of low glucose media and VK2 (100µM). This is marginally above the 68% decrease in CI values seen when cells are cultured in high glucose with VK2 (100µM) and is statistically significant (Figure 3.1.5 (b)).
3.3.2 Utilizing the RTCA xCELLigence platform to investigate the effect of PRIMA-1\textsuperscript{Met} on breast cancer cells.

3.3.2.1 PRIMA-1\textsuperscript{Met} has no effect on the growth of MCF-7 breast cancer cells.

The next objective was to use the RTCA xCELLigence platform to validate results obtained in the laboratory of Professor Joe Duffy, The Conway Institute, UCD. They had investigated the efficacy of the small molecule PRIMA-1\textsuperscript{Met} on a panel of breast cancer cells using the more traditional MTT assay. We took 2 cell lines that they had found to be resistant and 2 cell lines that they found to be sensitive to PRIMA-1\textsuperscript{Met} and mimicked their experimental conditions as much as possible to validate their results on the xCELLigence platform. MCF-7 cells were plated on Day 0 in regular DMEM media with 10% FBS at a density of 10,000 cells per well in an E-plate. On Day 1, cells were treated with the highest concentration of PRIMA-1\textsuperscript{Met} (25µM). Readings were taken every minute for the first ten hours and then every 15 minutes for the duration of the experiment. Data was normalised to the point before addition of the compound. As shown in Figure 3.2.1, PRIMA-1\textsuperscript{Met} had no effect on the viability of MCF-7 cells when used at a concentration of 25µM over 96 hours. This is shown in the representative graph from the xCELLigence system (a) and by determining the percentage viability of cells by comparing the CI of the control (untreated MCF-7 cells) to the CI of treated MCF-7 cells over 96 hours (b). No difference was detected between groups so we determined that MCF-7 cells are resistant to PRIMA-1\textsuperscript{Met}. This was in agreement with the results obtained by Professor Joe Duffy’s laboratory and is not surprising as MCF-7 cells are known to have wild type p53 status (see Table 3.1). It is also in agreement with a study which found the closely related small molecule, PRIMA-1 to have little to no effect of MCF-7 cells in normoxia (Rieber and Strasberg-Rieber 2012).
Figure 3.2.1: PRIMA-1\textsuperscript{Met} has no effect on the growth of MCF-7 breast cancer cells. (a) MCF-7 cells were plated on day 1 in culture media at a density of 10,000 cells per well in an E-plate. On day 2, cells were treated with the top concentration PRIMA-1\textsuperscript{Met} of 25µM. Readings were taken every minute for the first ten hours and then every 15 minutes for the duration of the experiment. The xCELLigence graph is representative of the average of duplicate wells and shows the experiment over 96 hours normalised to 15 minutes before addition of the compound. (b) Cell Index (CI) over 96 hours was used to determine percentage viability of cells by comparing the control (untreated MCF-7 cells) to the treated MCF-7 cells. Data is represented as mean ± SEM. A statistical analysis was performed using SPSS 20 statistical package. No difference was detected between groups using Student’s T Test. A p value of less than 0.05 was considered statistically significant. ***p<0.001. N=3.
3.3.2.2 Hs578T(i8)2 breast cancer cells show resistance to PRIMA-1\textsuperscript{Met}.

Our next cell line to be tested for sensitivity to PRIMA-1\textsuperscript{Met} were Hs578T(i8)2 cells. Cells were plated on Day 0 in regular RPMI with 10% FBS at a density of 10,000 cells per well in an E-plate. On Day 1, cells were treated with 0, 5, 10 and 25µm concentrations of PRIMA-1\textsuperscript{Met}. Readings were taken every minute for the first ten hours and then every 15 minutes for the duration of the experiment. Data was normalised to point before addition of the compound. As shown in Figure 3.2.2 (a), PRIMA-1\textsuperscript{Met} had no effect on the viability of Hs578T(i8)2 cells when used at concentrations of 5µM and 10µM over 96 hours. However, it did inhibit cell viability when used at the highest concentration of 25µM. CI over 96 hours was used to determine percentage viability of cells by comparing the control (untreated Hs578T(i8)2 cells) to the treated Hs578T(i8)2 cells (Figure 3.2.2 (b)). These results show that Hs578T(i8)2 cells known to have p53 mutant status (see Table 3.1) exhibit a resistance to the PRIMA-1\textsuperscript{Met}. This correlates well with our collaborators in UCD.

3.3.2.3 PRIMA-1 Met has an inhibitory effect on the growth of MDA-MB-468 breast cancer cells.

MDA-MB-468 cells were plated on Day 0 in regular RPMI with 10% FBS at a density of 10,000 cells per well in an E-plate. On Day 1, cells were treated with 0, 5, 10 and 25µM concentrations of PRIMA-1\textsuperscript{Met}. Readings were taken every minute for the first ten hours and then every 15 minutes for the duration of the experiment. As shown in Figure 3.2.3, PRIMA-1\textsuperscript{Met} has a significant effect on cell viability over 120 hours at both 10 and 25µM concentrations. Data is normalised to the point before addition of the compound (Figure 3.2.3 (a)). CI over 120 hours was used to determine percentage viability of cells by comparing the control (untreated MDA-MB-468 cells)
Figure 3.2.2: Hs578T(i8)2 breast cancer cells show resistance to PRIMA-1\textsuperscript{Met}. (a) Hs578T(i8)2 cells were plated on day 1 in culture media at a density of 10,000 cells per well in an E-plate. On day 2, cells were treated with 0, 5, 10 and 25µM concentrations of PRIMA-1\textsuperscript{Met}. Readings were taken every minute for the first ten hours and then every 15 minutes for the duration of the experiment. The xCELLigence graph is representative of the average of duplicate wells and shows the experiment over 96 hours normalised to point before addition of the compound. (b) CI over 96 hours was used to determine percentage viability of cells by comparing the control (untreated Hs578T(i8)2 cells) to the treated Hs578T(i8)2 cells. Data is represented as mean ± SEM. Differences between groups were determined using Welsh Anova and multiple groups were compared using Bonferroni correction. A p value of less than 0.05 was considered statistically significant. ***p<0.001. N=3.
to the treated MDA-MB-468 cells (Figure 3.2.3 (b)). Our results show that MDA-MB-468 cells (known to have p53 mutant status (see Table 3.1)) are sensitive to PRIMA-1\(^{\text{Met}}\) at concentrations of 10 and 25µM. Our results correlate with results from Prof. Duffy’s team.

### 3.3.2.4 PRIMA-1\(^{\text{Met}}\) has an inhibitory effect on the growth of MDA-MB-453 breast cancer cells.

MDA-MB-453 cells were plated on Day 0 in regular RPMI with 10% FBS at a density of 10,000 cells per well in an E-plate. On Day 1, cells were treated with 0, 5, 10 and 25µM concentrations of PRIMA-1\(^{\text{Met}}\). Readings were taken every minute for the first ten hours and then every 15 minutes for the duration of the experiment. As shown in Figure 3.2.4, PRIMA-1\(^{\text{Met}}\) has a significant effect on cell viability over 120 hours at all concentrations tested, 5, 10 and 25µM. The growth profile of MDA-MB-453 cells was altered after treatment with PRIMA-1\(^{\text{Met}}\) at these concentrations showing that they are sensitive to the compound. Data is normalised to the point before addition of the compound (Figure 3.2.4 (a)). CI over 120 hours was used to determine percentage viability of cells by comparing the control (untreated MDA-MB-453 cells) to the treated MDA-MB-453 cells (Figure 3.2.4 (b)). MDA-MB-453 cells have p53 mutant status (see Table 3.1) and again, these results correlate well with our collaborators in U.C.D.
Figure 3.2.3: PRIMA-1\textsuperscript{Met} has an inhibitory effect on the growth of MDA-MB-468 breast cancer cells: (a) MDA-MB-468 cells were plated on day 1 in culture media at a density of 10,000 cells per well in an E-plate. On day 2, cells were treated with 0, 5, 10 and 25\textmu M concentrations of PRIMA-1\textsuperscript{Met}. Readings were taken every minute for the first ten hours and then every 15 minutes for the duration of the experiment. The xCELLigence graph is representative of the average of duplicate wells and shows the experiment over 120 hours normalised 15 minutes before addition of the compound. (b) CI over 120 hours was used to determine percentage viability of cells by comparing the control (untreated MDA-MB-468 cells) to the treated MDA-MB-468 cells. Data is represented as mean ± SEM. Differences between groups were determined using Welsh Anova and multiple groups were compared using Bonferroni correction. A p value of less than 0.05 was considered statistically significant. ***p<0.001. N=3.
Figure 3.2.4: PRIMA-1\textsuperscript{Met} has an inhibitory effect on the growth of MDA-MB-453 breast cancer cells: (a) MDA-MB-453 cells were plated on day 1 in culture media at a density of 10,000 cells per well in an E-plate. On day 2, cells were treated with 0, 5, 10 and 25µM concentrations of PRIMA-1\textsuperscript{Met}. Readings were taken every minute for the first ten hours and then every 15 minutes for the duration of the experiment. The xCELLigence graph is representative of duplicate wells and shows the experiment over 120 hours normalised to point before addition of the compound. (b) CI over 120 hours was used to determine percentage viability of cells by comparing the control (untreated MDA-MB-453 cells) to the treated MDA-MB-453 cells. The relative IC50 = 15.8µM. Data is represented as mean ± SEM. Differences between groups were determined using Welsh Anova and multiple groups were compared using Bonferroni correction. A p value of less than 0.05 was considered statistically significant. ***p<0.001. N=3.
3.4 Discussion.

Cell-based assays to monitor breast cancer cells behavioural response to novel compounds are essential in the search to find potential, safe, therapeutic strategies against the disease (Kustermann et al. 2013, Xi et al. 2008). Many of the traditional cell-based assays are hindered by dependence on end-point analysis. The RTCA xCELLigence platform is highly advantageous in comparison to these assays as it facilitates label-free, continuous monitoring of cellular response to compounds (Abassi et al. 2009, Ke et al. 2010). It has proven to be a highly accurate platform to monitor cell behaviour (Dowling et al. 2014, Dwane et al. 2013, Hou et al. 2014) and is a robust and reliable system for measuring response to novel compounds and nanoparticles (Darbre et al. 2013, Ramis et al. 2013). It has been shown to correlate very well with the conventional adhesion, viability, migration and invasion assays (Limame et al. 2012).

In our study, we utilized the RTCA xCELLigence platform to determine the effect of two compounds, VK2 and PRIMA-1\textsuperscript{Met} on breast cancer cells. Not only involved in blood coagulation, VK2 has been shown to regulate bone homeostasis through activation of the steroid receptor, SXR (Ichikawa et al. 2006) and has already shown promise as a safe anti-cancer agent (Karasawa et al. 2013). PRIMA-1\textsuperscript{Met} has been investigated as an anti-cancer agent in a number of cancer types (Zandi et al. 2011, Ali et al. 2011, Saha et al. 2013) and has also been the subject of a clinical trial to establish its safety in the treatment of haematological malignancies and prostate cancer (Lehmann et al. 2012). However, in the case of both VK2 and PRIMA-1\textsuperscript{Met}, studies on their effects on breast cancer cells remain a largely unexplored area.

We chose to investigate the effects of VK2 on TNBC cells as this subtype of the disease is associated with a more aggressive phenotype, poor survival, high rates of
recurrence, visceral metastasis and a younger age profile (Oakman et al. 2010). As TNBC cells are devoid of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (Her2/neu receptor), hormonal and targeted therapies which can confer significant long-term survival benefits are unavailable in TNBC (Yuan et al. 2014). The poor prognosis of patients with TNBC is due to lack of targeted therapies therefore TNBC studies are on-going to improve outcomes for patients diagnosed with TNBC.

To investigate the effect of VK2 on TNBC cells, we began by establishing the optimum growth conditions for MDA-MB-231 cells on our system by using the slope of the growth curves obtained by using different cell numbers. This allowed us to be confident in our choice of 20,000 cells per well as the optimum cell number to perform our experiments to monitor the effect of VK2 on TNBC cells. When TNBC cells were exposed to VK2 at a range of concentrations, there was a significant decrease in CI over the first 8 hours which correlates with a decrease in adhesion and spreading (Figure 3.1.2). When comparing the percentage difference in mean cell index between untreated cells and cells treated with different concentrations of VK2 we showed that CI values are reduced by 20% to over 95% when VK2 concentrations were increased (100µM-150µM) as seen in Figure 3.1.2 (c).

VK2 has been shown to reduce prostate cancer cell proliferation and a number of different mechanisms including activation of the pro-apoptotic protein caspase-3, downregulation of phosphorylated Akt, inhibition of NFκB and reduction of androgen receptor expression have been shown to be causing this (Samykutty et al. 2013). In order for us to look at the effect of VK2 on the proliferation of TNBC cells, the study was extended over 48 hours of continuous monitoring which determined a significant decrease in CI over 48 hours correlating with a decrease in cell proliferation.
or cell viability (Figure 3.1.3) Comparing the percentage difference in mean cell index between untreated cells and cells treated with different concentrations of VK2 we showed that CI values are reduced by 30% to almost 70% with increasing concentration of VK2 used (100µM-150µM) as seen in Figure 3.1.3 (b).

Having determined that VK2 does have an inhibitory effect on TNBC, we then wanted to determine if reducing the glucose concentration in the culture media would have an additive effect on cell inhibition in combination with VK2. It has long been established that cancer cells avidly consume glucose in much larger quantities than normal cells (Warburg 1956, Kim and Dang 2006). CR has also been well studied as an anti-cancer strategy (Longo and Fontana 2010, Longo and Mattson 2014, Meynet and Ricci 2014, Hursting et al. 2010). We determined that TNBC cells cultured in low glucose media adhered and grew significantly slower than TNBC cells cultured in the regular DMEM which has a higher glucose concentration (Figure 3.1.4 and 3.1.5). We then wanted to investigate whether addition of VK2 could have an additional inhibitory effect on TNBC cell adhesion and proliferation.

TNBC cells were plated in an E-plate in either high glucose media with VK2 (100µM) or low glucose media with VK2 (100µM). Data from the first 8 hours of the experiment was extracted to determine the effect on adhesion and spreading. This was assessed over 2, 4, 6 and 8 hours (Figure 3.1.4 (b)) by comparing the percentage difference in CI values of the treated cells compared to the untreated control (which was cells cultured in the high glucose, regular DMEM media). Data was also assessed over the full 8 hours by analysing the decrease in CI values of the treated cells and comparing to the untreated control. Our results show that culturing the cells in both low glucose media and VK2 (100µM) has the largest inhibitory effect when compared to
treating the cells with high glucose media with VK2 (100µM) or treating the cells with either high or low glucose media alone (**Figure 3.1.4 (a)**).

Again we extended our study over 48 hours of continuous monitoring to determine the effect on proliferation. Comparing the percentage difference in CI values of the treated cells compared to the untreated control (which was cell culture in the high glucose, regular DMEM media) over 48 hours determined that there was a significant decrease in CI over 48 hours which correlates with a decrease in cell proliferation or cell viability. Our results show that culturing the cells in both low glucose media and VK2 (100µM) has the largest inhibitory effect when compared to treating the cells with high glucose media with VK2 (100µM) or treating the cells with either high or low glucose media alone (**Figure 3.1.5 (b)**).

Having successfully used the RTCA xCELLigence platform to monitor the effect of VK2 on TNBC cells, the system was also utilized to determine the effect of the small molecule PRIMA-1Met on a panel of breast cancer cell lines which represent most of the recognised breast cancer subtypes. Cell lines were selected based on both subtype and mutant p53 status to investigate the sensitivity of these cell lines to PRIMA-1Met. The duration of the experiments was cell line dependant and was based on previous experiments completed the laboratory of Prof. Joe Duffy which were run over longer periods of time to determine the time points at which the untreated control cells started to die off from other causes i.e. lack of space in the well and/or lack of nutrients.

The MCF-7 breast cancer cell line represents the luminal breast cancer subtype and has wild type p53 status (Edlund et al. 2012). There was no effect on the growth of the cells at any point over the duration of the experiment when treated with PRIMA-1Met (25µM) so we have determined that these cells are resistant to the anti-cancer effect of PRIMA-1Met. Hs578T(i8)2 cells are representative of the TNBC subtype. They were
found to harbour a p53 mutation (see **Table 3.1**). Our results show that treatment of the cells with PRIMA-1\textsuperscript{Met} has an inhibitory effect on the growth of the TNBC cells however this is seen only when the highest concentration of PRIMA-1\textsuperscript{Met} tested (25µM) suggesting a lower sensitivity to the compound.

MDA-MB-468 cells are also representative of the TNBC subtype and harbour a p53 mutation (see **Table 3.1**). Our results show that treatment of the cells with PRIMA-1\textsuperscript{Met} has an inhibitory effect on the growth of these TNBC cells at the top concentrations tested (10µM and 25µM). This confirms the sensitivity of these cells to PRIMA-1\textsuperscript{Met}.

MDA-MB-453 cells are representative of the Her2+ breast cancer subtype and harbour a p53 mutation (see **Table 3.1**). Our results show that treatment of the cells with PRIMA-1\textsuperscript{Met} has an inhibitory effect on the growth of these Her2+ cells at all concentrations tested (5µM, 10µM and 25µM) which confirms the sensitivity of these cells to PRIMA-1\textsuperscript{Met}.

These results are the first to show the anti-cancer effect of VK2 in real time both alone and in combination with a reduced glucose culture media on triple negative breast cancer cells. This work expands the body of knowledge about VK2 as an anti-cancer agent and strengthens the need for further study with regard to the potential VK2 has as a therapeutic strategy in cancer. Due to the low toxicity associated with VK2 and the urgent need for new strategies in the treatment of triple negative breast cancer, VK2 is an attractive possibility as an anti-cancer agent. We have also shown the effect of PRIMA-1\textsuperscript{Met} on the growth and viability of breast cancer cells in real time. PRIMA-1\textsuperscript{Met} has shown great potential as an anti-cancer strategy in other cancer types including prostate cancer (Lehmann et al. 2012). To date, PRIMA-1\textsuperscript{Met} treatment of breast cancer has remained a largely unexplored area. This study indicates that PRIMA-1\textsuperscript{Met} has potential as an anti-cancer strategy in certain breast cancer types where a p53 mutation
is detected and warrants further study. This study also re-enforces the RTCA xCELLigence platform as an excellent tool to determine the sensitivity of cancer cell lines to novel compounds and this is demonstrated by the work presented here.
Chapter 4:

Disruption of the RACK1/PP2A complex has implications for essential cell characteristics and PP2A activity.
4.1 Abstract

Conflicting reports implicate the scaffolding protein RACK1 in the progression of breast cancer. RACK1 has been identified as a direct binding partner of PP2A to regulate cell migration and stabilize PP2A activity. The objective of this study was to further characterise the interaction between PP2A and RACK1 in breast cancer cells. We examined how the PP2A holoenzyme is assembled in MCF-7 cells and found that both the C subunit and A subunit of PP2A are assembled on the RACK1 scaffold. We used immobilized peptide arrays representing the entire PP2A-Catalytic protein to identify amino acids on the C subunit of PP2A that are required for the binding of RACK1. The interaction sites between RACK1 and the PP2A C subunit were used to generate stable cell lines of PP2A mutants to disrupt the RACK1/PP2A complex. These HA-tagged PP2A mutant cell lines exhibited reduced PP2A phosphatase activity, confirming the role for RACK1 in stabilizing PP2A activity. The cell lines were used to determine that disruption of the RACK1/PP2A complex also reduces the adhesion, proliferation, migration and invasion of breast cancer cells. The work has significantly advanced our understanding of the RACK1/PP2A complex and indicates a pro-carcinogenic role for the RACK1/PP2A complex, highlighting a potential therapeutic target in the treatment of breast cancer.
4.2 Introduction

RACK1 plays a critical role in many fundamental cellular processes including cell adhesion, proliferation, migration and protein synthesis through its ability to act as a scaffold within signalling pathways. RACK1 is known to interact with a diverse array of proteins and works to recruit and shuttle these proteins to their substrates or other binding partners (Adams et al. 2011, McCahill et al. 2002).

Alterations in RACK1 expression and function are associated with a variety of disease states including cancer, Alzheimer’s disease and bipolar disorder. RACK1 plays a critical part in cell adhesion and migration in particular through its work in recruiting focal adhesion kinase (FAK) (Kiely et al. 2009). RACK1 is a component of the signalling pathways downstream of FAK and PDE4D5 that control both cell spreading and direction sensing to establish cell polarity which is an important element in the process of cell migration (Serrels et al. 2010, Serrels et al. 2011). RACK1 complexes with vimentin to regulate FAK in the process of cell invasion (Dave et al. 2013). RACK1 has also been shown to promote both cell migration and invasion in a number of cancer types including oesophageal and lung cancers through a variety of different signalling mechanisms (Li et al. 2012, Shi et al. 2012, Wang et al. 2009).

Expression of RACK1 is up-regulated in a range of cancers including lung, liver and oesophageal squamous cell carcinoma (Shi et al. 2012, Ruan et al. 2012, Hu et al. 2013) while it has been found to be down-regulated in gastric cancer (Deng et al. 2012). Conflicting reports suggest a role for RACK1 in breast cancer. In one study, high RACK1 expression in breast cancer patients is reported to correlate with poor clinical outcome (Xi-Xi Cao et al. 2010). RACK1 was also found to promote breast cancer proliferation and invasion both in vitro and in mouse models through interaction with
RhoA and activation of the RhoA/Rho kinase pathway (Xi-Xi Cao et al. 2010) This has led to the suggestion that RACK1 has potential as a valuable prognostic indicator of advanced disease in breast cancer. However, other studies report findings that are in direct contrast to this. It is reported that high RACK1 expression has been detected in breast cancer patients and this has been associated with good clinical outcome in a follow-up study over a decade (Al-Reefy and Mokbel 2010). This apparent conflict in findings could be due to the heterogeneous nature of breast cancer and information on breast cancer subtype and patient demographics may help to unravel some of the inconsistencies that appear to exist (Li and Xie 2014). Also, because RACK1 is involved in the scaffolding of such a large number of proteins within such diverse signalling pathways, it is acknowledged that any changes either up or down in expression of RACK1 has the potential to have serious consequences for the tight regulation of these pathways and as a result, has the potential to have serious consequences for the development and progression of cancer (Adams et al. 2011). It is clear that more work needs to be done to nail down the role of RACK1 in breast cancer.

PP2A negatively regulates growth factor signalling and downstream MAP kinase activation. This regulatory role involves a number of growth factors including insulin, epidermal growth factor (EGF) and IGF-1 (Ugi et al. 2002). PP2A directly interacts with RACK1 in an IGF-1 dependent manner (Kiely et al. 2006, Kiely et al. 2008). RACK1 has been found to stabilize PP2A activity as a reduction in RACK1 expression decreases the phosphatase activity of PP2A. This decrease was shown to promote cell migration in cancer cells. This indicates that RACK1 has a role to play in keeping a specific pool of PP2A ‘active’ thus facilitating the dephosphorylation properties of PP2A. Tyr302 in RACK1 is required for the interaction between RACK1 and PP2A and a mutation of this amino acid has been shown to decrease proliferation of
breast cancer cells (Kiely et al. 2008). However, the interaction between RACK1 and PP2A has remained a largely unexplored area.

PP2A has a well-established role in cancer. It is largely recognised as a tumour suppressor and has been found to be mutated in many cancer types (Perrotti and Neviani 2013, Kurimchak and Graña 2012b, Shih et al. 2011, Ruediger et al. 2001b). However, in certain cell models, inhibition of PP2A has shown great potential as an anti-cancer strategy (Wei Li et al. 2010, Duong et al. 2014, Lu et al. 2009, Boudreau et al. 2007). This is mostly attributed to the anti-apoptotic role of PP2A which is in direct contrast to its more established role as a tumour suppressor.

The objective of this study was to further characterise the interaction between RACK1 and PP2A. Having identified interaction sites between RACK1 and the catalytic subunit of PP2A, the work focused on identifying the role that the RACK1/PP2A complex has in breast cancer cells. Cell lines were used that expressed mutated amino acids at the interaction sites between RACK1 and PP2A. The ways in which the RACK1/PP2A complex is essential for PP2A activity and how disruption of this complex contributes to the maintenance and progression of cancer were investigated.
4.3 Results.

4.3.1 RACK1 and PP2A C subunit are in a complex together in MCF-7 cells.
To characterise the interaction between RACK1 and PP2A, it was required to demonstrate that RACK1 is in a complex with the PP2A C subunit. To do this, HA-tagged Empty Vector and PP2A Wild Type were transfected into MCF-7 cells. The cells were lysed and a RACK1 IP was performed. The IP samples were run on a 12% SDS-PAGE gel and analysed for associated RACK1 and HA-tagged PP2A C subunit by Western blotting. As seen in Figure 4.1, RACK1 and PP2A C subunit were confirmed to be in a complex together.

4.3.2 Identification of RACK1 interaction sites on PP2A C subunit peptide arrays.
In order to characterise the interaction between RACK1 and PP2A, it was necessary to identify the binding sites between RACK1 and the PP2A C subunit. To do this, Dr Patrick Kiely employed peptide array technology. Peptide arrays of immobilised overlapping 18-mer peptides, each shifted to the right by 5 amino acids encompassing the entire PP2A C subunit were generated. Arrays were probed with GST-RACK1, which was detected by immunoblotting with anti-GST antibody. Two arrays gave a similar pattern of RACK1 binding. Positively interacting peptides generated dark spots and non-interacting peptides left blank spots. Spots that were generated on the GST control were disregarded as non-specific binding. Two areas of positive binding were identified, from peptide spot 11-15 and peptide spot 40-44 (Figure 4.2).

4.3.3 Modelling of potential RACK1 interaction sites on the PP2A C subunit.
To narrow down the amino acids involved in the interaction between RACK1 and the PP2A C subunit, we further analysed positive spots identified on the arrays in Figure
Figure 4.1: RACK1 and PP2A C subunit are in a complex together in MCF-7 cells. HA-tagged Empty Vector and PP2A WT were transfected into MCF-7 cells using the Lipofectamine transfection method. The cells were lysed and a RACK1 IP was performed. IP samples were run on a 12% SDS-PAGE gel and analysed for associated PP2A C subunit by western blotting. Panel on the right are the corresponding cell lysates. N=3.
Figure 4.2: Identification of RACK1 interaction sites on PP2A C subunit peptide arrays. The amino acid sequence of the entire PP2A Cα subunit is shown schematically (1-309). Peptide arrays of immobilised overlapping 18-mer peptides, each shifted to the right by 5 amino acids encompassing the entire PP2A C subunit were generated. Arrays were probed with GST-RACK1, which was detected by immunoblotting with anti-GST antibody. Two arrays gave a similar pattern of RACK1 binding. Positively interacting peptides generated dark spots and non-interacting peptides left blank spots. Spots that were generated on the GST control were disregarded as non-specific binding.

<table>
<thead>
<tr>
<th>18-mer peptide sequence</th>
<th>Residues</th>
<th>Peptide Spot</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVTVCGDVHGQFHDLMELFRIGGKS</td>
<td>44-68</td>
<td>11</td>
</tr>
<tr>
<td>GDVHGQFHDLMELFRIGGKSPDTNY</td>
<td>49-74</td>
<td>12</td>
</tr>
<tr>
<td>QFDLMELFRIGGKSPDTNYLFMGE</td>
<td>55-79</td>
<td>13</td>
</tr>
<tr>
<td>MELFRIGGKSPDTNYLFMGEVDYVDRG</td>
<td>60-85</td>
<td>14</td>
</tr>
<tr>
<td>IGGKSPDTNYLFMGEVDYVDRGYSVE</td>
<td>65-90</td>
<td>15</td>
</tr>
<tr>
<td>CDLLWSDPDDRGGGISPRGAGYTF</td>
<td>196-220</td>
<td>40</td>
</tr>
<tr>
<td>SDPDDRGGGISPRGAGYTFGQDIS</td>
<td>201-225</td>
<td>41</td>
</tr>
<tr>
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<td>206-230</td>
<td>42</td>
</tr>
<tr>
<td>ISPRGAGYTFGQDISETFNHANGL</td>
<td>211-235</td>
<td>43</td>
</tr>
<tr>
<td>AYTFGQDISETFNHANGLTLVSRA</td>
<td>216-240</td>
<td>44</td>
</tr>
</tbody>
</table>
Figure 4.3: Modelling of potential RACK1 interaction sites on the PP2A C subunit. Positive spots were further analysed using alanine substitution. Arrays were synthesized in which 18 amino acids of the PP2A C subunit were sequentially substituted with alanine along the entire sequence of the 18-mer parent peptide. The array was probed with GST-RACK1, which was detected by immunoblotting with anti-RACK1 antibody. A decrease in intensity in binding to the peptides after alanine substitution is indicative of decreased binding of the PP2A C subunit to RACK1. The binding of RACK1 to each alanine-substituted PP2A C subunit peptide was quantified by densitometry and presented as a percentage of the control “parent” sequence. A cut off of less than 50% binding was applied. The arrays shown are representative of just one of three arrays analysed. Final result is average densitometry score of all three arrays. Modelling completed by Prof. Dave Adams, Heriot-Watt University, Edinburgh, confirmed that the sites (a) F63, R64, (b) R214 and Y218, identified in alanine substitution have potential as interaction sites between RACK1 and the PP2A C subunit as they all showed an average of 50% less binding over the three experiments. Other sites are highlighted just to give orientation.
4.2 using alanine substitution arrays. Peptides were generated, derived from the 18-mer parent peptides combining positive spots obtained on the arrays. For each parent peptide, 18 progeny were generated where each new peptide in the array had a single alanine substitution in successive amino acids in the sequence as described in Materials and Methods. A decrease in intensity in binding to the peptides after alanine substitution is indicative of decreased binding of the PP2A C subunit to RACK1. The binding of RACK1 to each alanine-substituted PP2A C subunit peptide was quantified by densitometry and presented as a percentage of the control ‘parent’ sequence. A cut off of less than 50% binding was applied. RACK1 binding to these peptides was either severely attenuated or ablated by alanine substitution of phenylalanine 63 (F63), arginine 64 (R64) (Figure 4.3 (a)), arginine 214 (R214) and tyrosine 218 (Y218) (Figure 4.3 (b)). The interaction of RACK1 with the peptides was quantified by densitometry and these amino acids had an average of less than 50% over the three arrays analysed. Modelling of the PP2A C subunit (provided by Prof Dave Adams, Heriot-Watt University, Edinburgh) suggested that interaction of RACK1 at the positions F63 and R64 on the PP2A C subunit allowed them to preclude the binding of other B subunits (Figure 4.3 (a)). Modelling identified R214 and Y218 as cell surface exposed amino acids that had the potential to be interaction sites between RACK1 and the PP2A C subunit (Figure 4.3 (b)). Together, these analyses confirm that the binding of RACK1 to PP2A C subunit is direct and demonstrates the key amino acids required for binding of the two proteins.

4.3.4 Generation of PP2A mutants that disrupt the RACK1/PP2A complex.

Having determined the interaction sites between RACK1 and PP2A C subunit, we wanted to generate stable cell lines of HA-tagged PP2A mutants. We used the
Figure 4.4: Generation of PP2A mutants that disrupt the RACK1/PP2A complex. HA-tagged PP2A mutants were transfected into MCF-7 cells using the Lipofectamine 2000 transfection method and stable cell lines were selected using G418. Lysates were run on a 12% SDS-PAGE gel and analysed for associated HA and actin by Western blotting. N=3
Lipofectamine 2000 transfection method to generate HA-Empty Vector, HA-PP2A WT, HA-PP2A F63R64/AA, HA-PP2A R214A and HA-PP2A Y218F in MCF-7 cells. Transfected cells were selected using G418. Western blot analysis indicates that the mutants were expressing equal levels in the cells (Figure 4.4).

4.3.5: Disruption of the RACK1/PP2A complex decreases PP2A activity.

Generation of HA-tagged PP2A stable mutant cell lines facilitated an investigation into whether the interaction between RACK1 and the PP2A C subunit is required for stabilisation of PP2A activity. An assay was completed to determine whether disruption of the RACK1/PP2A complex at the FR/AA site (amino acids 63 and 64) and the R214 and Y218 sites identified by alanine substitution assay (Figure 4.3 (a)) would have an effect on PP2A activity. Cellular PP2A activity of the stable HA-tagged PP2A mutant cell lines HA-PP2A WT, HA-PP2A FR/AA, HA-PP2A R214A and HA-PP2A Y218F was measured using a PP2A immunoprecipitation phosphatase assay kit as described in Materials and Methods. The activity of these stable HA-tagged PP2A mutant cell lines HA-PP2A FR/AA, HA-PP2A R214A and HA-PP2A Y218F was decreased in comparison to the activity levels of the HA-PP2A WT control (Figure 4.5). Data is represented as mean ± SEM. A statistical analysis was performed using SPSS 20 statistical package. Statistical significance was determined by Student T Test. Differences between groups were determined using Welsh Anova and multiple groups were compared using Bonferroni correction. A p value of less than 0.05 was considered statistically significant. *p<0.01. N=3. This data supports the hypothesis that RACK1 plays a role in keeping PP2A ‘active’ in cells. It shows that disruption of the interaction between RACK1 and PP2A at these sites has a negative effect on PP2A activity by
reducing activity levels by over 50% in the HA-tagged PP2A mutant cell lines HA-PP2A FR/AA, HA-PP2A R214A and HA-PP2A Y218F (Figure 4.5).

Further analysis using the stable PP2A mutant cell line HA-PP2A FR/AA showed that disruption of the RACK1/PP2A complex at this F63R64 site had no effect on cell adhesion or proliferation (data not shown). This was despite a reduced level of colocalisation seen upon disruption of the complex at this site compared to the Wild Type control (data not shown) in a similar way to the reduction in colocalisation reported in the HA-PP2A R214A and HA-PP2A Y218F cell lines when compared to the Wild Type control in Figure 4.6 so this interaction site is thought not to be playing a role in the progression or development of cancer in these cancer cells. For this reason, it is not included in any further analysis from this point forward in the study. However, the reduction in PP2A activity upon disruption of the complex at this site shows that RACK1 is having a regulatory effect on PP2A activity by binding at F63R64. More recent modelling of a PP2A holoenzyme (PDB 4I5L) shows the FR site to be more embedded in the structure and not as easily accessible as was previously thought when the model in Figure 4.3 was analysed (Wlodarchak et al. 2013). This could potentially be the reason why a mutation at the FR site (amino acids 63 and 64) is not having an effect on the cell characteristics being examined in this study.
Figure 4.5: Disruption of the RACK1/PP2A complex decreases PP2A activity. Cellular PP2A activity was measured using a PP2A immunoprecipitation phosphatase assay kit. MCF-7 cells expressing HA-PP2A WT, HA-PP2A FR/AA, HA-PP2A R214A and HA-PP2A Y218F were immunoprecipitated using an anti-HA antibody and the samples were analysed in a colorimetric assay at 650nm. PP2A activity levels of MCF-7 cells expressing HA-PP2A FR/AA, HA-PP2A R214A and HA-PP2A Y218F were compared to activity levels of HA-PP2A WT. Data is represented as mean ± SEM. A statistical analysis was performed using SPSS 20 statistical package. Differences between groups were determined using Welsh Anova and multiple groups were compared using Bonferroni correction. A p value of less than 0.05 was considered statistically significant. *p<0.01. N=3.
4.3.6 Characterisation of the stable PP2A mutant cell lines.

Having generated the stable HA-tagged PP2A mutant cell lines, these cell lines were then employed to determine whether RACK1 and PP2A colocalised differently in the cells which had disruption to the RACK1/PP2A complex when compared to the wild type control. HA-PP2A WT, HA-PP2A R214A and HA-PP2A Y218F were seeded at a density of 5,000 cells on 10 mm glass coverslips. The cells were fixed and stained as described in Materials and Methods. The level of colocalisation was measured using Pearson’s coefficient. Pearson’s coefficient is a standard measure of pattern recognition and is used to measure the strength of the linear relationship between two fluorescence images. Significant colocalisation of HA (Red) and RACK1 (green) was detected in the cell membrane of cells expressing HA-PP2A WT (Pearson’s coefficient = 0.514 ± 0.03) (Figure 4.6). However, in the case of the PP2A stable cell lines expressing PP2A R214A and HA-PP2A Y218F, colocalisation was detected at much lower levels (Pearson’s coefficient = 0.32 ± 0.06 for PP2A R214A and 0.228 ± 0.04 HA-PP2A Y218F) (Figure 4.5). This reduction in colocalisation in both stable PP2A mutant cell lines indicates reduced interaction between HA and RACK1 where interaction sites between PP2A and RACK1 have been disrupted.

4.3.7 Disruption of the RACK1/PP2A complex decreases cellular adhesion.

Next, it was investigated if disruption of the RACK1/PP2A complex had an effect on cellular adhesion and spreading. To investigate adhesion, 2,000 cells of the HA-tagged PP2A mutant stable cell lines HA-PP2A WT, HA-PP2A R214A and HA-PP2A Y218F were plated in pre-prepared collagen coated wells. The plates were incubated for
Figure 4.6: Characterisation of the HA-tagged stable PP2A mutants. HA-tagged PP2A mutant stable cell lines HA-PP2A WT, HA-PP2A R214A and HA-PP2A Y218F were plated on coverslips in DMEM. Cells were fixed with 4% PFA, permeabilised with PHEM/0.1% Triton X, blocked with PHEM/5% goat serum and co-stained with HA (red), RACK1 (green) and nucleus (blue). Merge and zoom images are shown in the right columns. All images were acquired sequentially and images merged using ImageJ. Level of colocalisation was measured at points of the cell membrane using Pearson’s coefficient: HA-PP2A WT = 0.514 ± 0.03, HA-PP2A R214A = 0.32 ± 0.06, HA-PP2A Y218F =0.228 ± 0.04, N=12.
Figure 4.7: Disruption of the RACK1/PP2A complex decreases cellular adhesion. 2,000 cells of the PP2A mutant stable cell lines HA-PP2A WT, HA-PP2A R214A and HA-PP2A Y218F were plated in collagen coated wells. The plates were incubated for one hour. Cells were then washed, fixed in 100μl methanol and stained with 0.1% crystal violet. Cells were washed again and Triton X was added to the wells. After drying, the plates were read at 590nm. Adhesion of HA-PP2A R214A and HA-PP2A Y218F cells were compared to adhesion of HA-PP2A WT. Data is represented as mean ± SEM. A statistical analysis was performed using SPSS 20 statistical package. Differences between groups were determined using Welsh Anova and multiple groups were compared using Bonferroni correction. A p value of less than 0.05 was considered statistically significant. ***p<0.001. N=3.
1 hour and processed as described in 2.2.8. After drying, absorbance was read at 590nm in a spectrophotometer. The results highlight differences in adhesion between the HA-tagged PP2A mutant stable cell lines HA-PP2A WT, HA-PP2A R214A and HA-PP2A Y218F (Figure 4.7). In the case of both HA-PP2A R214A and HA-PP2A Y218F, adherence was significantly slower over the course of the hour when compared to the adhesion detected in the wild type control. This data suggests that the RACK1/PP2A interaction is required for MCF-7 breast cancer cells to adhere.

4.3.8 Disruption of the RACK1/PP2A complex decreases adhesion and spreading. Cellular adhesion and spreading was monitored in real time using the xCELLigence system. Readings were automatically taken every minute over 8 hours with readings expressed as Cell Index (CI) values. The xCELLigence graph is representative of duplicate wells comparing cellular adhesion and spreading of the HA-tagged PP2A mutant stable HA-PP2A WT cells with HA-PP2A R214A and HA-PP2A Y218F cells (Figure 4.8 (a)). The RTCA xCELLigence system is a very effective system to study the cellular characteristics of adhesion and spreading. Our group has recently used this technology to analyse changes in adhesion when studying protein-protein interactions in neuronal models and also to study the effect of fibroblast media on the adhesion of colon cancer cells (Dowling et al. 2014, Dwane et al. 2013). Data shown in (Figure 4.8 (b)) compares the percentage difference in mean cell index between the cellular spreading of HA-PP2A WT, HA-PP2A R214A, and HA-PP2A Y218F cells. Data is represented as mean ± SEM. A statistical analysis was performed using SPSS 20 statistical package. Differences between groups were determined using Welsh Anova and multiple groups were compared using Bonferroni correction. A p value of less than 0.05 was considered statistically significant. ***p<0.001. N=3. Overall, the findings
Figure 4.8: Disruption of the RACK1/PP2A complex decreases cellular adhesion and spreading. Cellular spreading was monitored using the xCELLigence system. Readings were taken every minute over 8 hours and expressed as CI values. (a) The xCELLigence graph is representative of the average of duplicate wells from one experiment comparing cellular spreading of PP2A mutant stable cell lines HA-PP2A WT cells to the cellular spreading of HA-PP2A R214A and HA-PP2A Y218F cells. (b) Percentage difference in mean cell index between the cellular spreading of HA-PP2A WT (control) cells and both HA-PP2A R214A and HA-PP2A Y218F cells. Data is represented as mean ± SEM. Differences between groups were determined using Welsh Anova and multiple groups were compared using Bonferroni correction. A p value of less than 0.05 was considered statistically significant. ***p<0.001. N=3.
Figure 4.9: Disruption of the RACK1/PP2A complex decreases cellular proliferation. Proliferation was monitored in real time using the xCELLigence system. Readings were taken every minute over 10 hours and every 15 minutes for the remainder of the experiment. Readings expressed as CI values. (a) The xCELLigence graph is the average of duplicate wells comparing proliferation of PP2A mutant stable cell lines HA-PP2A WT cells compared to HA-PP2A R214A cells and HA-PP2A Y218F cells over 48 hours. (b) Comparison of the CI over 12, 24 and 48 hours. Data is represented as mean ± SEM. (c) The bar graph represents a comparison of the percentage difference in mean cell index of PP2A mutant stable cell line HA-PP2A WT with HA-PP2A R214A and HA-PP2A Y218F cells. Differences between groups were determined using Welsh Anova and multiple groups were compared using Bonferroni correction. A p value of less than 0.05 was considered statistically significant. ***p<0.001. N=3.
here show that cells expressing PP2A mutants PP2A R214A and HA-PP2A Y218F which are deficient in RACK1 binding have altered cell adhesion and spreading.

4.3.9 Disruption of the RACK1/PP2A complex decreases cellular proliferation.

Our next objective was to determine whether disruption of the RACK1/PP2A complex had an effect on cellular proliferation. Proliferation was monitored in real time using the xCELLigence system. Readings were automatically taken every minute for the first 10 hours and every 15 minutes for the remainder of the experiment. Readings are expressed as Cell Index (CI) values. The xCELLigence graph shown in Figure 4.9 (a) is representative of duplicate wells comparing proliferation of HA-PP2A WT, HA-PP2A R214A and HA-PP2A Y218F cells over 48 hours. Figure 4.9 (b) shows a comparison of the CI over 12, 24 and 48 hours. Figure 4.9 (c) shows comparisons of the percentage difference in mean cell index between the proliferation of HA-PP2A WT, HA-PP2A R214A and HA-PP2A Y218F cells. Data is represented as mean ± SEM. A statistical analysis was performed using SPSS 20 statistical package. Differences between groups were determined using Welsh Anova and multiple groups were compared using Bonferroni correction. As above, a p value of less than 0.05 was considered statistically significant. ***p<0.001. N=3. Our data here indicates that the RACK1/PP2A complex has a role to play in cell proliferation. Disruption of the interaction between RACK1 and PP2A using the HA-tagged PP2A mutants shows a reduced pattern of proliferation when compared to the HA-PP2A WT control showing that this complex is implicated in the proliferation of breast cancer cells.

4.3.10 Disruption of the RACK1/PP2A complex decreases cellular migration.

Next, we investigated if disruption of the RACK1/PP2A complex had an effect on cellular migration. To execute this, 20,000 cells were plated into the wells containing
Figure 4.10: Disruption of the RACK1/PP2A complex decreases cellular migration. (a) 20,000 cells were plated into wells containing culture inserts. The insert was removed after 24 hours and the cells were photographed at 0 hours and 24 hours. The migration of cells was analysed using Ibidi® Quantitative Image Analysis. (b) Percentage wound closure of HA-PP2A WT was compared to the percentage wound closure of HA-PP2A R214A and HA-PP2A Y218F after 24 hours. Differences between groups were determined using Welsh Anova and multiple groups were compared using Bonferroni correction. A p value of less than 0.05 was considered statistically significant. ***p<0.001. N=3.
culture inserts and left overnight to adhere fully. The insert was then removed to leave a cell free gap of 500µm ± 50µm. The cells were photographed using a Nikon microscope x63 lens at 0 hours and 24 hours (Figure 4.10 (a)). Data is presented by comparing the percentage of wound that was closed after 24 hours (Figure 4.10 (b)). Migration of cells was analysed using Ibidi® Quantitative Image Analysis. It was found that the HA-tagged PP2A mutant stable cell line HA-PP2A WT migrated faster than both HA-PP2A R214A and HA-PP2A Y218F cells. This indicates a role for the RACK1/PP2A complex in the fundamental process of cell migration. The role of RACK1 as a scaffolding protein involved in signalling pathways important for cell migration has been well studied (Adams et al. 2011). Our data serves to strengthen this view that RACK1 has a prominent role to play in recruiting and shuttling proteins to their substrates for activation of downstream signalling pathways involved in cell migration. It specifically indicates the role RACK1 plays in cell migration when in a complex with PP2A.

4.3.11 Disruption of the RACK1/PP2A complex decreases cellular invasion.

Next, cell invasion was monitored in real-time with the xCELLigence system CIM-plates (Figure 4.11). The CIM plate protocol was followed as in Materials and Methods. The impedance value of each well was automatically monitored by the xCELLigence system for duration of the experiment and expressed as a CI value (Figure 4.11 (a)). In the initial stages (0-2 hours), no reading is recorded due to the time required for the cells to migrate through the Matrigel™ layer. A number of cells reach the electrodes after this initial phase and cause enough impedance to result in a CI read out. Percentage difference in cell index of HA-PP2A WT was compared to HA-PP2A R214A and HA-PP2A Y218F over 72 hours (Figure 4.11 (b)). Invasion was also compared over 24, 36,
Figure 4.11: Disruption of the RACK1/PP2A complex decreases cellular invasion. Cell invasion of PP2A mutant stable cell lines HA-PP2A WT, HA-PP2A R214A and HA-PP2A Y218F was monitored in real-time with xCELLigence system CIM-plates. (a) 20,000 cells were seeded in the upper chamber in serum-free media. DMEM media containing 10% FBS was added to the lower chamber. The impedance value of each well was automatically monitored by the xCELLigence system for duration of the experiment and expressed as a CI value. (b) Percentage difference in cell index of HA-PP2A WT compared to HA-PP2A R214A and HA-PP2A Y218F over 72 hours. Multiple groups were compared using Welsh Anova and Bonferroni correction. A p value of less than 0.05 was considered statistically significant. ***p<0.001. N=3.
Figure 4.11: Disruption of the RACK1/PP2A complex decreases cellular invasion. (c) Invasion of HA-PP2A WT compared to HA-PP2A R214A and HA-PP2A Y218F was compared over 24, 36, 48, 60 and 72 hours. Multiple groups were compared using Welsh Anova and Bonferroni correction. A p value of less than 0.05 was considered statistically significant. ***p<0.001. N=3.
48, 60 and 72 hours (Figure 4.11 (c)). A statistical analysis was performed as described in Materials and Methods. ***p<0.001, N=3. Similarly to our cell migration study, data extracted shows that the ability of breast cancer cells to invade was reduced when the RACK1/PP2A complex is disrupted. HA-tagged PP2A mutant stable cell line HA-PP2A WT invaded faster than both HA-PP2A R214A and HA-PP2A Y218F cells.

4.3.12 Disruption of the RACK1/PP2A complex decreases plating efficiency.
Decreased cell adhesion, spreading, proliferation, migration and invasion in cells where RACK1 and PP2A binding is deficient suggests that the RACK1/PP2A complex has a role to play in maintaining the transformed phenotype in breast cancer. In order to investigate this hypothesis further, our next objective was to determine whether disruption of the RACK1/PP2A complex had an effect on plating efficiency and/or the cells ability to form spheroids in soft agar. To investigate plating efficiency, HA-PP2A WT, HA-PP2A R214A and HA-PP2A Y218F cells were harvested with trypsin/EDTA, washed and counted using a haemocytometer. 500 cells of each were plated per well of a 6 well plate. The plates were incubated at 37° in 5% CO₂ for 10 days. After 10 days, the cells were fixed in 96% ethanol and subsequently stained with 0.05% crystal violet. The wells were washed and allowed to dry. As shown in Figure 4.12, colonies were counted and recorded. Results from this study indicate that cells which have a disruption to the RACK1/PP2A complex are deficient in plating efficiency. This supports our hypothesis that the interaction between RACK1 and PP2A helps to maintain the transformed phenotype in breast cancer cells.

4.3.13 Disruption of the RACK1/PP2A complex decreases the cells ability to form spheroids in soft agar.
To determine the HA- tagged PP2A mutant cell lines ability to form spheroids in soft
Figure 4.12: Disruption of the RACK1/PP2A complex decreases plating efficiency. MCF-7 cells stably expressing HA-PP2A WT, HA-PP2A R214A and HA-PP2A Y218F were harvested with trypsin/EDTA, washed with DMEM and counted using a haemocytometer. (a) 500 cells were plated per well and incubated at 37°C in 5% CO₂ for 10 days. After 10 days, the cells were fixed in 96% ethanol and subsequently stained with 0.05% crystal violet. The wells were washed and colonies greater than 50 cells were counted and recorded. (b) Difference in colony number between HA-PP2A WT, HA-PP2A R214A and HA-PP2A Y218F. A statistical analysis was performed using SPSS 20 statistical package. Differences between groups were determined using Welsh Anova and multiple groups were compared using Bonferroni correction. A p value of less than 0.05 was considered statistically significant. ***p<0.001. N=3.
Figure 4.13: Disruption of the RACK1/PP2A complex decreases the ability to form spheroids in soft agar. 20,000 HA-PP2A WT, HA-PP2A R214A and HA-PP2A Y218F cells were plated in 0.3% agarose on to a solidified layer of 0.6% agarose. The cells were incubated at 37°C in 5% CO₂ for 14 days. Cells were then stained with 0.01% crystal violet overnight and counted. Differences between groups were determined using Welsh Anova and multiple groups were compared using Bonferroni correction. A p value of less than 0.05 was considered statistically significant. ***p<0.001. N=3.
agar, 20,000 HA-PP2A WT, HA-PP2A R214A and HA-PP2A Y218F cells were plated in 0.3% agarose on top of a layer of 0.6% agarose and processed as described in Materials and Methods. Statistical analysis was performed as described in Materials and Methods. ***p<0.001. N=3. The result from this data is that cells expressing a disruption to the RACK1/PP2A complex have deficient ability to form colonies in soft agar when compared to the Wild Type control (Figure 4.13).

4.3.14 Mapping the interaction between RACK1 and PP2A A subunit.

Our hypothesis is that RACK1 is functioning as a regulatory subunit of PP2A. If this is the case, RACK1 would be required to bind to both the PP2A A and C subunit to stabilize the PP2A holoenzyme. In order to identify any binding sites of RACK1 on the PP2A A subunit, we utilised peptide arrays which have previously been used to identify the binding sites of RACK1 on PP2A C subunit (Figure 4.1). A library of overlapping peptides (18-mers) each shifted by 4 amino acid residues encompassing the entire sequence of the PP2A A subunit was spot-synthesized on nitrocellulose membranes to generate PP2A A subunit arrays. These arrays were then probed with recombinant GST or GST-RACK1 as described in Materials and Methods. Any spots that GST bound to within the PP2A A subunit array were subtracted from the array probed with GST-RACK1. A number of positive reaction patterns were identified on two areas of the array (peptides 83, 84, 85, 87 and peptides 129-131) (Figure 4.14). RACK1 bound to a number of peptides with the positive reactions indicated by dark spots. These positive reactions confirm interaction between RACK1 and the PP2A A subunit.

4.3.15 Identification of RACK1 interaction sites on PP2A A subunit alanine substitution arrays.

We wanted to demonstrate that RACK1 is in a complex with the core PP2A
<table>
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<th>Interaction</th>
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<td>Peptide 88</td>
<td>+</td>
</tr>
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<tr>
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<td>521-538</td>
<td>Peptide 131</td>
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**Figure 4.14: Mapping the interaction between RACK1 and the PP2A A subunit.**

(a) The amino acid sequence of the entire PP2A Aα subunit is shown schematically (1-589). Peptide arrays of immobilised overlapping 18-mer peptides, each shifted to the right by 4 amino acids encompassing the entire PP2A A subunit were generated. Arrays were probed with GST-RACK1, which was detected by immunoblotting with anti-GST antibody. The arrays shown are representative of two arrays which gave a similar pattern of RACK1 binding. Positively interacting peptides generated dark spots and non-interacting peptides left blank spots. Spots that were generated on the GST control were disregarded as non-specific binding.
Figure 4.15: Identification of RACK1 interaction sites on the PP2A Aα subunit alanine substitution arrays. (a) MCF-7 cells lysed and a PP2A C subunit IP was performed. IP samples were run on a 12% SDS-PAGE gel and analysed for associated RACK1 and PP2A A subunit by western blotting. (b) Arrays were synthesized in which 18 amino acids in peptide 85 (A337-P354) of the PP2A Aα subunit were sequentially substituted with alanine along the entire sequence of the 18-mer parent peptide. (c) Arrays were synthesized in which 18 amino acids in peptide 130 (T517-A534) of PP2A A subunit were sequentially substituted with alanine along the entire sequence of the 18-mer parent peptide. The array was probed with GST-RACK1, which was detected by immunoblotting with anti-RACK1 antibody. A decrease in intensity in binding to the peptides after alanine substitution is indicative of decreased binding of the PP2A Aα subunit to RACK1. The binding of RACK1 to each alanine-substituted PP2A Aα subunit peptide was quantified by densitometry and presented as a percentage of the control “parent” sequence. N=3
holoenzyme (both the PP2A A and C subunits). To do this, MCF-7 cells were serum-starved, lysed and a PP2A C subunit IP was performed. The IP samples were run on a 12% SDS-PAGE gel and analysed for associated RACK1 and PP2A A subunit by western blotting. Using immunoprecipitation, RACK1 and PP2A A subunit were confirmed to be in a complex with the PP2A C subunit (Figure 4.15 (a)).

After narrowing down the binding sites of RACK1 on the PP2A A subunit (Figure 4.14), alanine substitution arrays were used to identify the specific amino acids within these peptides required for RACK1 binding. An array of peptides were generated derived from the 18-mer parent peptides corresponding to peptide spot 85 (A337-P354) and peptide spot 130 (T517-534A). For each parent peptide, 18 progeny were generated where each new peptide in the array had a single alanine substitution in successive amino acids in the sequence. These two alanine-scanning peptide arrays were then probed with recombinant GST-RACK1. Results show that RACK1 binding to these peptides was either severely attenuated or ablated by alanine substitution of lysine 342 (K342) in peptide 85 and lysine 519 (K519) in peptide 130. The interaction of RACK1 with the peptides was quantified by densitometry and is presented as a percentage of binding of RACK1 to the control parent peptide (Figure 4.15 (b and c)). Together, these analyses confirm that the binding of RACK1 to PP2A A subunit is direct and suggests the key amino acids required for binding of the two proteins.

4.3.16: Modelling of the potential RACK1 binding sites on the PP2A A subunit.

As two amino acids were identified that had the potential to be the binding site on the PP2A A subunit for RACK1 using alanine substitution assays (Figure 4.14), we wanted to model these sites on the crystal structure of the PP2A A subunit to determine whether the amino acids are on the surface and suitable as interaction sites. Using the crystal
Figure 4.16: Modelling of the potential RACK1 binding sites on the PP2A A subunit. Crystal structure (PDB code 1B3U) of *H.Sapien* PP2A A subunit (Groves et al. 1999) was used to map the location of RACK1 binding sites on the protein. (a) The crystal structure of PP2A A subunit with both sites, K342 and K519 identified in the alanine substitution array (Figure 4.15) highlighted in green. (b) The crystal structure of PP2A A subunit with the location of amino acid K342 in green (identified in the alanine substitution assay Figure 4.15). (c) The crystal structure of the PP2A A subunit with the location of amino acid K519 in green (identified in the alanine substitution assay Figure 4.15).
structure of *H. sapiens* PP2A A subunit (PDB code 1B3U) (Groves et al. 1999) *(Figure 4.16 (a))*, we mapped the locations of K342 and K519 using PyMol. Both amino acids are highlighted in green. K342 is highlighted in green *(Figure 4.16 (b)) and K519 is highlighted in green *(Figure 4.16 (c)). Both amino acids are visible on the surface of the PP2A A subunit and therefore, either of these amino acids could be important for RACK1 binding.
4.4 Discussion.

RACK1 has over 90 known binding partners (McCahill et al. 2002, Adams et al. 2011). Although some of these interactions are yet to be characterised, it is clear that RACK1 has distinct roles to play in cells. RACK1 acts as a scaffold for a diverse array of binding partners in several signalling pathways. In this way, RACK1 integrates a wide array of signalling events in the cell. Central to this is the role played by RACK1 in growth factor signalling. RACK1 also has the capacity to stabilize enzymatic activity of its binding partners without having any intrinsic enzymatic capacities itself. This has already been shown with PP2A where RACK1 stabilizes PP2A activity (Kiely et al. 2006, Kiely et al. 2008) and also maintains PKCβII in its active conformation (Ron et al. 1994, Stebbins and Mochly-Rosen 2001, Ron et al. 1999).

As RACK1 expression is reported to be increased in many cancer types including oral squamous carcinoma and lung cancer (Li et al. 2012, Shi et al. 2012, Wang et al. 2009, Xi-Xi Cao et al. 2010) and PP2A can be either a tumour suppressor or show carcinogenic properties (Perrotti and Neviani 2013, Boudreau et al. 2007), we wanted to determine exactly how RACK1 and PP2A interact and determine the consequences for the cells when the RACK1/PP2A complex was disrupted. To do this, we used immobilised peptide array technology to identify three potential binding sites between RACK1 and the PP2A C subunit; FR which are amino acids 63 and 64, R which is amino acid 214 and Y which is amino acid 218. Modelling of these sites revealed that the binding sites are surfaced exposed on the PP2A C subunit so could function as interaction sites between the RACK1 and PP2A. R214 is located close to the active site on the PP2A C subunit. Y218 is located quite near to the catalytic pocket and interestingly in one crystal structure (PDB code 3FGA), Y218 forms part of the surface...
contact with the shugoshin protein Sgo1 (Xu et al. 2009). Although it is not thought that the interaction between RACK1 and PP2A mimics this interaction precisely (because RACK1 is unlikely to reorganise so as to present a helical motif), it does establish a precedent for Y218 as part of a binding surface for a partner protein.

In order to determine the role that the RACK1/PP2A complex was playing in breast cancer cells, we generated HA-tagged stable PP2A mutant cell lines containing mutations at the three interaction sites identified. These were a mutation from phenylalanine to alanine at amino acid 63, a mutation from arginine to alanine at amino acid 64, a mutation from alanine to arginine at amino acid 214 and a mutation from tyrosine to phenylalanine at amino acid 218. Molecular modelling (in collaboration with Prof Dave Adams Heriot-Watt University, Edinburgh, Scotland) suggested that binding of RACK1 at amino acid sites 63 and 64 would preclude the binding of other regulatory B subunits to the core PP2A subunit. We also have evidence demonstrating that disruption at this F63R64 site has a negative effect on PP2A activity. However, when we disrupted this site we detected no other change in cellular behaviour. Modelling of all three sites does suggest that it would be possible for two RACK1 molecules to bind to the PP2A core enzyme at the same time suggesting that there may be more than one role for RACK1 depending on which site it binds to. We also carried out a series of experiments with the other two mutants, HA-PP2A R214A and HA-PP2A Y218F. We used these mutants to characterize the interaction between RACK1 and PP2A using immunofluorescence and found that RACK1 and PP2A colocalised less in cells that stably expressed either of the PP2A mutants compared to the wild type control. This was further evidence that these mutants were disrupting the RACK1/PP2A complex.

Immunoprecipitation of RACK1 from HA-PP2A R214A and HA-PP2A Y218F as well as the Wild Type control were attempted on numerous occasions to determine
there was a reduction in the interaction of RACK1 and PP2A upon disruption of the complex. Despite a number of attempts, it proved to be to technically difficult to immunoprecipitate the same amount of RACK1 from each cell lysate so data for this cannot be presented.

It is already known that RACK1 stabilizes PP2A activity (Kiely et al. 2006, Kiely et al. 2008). We used our HA-tagged stable PP2A mutant cell lines to determine whether disruption of the interaction sites we had identified would be sufficient to disrupt the complex and have a similar effect on PP2A activity. In the case of both mutations, we saw a reduction of PP2A phosphatase activity of over 50% when compared to the control. We then generated stable cell lines expressing the PP2A mutants and used the HA-tagged stable PP2A mutant cell lines to determine the consequences of disrupting the RACK1/PP2A complex on a number of essential cell characteristics that play an important role in the development and progression of cancer. To begin, we investigated the effects on adhesion by using both a traditional cell adhesion assay and also a real time cell analysis platform (xCELLigence system). Both assays showed decreased cell adhesion in cells where the RACK1/PP2A complex had been disrupted in comparison to the control. With regard to cell proliferation, studies using the RTCA xCELLigence system showed that the RACK1/PP2A complex has a role to play in driving breast cancer cell proliferation.

We next wanted to determine if the RACK1/PP2A complex was playing a contributory role in the promotion of cell migration and invasion (Kiely et al. 2009, Dave et al. 2013) Making further use of our PP2A mutant cell lines, we investigated the difference in cell migration between them and our control cells. We found that both mutants migrated slower than the control over 24 hours. For the invasion experiments, we utilized the Cell Invasion Migration (CIM) plates of the RTCA xCELLigence
system to monitor the cells ability to invade through a Matrigel™ layer (See Methods, Chapter 2). Results show that both mutants invaded slower compared to the control cells.

We now had a set of experiments showing that the RACK1/PP2A complex plays a role in a number of essential cell characteristics: cell adhesion, proliferation, migration and invasion. Collectively, these experiments suggest that the RACK1/PP2A complex is driving the cancer phenotype in these breast cancer cells. In order to gain further evidence to support this theory, we employed two more assays using our HA-tagged stable PP2A mutant cell lines. Firstly, we looked at the plating efficiency of the cell compared to the control. Both mutant cell lines displayed reduced ability to proliferate when seeded at such low cell densities compared to the control. Secondly, we investigated the ability of the cells to form colonies in soft agar. Again, the mutants had reduced proliferative capacity when compared to the control cell line. This work points towards a role for the RACK1/PP2A complex in the progression and maintenance of the cancer phenotype in this breast cancer cell model.

This work highlights a number of intriguing possibilities. It suggests that PP2A in a complex with RACK1 through interactions at amino acids 214 and 218 of the PP2A C subunit may not be functioning as a tumour suppressor. Rather, we show that inhibition of PP2A activity through disruption of the RACK1/PP2A complex reduces cell adhesion, proliferation, migration and invasion. This strongly points towards PP2A having a pro-carcinogenic role to play in this cancer cell model when in a complex with RACK1. RACK1 appears to have the ability to scaffold PP2A to sites and substrates involved in cancer progression while at the same time RACK1 also plays an essential role in stabilizing PP2A phosphatase activity. To this end, the RACK1/PP2A complex may be considered as a potential therapeutic target for breast cancer in the future.
Development of small molecules that disrupt the interaction between these two proteins could serve to slow down the development and progression of a malignancy. Inhibition or inactivation of PP2A is not a new concept and has been suggested as a potential therapy in cancer (Zimmerman et al. 2012, McDermott et al. 2014, Huang et al. 2009). Work has already begun on establishing small molecules that inhibit PP2A to improve efficacy to chemotherapy (Lu et al. 2009). However, this study implicates the RACK1/PP2A interaction as a potential site for targeted therapy. This work suggests that designing small peptides to disrupt the RACK1/PP2A interaction may be promising as anti-cancer drugs. In the next chapter of this thesis, we will focus on identifying the subset of proteins that bind to the RACK1/PP2A complex. In particular, we will concentrate on identifying proteins known to play a role in cancer.

To further elucidate the PP2A interaction with RACK1 we wanted to determine whether there are potential binding sites between RACK1 and the PP2A A subunit. To do this, we again utilized peptide array technology and peptide arrays of the PP2A A subunit were probed with GST-RACK1 to reveal two potential areas of interaction between the two proteins. Further analysis using alanine substitution assays identified two amino acids involved in the binding between RACK1 and PP2A A subunit. The A subunit is consistently pulled down in the RACK1/PP2A A subunit immunoprecipitations and we suggest that RACK1 may play a role in stabilising the formation of this complex. This will be a focus of future research projects in the laboratory.

In summary, we have identified the interaction sites between RACK1 and the core PP2A subunit. We generated stable breast cancer cells lines expressing mutants that disrupt the interaction between RACK1 and the PP2A C subunit. We used those cell lines to determine that disruption of the RACK1/PP2A complex decreases PP2A
phosphatase activity levels and also reduces the adhesion, proliferation, migration and invasion of the breast cancer cells. This work has significantly advanced our understanding of the RACK1/PP2A complex.
Chapter 5:

Identification of novel proteins interacting with the RACK1/PP2A complex using Mass Spectrometry analysis
5.1 Abstract

In its capacity as a shuttle and scaffolding protein, RACK1 facilitates the movement of its interacting partners, bringing them in close proximity to their substrates. RACK1 is a binding partner of PP2A and our data indicates that RACK1 plays a critical role in determining how PP2A functions in signalling pathways by modulating the activity and substrate specificity of PP2A, while regulating its distribution to specific cellular locations. A mass spectrometry screen of the RACK1/PP2A complex in breast cancer cells identified 66 novel binding partners of the complex. This diverse group of proteins is from a variety of cellular locations. TTBK-1 was identified for the first time in breast cancer cells and was found to be over-expressed in 70% of cancer samples analysed. This novel protein has the potential to be used as a prognostic indicator of disease in breast cancer. Metadherin was also identified in the mass spectrometry screen. It was chosen for further study because it is overexpressed in a significant number of breast cancer cases. Our study with a small cohort of Irish patients confirms this with 60% of our cohort having over-expression of Metadherin. This strengthens the evidence that Metadherin has potential as a prognostic factor in breast cancer. The interaction between Metadherin, RACK1 and PP2A was confirmed by both immunofluorescence and immunoprecipitation. Potential interaction sites between Metadherin and RACK1 have been revealed, providing evidence that the interaction between these two proteins is direct. This work also promotes our understanding of RACK1 in cancer and highlights RACK1 involvement with proteins known to be linked to the progression of cancer. It also highlights the value of considering scaffolding proteins as novel therapeutic targets.
5.2 Introduction

The scaffolding properties of RACK1 allow it to interact with proteins both directly and as part of a larger complex. It shuttles proteins around the cells and anchors them at locations in close proximity to their substrates (Adams et al. 2011). Now that the interaction between RACK1 and PP2A has been characterised (Chapter 4), we next wanted to identify the subset of proteins that are interacting with the RACK1/PP2A complex. Our hypothesis was that RACK1 facilitates the substrate specificity of PP2A by scaffolding it to its substrates where it can regulate their activity.

RACK1, as well as being highly expressed in most tissues (Chou et al. 1999), is found in many different subcellular locations, allowing it to interact with multiple proteins and regulate diverse functions. Subcellular compartments in which RACK1 has been identified include the cytosol, endoplasmic reticulum, ribosome, cytoplasm and cell membrane (Adams et al. 2011). It is involved in translation through its interaction with the ribosome (Coyle et al. 2009). RACK1 can also translocate to different subcellular compartments in response to stimuli, bringing its binding partners closer to their substrates. For example, RACK1 binds to Src via its SH2 domain to translocate Src to cellular compartments where it can function to regulate cell adhesion and migration (Chang et al. 2002, Doan and Huttenlocher 2007). This is known to be an IGF-1 dependant mechanism, regulated by IGF-1 at the IGF-IR signalling pathway (Kiely et al. 2005). RACK1 has also been shown to translocate to the nucleus where it has been identified in a number of complexes (Adams et al. 2011).

It is well established that RACK1 is essential for cell migration as it binds to many components of the cell migration machinery including kinases and phosphatases (e.g. PP2A and FAK) (Kiely et al. 2005, Kiely et al. 2006, Serrels et al. 2011, Doan and
Huttenlocher 2007, Dave et al. 2013) as well as binding to cytoplasmic domains of cell surface receptors (Adams et al. 2011). Cell migration is a fundamental process, required for embryonic development, wound healing and immune responses. Dysregulation of cell migration can have serious consequences including a malfunctioning immune system and materialisation of cancer. RACK1 is also a mediator of cellular spreading through its interactions with the extracellular matrix and growth factor receptors at adhesion sites (Hermanto et al. 2002).

RACK1 also scaffolds several proteins which are altered in cancer, including PP2A (Adams et al. 2011). The role for RACK1 in breast cancer is not clear, with some studies reporting high RACK1 expression in breast cancer patients correlating with poor clinical outcome (Xi-Xi Cao et al. 2010) and other studies report decreased RACK1 expression in breast cancer patients (Al-Reefy and Mokbel 2010).

The role of PP2A in cancer has been well studied. PP2A is most well known as a tumour suppressor and for this reason, is a target for many cancer causing viruses (Guergnon et al. 2011) (Eichhorn et al. 2009). PP2A expression is altered in many cancer types, however, PP2A has also been linked to an anti-apoptotic role in certain cellular responses (Wei Li et al. 2011). This highlights the very complex role PP2A plays in signalling pathways. The tight regulation of PP2A and its substrates is essential to stabilize activity of these proteins. We hypothesize that the ability of RACK1 to scaffold PP2A to its substrates is essential to facilitate PP2A activity and facilitate the binding of PP2A to bind to its substrates and maintain regulatory control of signalling pathways.

The aim of our research was to identify novel interacting binding partners of the RACK1/PP2A complex in breast cancer cells. Once identified, our aims were to
categorise a subset of these determine the role they play in the development and/or progression of breast cancer.
5.3 Results

5.3.1 Work-flow to identify novel interacting proteins of the RACK1/PP2A complex.

The hypothesis was that RACK1 facilitates the substrate specificity of PP2A by scaffolding it and bringing it in close proximity to its substrates where it can regulate their activity. To identify these substrates, a double immunoprecipitation protocol was developed and refined which allowed us to pull down RACK1 in a complex with the PP2A C subunit and all the other proteins which are in a complex with both RACK1 and PP2A. MCF-7 cells were cultured for ten days in 3D using Matrigel\textsuperscript{TM}. The cells were lysed and a RACK1 IP was performed. This pulled down RACK1 and all proteins interacting with RACK1. The sample was then re-incubated for 3 hours with an anti-PP2A C subunit antibody to more specifically pull down the proteins that are in a complex with both RACK1 and the PP2A C subunit. Western blot analysis showed that RACK1 and the subset of the PP2A C subunit that is bound to RACK1 had been pulled down from the 3D culture. When satisfied with the protocol, the samples were ran on a 12% SDS-PAGE gel (Figure 5.1). A control IP was also performed to rule out non-specific binding. Bands were cut from the gel and sent to University of St Andrews, Fife, Scotland for mass spectrometry analysis. 66 novel interacting binding partners of the RACK1/PP2A complex were identified.

5.3.2 Subcellular location and function of novel interacting proteins of the RACK1/PP2A complex.

Having determined a list of proteins that bind to the RACK1/PP2A complex in MCF-7 cells (Table 5.1), we wanted to find proteins that could be involved in the development or progression of breast cancer. Protein hits were analysed and grouped in bar charts.
Figure 5.1: Work-flow to identify novel interacting proteins of the RACK1/PP2A complex. MCF-7 cells were cultured in 3D using Matrigel™. The 3D cultures were lysed and a RACK1 IP was performed. The sample was then re-incubated with an anti-PP2A C subunit antibody for a ‘double IP’. Samples were run on a 12% SDS-PAGE gel. Western blot is representative of blots used in the development of the protocol to determine that the IP’s were successful in pulling down the PP2A C subunit that was in a complex with RACK1. For mass spectrometry, samples were run on a 12% SDS-PAGE gel. Bands were cut out and sent for mass spectrometry analysis.
Table 5.1 Identification of proteins interacting with the RACK1/PP2A complex using Mass Spectroscopy analysis.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Accession Number</th>
<th>No. of Peptides returned (95% confidence)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60S Ribosomal Protein L8</td>
<td>NP_150644</td>
<td>7</td>
</tr>
<tr>
<td>Ribosomal Protein L10</td>
<td>BAA28595</td>
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<tr>
<td>hCG2039812</td>
<td>EAW96631</td>
<td>4</td>
</tr>
<tr>
<td>Mitochondrial ATP synthase, H+ transporting F1 complex beta subunit</td>
<td>ABD77240</td>
<td>4</td>
</tr>
<tr>
<td>ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle.</td>
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<td>4</td>
</tr>
<tr>
<td>Heat shock 70kda protein 8 isoform 2 Variant.</td>
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<td>3</td>
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<tr>
<td>Ribosomal Protein L18.</td>
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<td>3</td>
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<tr>
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<tr>
<td>Ribosomal Protein L7a.</td>
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<tr>
<td>DEAD box polypeptide 17 isoform p82 variant.</td>
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</tr>
<tr>
<td>Prolactin-inducible protein precursor</td>
<td>NP_002643</td>
<td>1</td>
</tr>
<tr>
<td>A-Glucosidase 1</td>
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</tr>
<tr>
<td>Phosphoglycerate kinase 1.</td>
<td>NP_000282.1</td>
<td>1</td>
</tr>
<tr>
<td>Human elongation factor 1 delta</td>
<td>CAA79716</td>
<td>1</td>
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<td>hCG21218</td>
<td>EAW78873</td>
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<td>Septin 10</td>
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<td>CD68 Antigen Variant.</td>
<td>BAD96212</td>
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</tr>
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<td>NP_001958</td>
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<td>Protein Name</td>
<td>Accession Number</td>
<td>No. of Peptides returned (80% confidence)</td>
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</tr>
<tr>
<td>HnRNP U Protein.</td>
<td>CAA46472</td>
<td>2</td>
</tr>
<tr>
<td>Rab 7.(endosomal)</td>
<td>NP_004628</td>
<td>1</td>
</tr>
<tr>
<td>Brain Derived Tau Kinase.</td>
<td>NP_115927</td>
<td>1</td>
</tr>
<tr>
<td>Replication Factor C (activator 1) 4 37kDa</td>
<td>AAH24022</td>
<td>1</td>
</tr>
<tr>
<td>H2AFX</td>
<td>CAG33360</td>
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</tr>
<tr>
<td>KIAA0111</td>
<td>BAA04879</td>
<td>1</td>
</tr>
<tr>
<td>SCCA2/SCCA1 fusion protein. Isoform 1</td>
<td>AAQ04770</td>
<td>1</td>
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<td><strong>Protein Name</strong></td>
<td><strong>Accession Number</strong></td>
<td><strong>No. of Peptides returned (below 80% confidence)</strong></td>
</tr>
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<td>ATP Dependant RNA Helicase DDX1</td>
<td>Q92499</td>
<td>2</td>
</tr>
<tr>
<td>Replication Factor C (activator 1) 4 37kDa</td>
<td>AAH24022</td>
<td>2</td>
</tr>
<tr>
<td>Ribosomal Protein S13</td>
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<tr>
<td>Ribosomal Protein SA, 40S.</td>
<td>P08865</td>
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</tr>
<tr>
<td>Ribosomal Protein S5.</td>
<td></td>
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</tr>
<tr>
<td>AMY1A amylase.</td>
<td>AAI44453</td>
<td>1</td>
</tr>
<tr>
<td>Zinc finger protein 318</td>
<td>NP_055160</td>
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</tr>
<tr>
<td>Isoleucyl-trna synthetase, cytoplasmic variant</td>
<td>BAD92471</td>
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<tr>
<td>Histone H2B type 2-\isofrom B.</td>
<td>NP_001154806</td>
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<td>Haemoglobin beta.</td>
<td>CAA00182</td>
<td>1</td>
</tr>
<tr>
<td>Haemoglobin alpha-1 globin chain.</td>
<td>NP_000549</td>
<td>1</td>
</tr>
<tr>
<td>Dihydrolipoamide S-acetyltransferase.</td>
<td>AAH39084</td>
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</tr>
<tr>
<td>Glutaminyl-TRNA synthase variant.</td>
<td>P07814</td>
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<tr>
<td>V(D)J recombination-activating protein 1</td>
<td>NP_000439</td>
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</tr>
<tr>
<td>Citrate Synthase Protein</td>
<td>NP_004068</td>
<td>1</td>
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<tr>
<td>Albumin Like.</td>
<td>AAA64922</td>
<td>1</td>
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</tbody>
</table>
Table 5.1 Identification of proteins interacting with the RACK1/PP2A complex using Mass Spectroscopy analysis. MCF-7 cells were cultured for 10 days in 3D using Matrigel™. A RACK1 IP was performed and the sample was re-incubated with an anti-PP2A C subunit antibody. IP samples were run on a 12% SDS-PAGE gel. Bands were cut out and sent for mass spectrometry. Bands from a control IP were also sent to control for non-specific binding proteins. Mass spectrometry results were analysed and protein hits from the corresponding control lane were subtracted. 66 unique proteins were found to be bound to the RACK1/PP2A complex in MCF-7 cells cultured in 3D. These interacting proteins require further analysis to find potential interacting proteins of the RACK1/PP2A complex with links to the development or progression of breast cancer. Proteins that are unique binding partners of the RACK1/PP2A complex are listed with their NCBI accession number and the number of peptides returned from the mass spectroscopy analysis at the highest confidence level for each protein identified.
according to subcellular localisation (Figure 5.2 (a)) and function (Figure 5.2 (b)). A number of proteins were chosen for further study.

5.3.3 Focus on TTBK-1

A double IP of the RACK1/PP2A complex and a control IP were run on an SDS-PAGE gel and TTBK-1 was identified as a binding partner of the RACK1/PP2A complex in the RACK1/PP2A complex IP only. One matched TTBK-1 peptide was found: VATISPRR (Figure 5.3 (a)). The mass spectrometry graph is composed of b-type ions and y-type ions with intensity on the y-axis and mass to charge (m/z) ratio on the x-axis. The mass difference between the b-type ion peaks corresponds directly to the amino acid sequence and is read from left to right. The y-type ion peaks have different measured heights and are read from right to left. Theoretical m/z values of product ions are shown in bold red (Figure 5.3 (b)). We identified TTBK-1 as a protein that interacts with the RACK1/PP2A complex. This interaction was confirmed using immunofluorescence (Hayes S, Kiely M, Kiely P.A, in final preparation).

Tau TuBulin Kinase-1 (TTBK-1) is a protein with 1,321 amino acids and is located on chromosome 6p21.1 (Sato et al. 2006). TTBK-1 is reported to be a brain specific protein (Sato et al. 2006). It is a serine/threonine/tyrosine kinase, which is a member of the casein kinase 1 superfamily that has the ability to phosphorylate Tau, a protein that is involved in tubulin polymerisation (Sato et al. 2006). TTBK-1 phosphorylates Tau at a number of sites in the brain associated with Alzheimer’s disease and has been shown to induce Tau aggregation in a neuronal cell model (Sato et al. 2006). This aggregation, known as neurofibrillary tangles are strongly implicated in the onset and progression of neurodegenerative diseases including
Figure 5.2: Subcellular location and function of novel interacting proteins of the RACK1/PP2A complex. (a) Binding proteins of the RACK1/PP2A complex are grouped according to their subcellular localisation: endosome, extracellular, mitochondria, nuclear, ribosome, membrane, secretory and cytoplasm. Nuclear proteins were the most common. (b) Binding proteins of the RACK1/PP2A complex are grouped according to their functional role: metabolism, heat shock, immune system, histones, hormones, RNA helicases, transport and trafficking, DNA regulation, adhesion, protein synthesis, ribosomes, neuronal, blood and skin. Ribosomal proteins and proteins involved in metabolism were the most common.
Alzheimer’s disease (Ballatore et al. 2007). It is known that PP2A is a major Tau phosphatase and down-regulation of PP2A contributes to Tau hyper-phosphorylation in Alzheimer’s disease (Qian et al. 2010). To the best of our knowledge, TTBK-1 has never been linked to cancer. We chose TTBK-1 for further study because we were interested to investigate why it was expressed in breast cancer cells when it is normally brain specific.

5.3.4 TTBK-1 expression in breast cancer.

Our first objective was to determine if there was differential expression of TTBK-1 in breast cancer cell lines compared to a non-cancer cell line. RNA was extracted from cell lines using the Qiagen RNeasy Plus mini kit. The concentration and quality of the RNA was determined by reading a sample of the RNA on the NanoDrop (see Appendix B). RNA (1µg) was synthesised into cDNA using the Invitrogen Vilo cDNA synthesis kit. Quantitative real-time PCR was carried out on the cDNA using Taqman® assays in a thermocycler. All data was analysed using REST© software. Figure 5.4 (a) shows TTBK-1 to be up-regulated in both breast cancer cell lines tested when compared to expression in a non-cancer breast cancer cell line.

Core biopsies of breast tissue were collected from 12 patients undergoing surgery in University Hospital Limerick. Normal tissue from 1 patient was collected approximately 3cm from the tumour, dissected and confirmed as normal by a pathologist. RNA was extracted from the tissue using the Qiagen RNeasy Lipid Tissue mini kit. The concentration and quality of the RNA was determined by reading a sample of the RNA on the NanoDrop (see Appendix B). RNA (500ng) was synthesised into cDNA using the Invitrogen Vilo cDNA synthesis kit. Quantitative real-time PCR was carried out on the cDNA using Taqman® assays in a
Figure 5.3: Identification of Tau Tubulin Kinase-1 as a binding partner of the RACK1/PP2A complex. (a) Mass spectrometry analysis of the RACK1/PP2A complex identified Tau Tubulin Kinase-1 (TTBK-1) as a binding partner. The matched peptide has >80% confidence of positive protein identification. (b) The peptides found in the mass spectrometry analysis are listed in plots and tables of MS/MS/fragmentation of (TTBK-1) peptides.
Figure 5.4: TTBK-1 expression in breast cancer. RNA was extracted and quantitative real-time PCR was carried out with the cDNA using Taqman® assays. All data was analysed using REST© software. (a) Bar graph representing the fold change of the TTBK-1 gene in MCF-7 and MDA-MB-231 cell lines compared to the non-cancer MCF10A cell line. MCF-7 cells were normalized using the reference genes PMM1 and GUSB. MDA-MB-231 cells were normalized using the reference genes PMM1 and TBP. (b) Core biopsy breast tissue samples were collected from 12 patients undergoing surgery in University Hospital Limerick. Normal tissue from 1 patient was also collected approx 3cm from the tumour and confirmed as normal by a pathologist. Bar graph representing the fold change of TTBK-1 in each cancer tissue sample when compared to the normal tissue sample. Luminal A samples were normalized using the reference genes; PMM1, PGK1 and ATCB. Fibroadenoma samples were normalized using the reference genes; PMM1 and PGK1. TNBC samples were normalized using the reference genes; PMM1, PGK1 and PP1A. The HER2⁺ sample was normalised using the reference genes; PMM1, PGK1 and ATCB. *p<0.01. ***p<0.001. N=3.
thermocycler and data was analysed using REST© software. The patients were grouped according to disease subtype. Luminal A samples were normalized using the reference genes PMM1, PGK1 and ATCB. Fibroadenoma samples were normalized using the reference genes PMM1 and PGK1. TNBC samples were normalized using the reference genes PMM1, PGK1 and PP1A. The HER2^+^ sample was normalised using the reference genes PMM1, PGK1 and ATCB. Our results show that TTBK-1 gene was overexpressed in the fibroadenoma tissue of 1 out of the 2 patients analysed when compared to the normal tissue of 1 patient sample (Figure 5.4 (b)). TTBK-1 was found to be overexpressed in 70% of the cancer samples analysed (Figure 5.4 (b)). This includes 5 out of 7 patients (71.4%) in the Luminal A cancer tissue of when compared to the normal tissue of 1 patient sample. One TNBC sample and one HER2^+^ sample had overexpression of TTBK-1.

5.3.5 Focus on Metadherin.

Metadherin was identified in the mass spectrometry screen of proteins interacting with the RACK1/PP2A complex. Three names, Astrocyte Elevated Gene 1 (AEG-1)/Metadherin/LYSine Rich CEACAM1 coisolated (LYRIC) are used interchangeably for this novel protein identified by four independent laboratories in a period from 2002-2005 (Su et al. 2003, Brown and Ruoslahti 2004, Britt et al. 2004, Sutherland et al. 2004). I use the name Metadherin throughout this thesis. Metadherin is a 64kDa protein with a 562 amino acid sequence (Kang et al. 2005). Metadherin homologues have been identified in other mammals apart from humans as well as in some vertebrates but it is not seen in invertebrates (Yoo et al. 2011a). The human Metadherin gene (MTDH) is located on chromosome 8q22. This site has already been shown to be the site of genomic alterations in hepatocellular carcinoma (HCC) cells and breast cancer cells.
Analysis of the gene has revealed relatively few protein domains and this hampers the ability to determine the function of the gene based on structural analysis (Yoo et al. 2011a). Prediction analysis indicates that Metadherin is a single-transmembrane protein with a putative transmembrane domain at amino acid residues 51-72 (Kang et al. 2005, Britt et al. 2004). The gene is rich in lysine (12.3%) and serine residues (11.6%). These are targets for a variety of post translational modifications including acetylation of lysines and phosphorylation of serines (Thirkettle et al. 2009). Sites for phosphorylation of tyrosine and threonine residues are present also on this gene (Yoo et al. 2011a).

Metadherin mRNA is ubiquitously expressed in all normal tissue with relatively higher expression levels reported in skeletal, cardiac, liver and some endocrine glands e.g. the adrenal gland (Kang et al. 2005). Metadherin has a potential role in embryogenesis and development (Jeon et al. 2010). It colocalises with proliferation marker Ki67 at specific stages of embryogenesis which suggests a role for Metadherin in cell proliferation during this process. Increased expression of Metadherin has been reported at stages E12.5 and E18.5 of embryogenesis in mice, specifically in brain, olfactory and skeletal systems indicating a potential role for Metadherin in the development of these organs. Metadherin is found in many cellular locations including the cytoplasm, cell membrane, endoplasmic reticulum and nucleus (Lee et al. 2013).

There is overwhelming evidence available in the literature confirming that Metadherin is an oncogene. Metadherin overexpression is reported in almost all cancer types. These studies have been completed in both cell lines and matched tissue samples and include breast cancer, HCC, cancer of the larynx, non-small cell lung cancer (NSCLC), colorectal cancer, oesophageal squamous cell carcinoma and lymphoma (Hu et al. 2009, Li et al. 2008, Poon et al. 2006, Liu et al. 2013, Song et al. 2009, Song et al.
2010, Yu et al. 2009, Li et al. 2014) In many cases, Metadherin overexpression correlates with clinical stage of disease, lymph node metastasis, disease free survival, overall survival and post-operative recurrence (Liu et al. 2013, Yu et al. 2009, Tokunaga et al. 2014). This accumulating evidence suggests that Metadherin has the potential to be a prognostic and diagnostic marker of metastasis, advanced stage of disease and poor patient prognosis.

To look specifically at breast cancer, analysis of over 1000 breast cancer tissue samples detected Metadherin overexpression in over 40% of cases compared to normal tissue. This was significantly correlated to staging of the disease through involvement in metastasis, survival and angiogenesis (Hu et al. 2009, Cong Li et al. 2011). For example, higher expression of Metadherin is reported in patients with metastatic breast cancer in comparison to patients with a primary tumour only. Metadherin overexpression is significantly associated with more advanced stage breast cancer and with TMN classification (Li et al. 2008).

The lung homing domain in Metadherin at amino acid residues 378-440 is a mediator of breast cancer cell adhesion to lung vasculature. When MTDH is silenced using siRNA, this metastasis is reduced (Brown and Ruoslahti 2004). It was shown that mice injected with a subline of MDA-MB-231 cells overexpressing Metadherin had accelerated metastasis to the lung and had decreased survival time (Hu et al. 2009). Conversely, suppression of Metadherin allowed increased survival.

The mechanism of action of Metadherin as an oncogene is linked to the processes of metastasis and invasion. Metadherin promotes metastasis in many cancer cell types. Two mechanisms are associated with this; angiogenesis and survival. Increased Metadherin leads to increased production of many angiogenic factors including VEGF, PIGF, FGFα (Yoo et al. 2010) and in nude mice CREF MTDH cells
produced extremely aggressive and angiogenic tumours (Emdad et al. 2009). Using 3D cell culture models, it has been shown that higher Metadherin expression leads to increased invasion in a number of cell types including HeLa cells, HCC cells and neuroblastoma cells (Emdad et al. 2006, Sarkar et al. 2008, Emdad et al. 2009). In breast cancer, Metadherin promotes invasion through a number of different methods. Firstly, it mediates lipopolysaccharide (LPS) induced invasion and migration of triple negative breast cancer. LPS is a proinflammation endotoxin so this would indicate a role for Metadherin in regulation of inflammation induced tumour progression (Zhao et al. 2011). Secondly, Metadherin mediates breast cancer cell invasion by inducing epithelial to mesenchymal transition (EMT) (Xiaoyan Li et al. 2011, Cao et al. 2013). Overexpression of Metadherin results in up-regulation of fibronectin, down-regulation of E-cadherin and nuclear accumulation of beta-catenin, all of which are known indicators of EMT.

MicroRNA’s also play a role in Metadherin’s involvement in carcinogenesis. The microRNA miR-30a has been established as a tumour suppressor due to its ability to suppress Metadherin in vitro and in vivo (N Zhang et al. 2013). This suppression of Metadherin inhibited breast cancer cell migration and invasion and inhibited metastasis in vivo. Microarray miR-375 plays a tumour suppressor role in head and neck carcinoma by regulating Metadherin (Hui et al. 2011, Nohata et al. 2011). miR-375 was significantly reduced in cancer samples whereas Metadherin was significantly increased in the same samples. Patients with increased Metadherin expression had lower survival rates and increased cases of relapse. Inhibition of Metadherin and reestablishment of miR-375 reversed this trend in vitro.

Knockdown of Metadherin has been shown to increase sensitivity of many cancer cell lines to a number of cancer drugs including cisplatin and doxorubicin (Hu et
al. 2009, Qian et al. 2011). This chemo resistance is thought to be due to the promotion of cancer cell survival. Metadherin overexpression in normal cells including melanocytes and human foetal astrocytes protects them from serum starvation related apoptosis suggesting that Metadherin is an anti-apoptotic protein (SG Lee et al. 2007). Knock down of Metadherin induces apoptosis in cancer cells through up regulation of FOX3A (Kikuno et al. 2007). Metadherin knockdown in LM2 breast cancer cells increased expression of pro apoptotic genes e.g TRAIL and decreased a number of chemo resistance related genes including aldehyde dehydrogenase (Hu et al. 2009). Metadherin expression has been positively correlated with chemo resistance to cisplatin in a number of cervical cancer cell lines (Zhang et al. 2013). The proposed mechanism for this chemo-resistance is through regulation of autophagy and activation of the Erk and the NFκB pathways. Metadherin knock down increases the sensitivity of the cells to AZD6244 which is an ATP-uncompetitive inhibitor of the MAPK pathway. This increased sensitivity is shown to be again through increased FOX03a expression through the MAP kinase pathway. The possibility of a DNA vaccine targeting Metadherin has been explored in the case of breast cancer in an effort to stem the lung metastasis associated with Metadherin (Qian et al. 2011). The vaccine showed success, resulting in slowing of tumour growth and metastasis in mouse models, prolonged survival for mice with tumours and increased chemo resistance to doxorubicin.

The numerous studies already completed on Metadherin convincingly argue a significant role for Metadherin as an oncogene through its ability to regulate and/or play a role in almost all events involved in tumourgenesis including proliferation, migration, invasion, metastasis, survival and chemo resistance. To be such a ubiquitous force driving the progression of cancer, Metadherin cross links many of the most well-known and well-studied signalling pathways. The PI3K/AKT pathway has been shown to be
activated by Metadherin (SG Lee et al. 2007). This is a pathway responsible for cell survival and it is through this pathway that Metadherin promotes cell growth and survival. Metadherin activates this pathway and a number of downstream transcription factors of Akt including FOX3a and NFkB. This allows regulation of proliferation and also apoptosis through the down regulation of pro apoptotic proteins such as BAD and p21. Due to this regulation of these apoptotic proteins, activation of this pathway plays a role in the chemo resistance attributed to Metadherin.

The relationship between Metadherin and the NFkB pathways is thought to be critical in Metadherin related cancer progression. Metadherin interacts directly with NFkB on the p65 subunit and this interaction promotes its translocation to the nucleus. Metadherin activation of the NFkB pathways mediates induction of many downstream targets of NFkB known to play essential roles in the progression of cancer through their involvement in proliferation, inflammation and angiogenesis including ICAM-2 and ICAM-3 (involved in cell adhesion) and IL-6 and IL-8 (Emdad et al. 2006). Metadherin also activates the MAPK pathway, in particular ERK and p38 MAPK, to promote invasion. This activation of ERK leads to cross talk with the Wnt/β-catenin pathway which promotes cell proliferation. Specific inhibitors of the MAPK pathway are able to reverse the highly invasive effect of Metadherin in vitro (Yoo et al. 2009)

Metadherin is a multifaceted protein with many roles within the cell so it is essential that it interacts with other proteins to form complexes. It has been suggested that Metadherin can function as a scaffold protein with these complexes (Yoo et al. 2011a). Known binding partners of Metadherin are summarized in a table below.
Table 5.2. Known binding partners of MTDH.

<table>
<thead>
<tr>
<th>Name</th>
<th>Binding Region</th>
<th>Consequence of Interaction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFkB</td>
<td>a.a. 101 – 205 subunit P65 of NFkB</td>
<td>Activation of downstream targets to promote invasion and anchorage independent growth.</td>
<td>(Emdad et al. 2006)</td>
</tr>
<tr>
<td>BCCIPα</td>
<td>a.a 7 – 169 of MTDH</td>
<td>Reduction in the tumour suppressor abilities of BCCIPα</td>
<td>(Ash et al. 2008)</td>
</tr>
<tr>
<td>SND1</td>
<td>aa. 101 – 205 of MTDH</td>
<td>Mediation of RNAI–mediated gene silencing.</td>
<td>(Yoo et al. 2011b)</td>
</tr>
</tbody>
</table>

5.3.6 Mass Spectrometry data for Metadherin.

A double IP of the RACK1/PP2A complex and a control IP were run on an SDS-PAGE gel and Metadherin was found as a binding partner of the RACK1/PP2A complex in the RACK1/PP2A complex IP only. As shown in Figure 5.5 (a), one matched Metadherin peptide was found: TELGLDLGLEPK. The mass spectrometry graph is composed of b-type ions and y-type ions with intensity on the y-axis and mass to charge (m/z) ratio on the x-axis. The mass difference between the b-type ion peaks corresponds directly to the amino acid sequence and is read from left to right. The y-type ion peaks have different measured heights and are read from right to left. Theoretical m/z values of product ions are shown in bold red (Figure 5.5 (b)). We identified Metadherin as a
Figure 5.5: Identification of Metadherin as a binding partner of the RACK1/PP2A complex. (a) Mass spectrometry analysis of the RACK1/PP2A complex identified Metadherin as a binding partner. The matched peptide has >99% confidence of positive protein identification. (b) The peptides found in the mass spectrometry analysis are listed in plots and tables of MS/MS/ fragmentation of Metadherin peptides.
protein that interacts with the RACK1/PP2A complex; however, this interaction required further confirmation before further analysis of Metadherin could be performed. Metadherin was chosen for further study as it had previously been shown to be implicated in the progression of breast cancer (Li et al. 2008).

5.3.7 Metadherin colocalises with both RACK1 and PP2A C subunit in MCF-7 cells.

Having determined that Metadherin was a RACK1/PP2A complex binding protein, we wanted to confirm this interaction using immunofluorescence. MCF-7 cells were plated on coverslips and cultured in serum free DMEM for 4 hours. Cells were fixed with 4% PFA, permeabilized with PHEM/0.1% Triton X, blocked with PHEM/5% goat serum for 30 minutes. Cells were co-stained with RACK1 (red) and Metadherin (green) (Figure 5.6 (a)). RACK1 and Metadherin colocalise in the cell membrane of MCF-7 cells. Cells were co-stained with PP2A C subunit (red) and Metadherin (green) (Figure 5.6 (b)). PP2A C subunit and Metadherin colocalise in the cell membrane of MCF-7 cells. Merge and zoom images are shown in the bottom columns. All images were acquired sequentially and images merged using ImageJ. Level of colocalisation was measured using Pearson’s coefficient. Pearson’s coefficient is a standard measure of pattern recognition and is used to measure the strength of the linear relationship between two fluorescence images.

5.3.8 Confirmation of RACK1/PP2A/Metadherin interaction in MCF-7 cells.

Having determined that Metadherin was a RACK1/PP2A complex binding protein, we also wanted to confirm this interaction using immunoprecipitation. We performed a RACK1 IP and show that RACK1, PP2A C subunit and Metadherin coimmunoprecipitate in MCF-7 cells (Figure 5.7 (a)). We also performed a PP2A C
Figure 5.6: Metadherin colocalises with both RACK1 and PP2A C subunit in MCF-7 cells. MCF-7 cells were plated on coverslips and cultured in serum free DMEM for 4 hours. Cells were fixed with 4% PFA, permeabilized with PHEM/0.1% Triton X, blocked with PHEM/5% goat serum. (a) Cells were co-stained with RACK1 (red) and Metadherin (green). (b) Cells were co-stained with PP2A C subunit (red) and Metadherin (green). Merge and zoom images are shown in the bottom columns. Scatter graphs are representative of one section of the cell membrane analysed for colocalisation. All images were acquired sequentially and images merged using ImageJ. Level of colocalisation measured using Pearson’s coefficient: RACK1 and Metadherin = 0.71 ± 0.03 (sem) N=35, PP2A C subunit and Metadherin = 0.72 ± 0.04 (sem), N = 10.
Figure 5.7: Confirmation of RACK1/PP2A/Metadherin interaction in MCF-7 cells. (a) MCF-7 cells were lysed and a RACK1 IP was performed. A control IP was also performed to control for non-specific binding proteins. IP samples were run on a 12% SDS-PAGE gel and analysed for associated PP2A C subunit and Metadherin by western blotting. (b) MCF-7 cells were lysed and a PP2A C subunit IP was performed. A control IP was also performed to control for non-specific binding proteins. IP samples were run on a 12% SDS-PAGE gel and analysed for associated RACK1 and Metadherin by western blotting. N=3.
subunit IP and show again that RACK1, PP2A C subunit and Metadherin coimmunoprecipitate in MCF-7 cells. Together with the results obtained in Figure 5.6, the results in Figure 5.7 confirm the interaction of Metadherin with the RACK1/PP2A complex as found in the mass spectrometry analysis.

5.3.9 Identification of RACK1 interaction sites on Metadherin peptide arrays.

In order to narrow down the binding site of RACK1 on Metadherin, we utilised peptide arrays. A library of overlapping peptides (18-mers) each shifted by 3 amino acids encompassing the entire sequence of Metadherin was spot-synthesized on nitrocellulose membranes to generate Metadherin subunit arrays. These arrays were then probed with recombinant GST or GST-RACK1. Any spots that GST bound to within the Metadherin array were subtracted from the array probed with GST-RACK1. A number of positive reaction patterns were identified on an area of the array (peptides 178-180) (Figure 5.8 (a)). RACK1 bound to a number of peptides with the positive reactions indicated by dark spots. These positive reactions confirm interaction between RACK1 and the Metadherin subunit. The crystal structure for Metadherin has yet to be resolved so we were unable to do any modelling of the region.

5.3.10 Metadherin expression in breast cancer tissue.

Due to the prognostic potential of Metadherin in advanced breast cancer disease, (Li et al. 2008) we wanted to analyse the expression of MTDH (the gene coding for Metadherin) in an Irish cohort of patients to contribute to the growing body of evidence reporting that MTDH is overexpressed in breast cancer. Core biopsies of breast tissue were collected from 10 patients undergoing surgery in University Hospital Limerick. Normal tissue from 1 patient was also collected approx. 3cm from the tumour. RNA
Figure 5.8: Identification of RACK1 interaction sites on Metadherin peptide arrays. The amino acid sequence of the entire Metadherin protein is shown schematically (1-582). Peptide arrays of immobilised overlapping 18-mer peptides, each shifted to the right by 3 amino acids encompassing the entire Metadherin protein were generated. Arrays were probed with GST-RACK1, which was detected by immunoblotting with anti-GST antibody. The arrays shown are representative of two arrays which gave a similar pattern of RACK1 binding. Positively interacting peptides generated dark spots and non-interacting peptides left blank spots. Spots that were generated on the GST control were disregarded as non-specific binding.
Figure 5.9: MTDH expression in breast cancer tissue. Core biopsy breast tissue samples were collected from 11 patients undergoing surgery in University Hospital Limerick. Normal tissue from 1 patient was collected approx 3cm from the tumour. RNA was extracted and quantitative real-time PCR was carried out on the cDNA using Taqman® assays. All data was analysed using REST© software. Samples were characterised based on disease subtype. Patient samples with Luminal A disease were normalized using the reference genes; PMM1, PGK1 and ATCB. TNBC samples were normalized using the reference genes; PMM1, PGK1 and PP1A. HER2+ samples normalized using the reference genes; PGK1, ATCB and PP1A. Bar graph representing the fold change of MTDH in each cancer tissue sample when compared to the normal tissue sample. *p<0.01. N=3.
was extracted from the tissue using the Qiagen RNeasy Lipid Tissue mini kit. The concentration and quality of the RNA was determined by reading a sample of the RNA on the NanoDrop (See Appendix B). RNA (500ng) was synthesised into cDNA using the Invitrogen Vilo cDNA synthesis kit. Quantitative real-time PCR was carried out on the cDNA using Taqman® assays in a thermocycler. All data was analysed using REST© software. The patients were grouped according to disease subtype. Patient samples with Luminal A disease were normalized using the reference genes; PMM1, PGK1 and ATCB. TNBC samples were normalized using the reference genes; PMM1, PGK1 and PP1A. The HER2⁺ sample was normalized using the reference genes; PGK1, ATCB and PP1A. Our results show MTDH overexpression in five out of seven patients (71.4%) with Luminal A breast cancer (Figure 5.9). We had one tissue sample from a patient diagnosed with HER2⁺ disease. Analysis of this sample showed no change in MTDH expression when compared to normal tissue (Figure 5.9). Two patients with TNBC were analysed and MTDH was found to be overexpressed in one of the samples (50%) (Figure 5.9). Overall, MTDH was overexpressed in 60% of the cancer samples analysed when compared to normal tissue.

5.3.11 GNB2L1 expression in breast cancer tissue.

GNB2L1 is the gene that codes for the RACK1 protein. Core biopsies of breast tissue were collected from 10 patients undergoing surgery in University Hospital Limerick. Normal tissue from 1 patient was also collected approx. 3cm from the tumour. RNA was extracted from the tissue using the Qiagen RNeasy Lipid Tissue mini kit. The concentration and quality of the RNA was determined by reading a sample of the RNA on the NanoDrop (See Appendix B). RNA (500ng) was synthesised into cDNA using the Invitrogen Vilo cDNA synthesis kit. Quantitative real-
Figure 5.10: GNB2L1 expression in breast cancer tissue. GNB2L1 is the gene coding for RACK1. Core biopsy breast tissue samples were collected from 11 patients undergoing surgery in University Hospital Limerick. Normal tissue from 1 patient was collected approx 3cm from the tumour. RNA was extracted and quantitative real-time PCR was carried out on the cDNA using Taqman® assays. All data was analysed using REST© software. Samples were characterised based on disease subtype. Patient samples with Luminal A disease were normalized using the reference genes; PMM1, PGK1 and ATCB. TNBC samples were normalized using the reference genes; PMM1, PGK1 and PP1A. HER2⁺ samples normalized using the reference genes; PGK1, ATCB and PP1A. Bar graph representing the fold change of GNB2L1 in each cancer tissue sample when compared to the normal tissue sample. ***p<0.001. N=3.
Figure 5.11. Knock down of Metadherin reduces the invasive capabilities of breast cancer cells. (a) Bar graph representing expression of MTDH in MCF-7 cells compared to the non-cancer MCF10A cell line. MCF-7 cells were transfected with esiRNA Metadherin and a negative control using the Oligofectamine™ transfection protocol. Cell lines were normalized using the reference genes; B2M, PGK1 and PP1A. (b) Knockdown was optimised to 48 hours. (c) Cells were plated in 0.3% agarose on to a solidified layer of 0.6% agarose. The cells were incubated at 37°C in 5% CO2 for 10 days. Cells were then stained with 0.01% crystal violet overnight and counted. Differences between groups were determined using a T-Test. A p value of less than 0.05 was considered statistically significant. *p<0.01. N=2.
time PCR was carried out on the cDNA using Taqman® assays in a thermocycler. All data was analysed using REST© software. The patients were grouped according to disease subtype. Patient samples with Luminal A disease were normalized using the reference genes; PMM1, PGK1 and ATCB. TNBC samples were normalized using the reference genes; PMM1, PGK1 and PP1A. The HER2+ sample was normalized using the reference genes; PGK1, ATCB and PP1A. Analysis of GNB2L1 (the gene coding for RACK1) expression in the same cohort of patients showed GNB2L1 to be down-regulated in both TNBC samples (Figure 5.10). GNB2L1 was overexpressed in one Luminal A patient sample.

5.3.12 Knock down of Metadherin reduces the invasive capabilities of breast cancer cells.

Gene expression analysis has determined that Metadherin is overexpressed in MCF-7 breast cancer cells when compared to the non-cancer breast cell line MCF10A (Figure 5.11 (a)). In order to determine whether Metadherin has a role to play in the ability of MCF-7 cells to invade, we knocked down Metadherin using esiRNA and Oligofectamine™ as per the manufacturer’s instructions. Using 1.3µg of esiRNA for 48 hours resulted in an optimum 50% knockdown (Figure 5.11 (b)). These knockdown cells were plated in 0.3% agarose on to a solidified layer of 0.6% agarose. The cells were incubated at 37°C in 5% CO₂ for 10 days. Cells were then stained with 0.01% crystal violet overnight and counted. Our results show that reducing Metadherin expression in MCF-7 cells decreased the invasive abilities of the cells. The colony number is reduced by 35% in cells where Metadherin has been knocked down in comparison to the control cells (Figure 5.11 (c)).
5.4 Discussion

The role of RACK1 as a scaffolding protein within signalling pathways has been well established. It is known that RACK1 binds both directly and indirectly to an array of proteins, bringing them in close proximity to their substrates. In this study, we wanted to identify the binding proteins/substrates of the RACK1/PP2A complex. We had a particular interest in the proteins that had a role in cancer.

In order to identify this group of proteins, we cultured MCF-7 cells in 3D using Matrigel™. It is well established that 3D cell culture is a more physiologically relevant method of cell culture as cells grown in this manner are able to respond to environmental signals from the extracellular matrix which are known to be important for survival, growth and metastasis (Bissell et al. 2005, Kenny et al. 2007). The cells were lysed and a ‘double IP’ was performed which involved, firstly, performing a RACK1 IP and then re-incubated the sample with an anti-PP2A C subunit in order to pull down the specific subset of proteins that were interacting with the RACK1/PP2A complex. Mass spectrometry analysis identified over 60 novel proteins binding to the RACK1/PP2A complex. These proteins were quite diverse and were located in a variety of cellular locations. A literature review was completed on each protein so that we could narrow our study to a number of proteins of particular interest in the context of breast cancer. Two proteins, TTBK-1 and Metadherin were chosen for further study. However there were a couple of other candidates that also looked interesting. For example, Phosphoglycerate Kinase-1 PGK1) is a protein involved in glycolysis and enhances the metastatic rate in both gastric and prostate cancer (Wang et al. 2010, Zieker et al. 2008). PGK1 has been linked to inducing a cancer associated fibroblast phenotype in the stroma possibly by increasing metabolism or cell cycling. This suggests that PGK1 has
a role to play in the interactions between cancer and its microenvironment (Wang et al. 2010). PGK1 is also reported to be up-regulated in HER2+ breast cancer cases (Zhang et al. 2005) so it is definitely a protein to take a closer look at in the future. There were a large amount of ribosomal proteins identified in the mass spectrometry analysis suggesting a potential role for the RACK1/PP2A complex in the ribosome. Similarly, a large subset of proteins associated with metabolism were identified as interacting partners of the RACK1/PP2A complex and this warrants further investigation in the future.

TTBK-1 was chosen from our list of novel interacting binding partners of the RACK1/PP2A complex for further study. It is reported to be a brain specific protein (Sato et al. 2006) so we wanted to investigate why it had been detected in breast cancer cells. It is known that PP2A is a major Tau phosphatase and downregulation of PP2A contributes to Tau hyperphosphorylation in Alzheimer’s disease (Qian et al. 2010). However, to the best of our knowledge, this is the first time that PP2A and TTBK-1 have been identified together in a complex. It is also the first time that TTBK-1 has been implicated in breast cancer. Work done by a colleague in the laboratory has confirmed and characterised the interaction between TTBK-1 and RACK1 (Hayes S, Kiely M, Kiely P.A, in final preparation).

We compared the expression of TTBK-1 in two breast cancer cells lines (MCF-7 and MDA-MB-231) to a non-cancer breast cell line (MCF10A) and found that TTBK1 was undetectable in MCF10A cells. In comparison to this, TTBK-1 expression was up-regulated in both cancer cell lines tested. Expression of TTBK-1 was higher in the triple negative, more aggressive, MDA-MB-231 cell line compared to the MCF-7 cells. Based on these results, we wanted to determine whether this up-regulation of TTBK-1 was also seen in breast cancer tissue. We analysed the expression of the TTBK-1 gene in
tissue samples obtained from patients being treated in University Hospital Limerick and found that it is over expressed in one out of two fibroadenomas (50%). TTBK-1 is overexpressed in five out of seven patients with Luminal A disease (71.4%) compared to normal and in one out of two samples of TNBC disease (50%). It was also overexpressed in the one HER2+ sample we analysed. TTBK-1 was quite lowly expressed in the normal sample. Although our data set is small, this work is very important as it shows that TTBK-1 is turned on in breast cancer and has the potential to be a prognostic marker of disease. Up-regulation of TTBK-1 has the potential to cause hyper-phosphorylation of its substrates leading to dysregulation within tightly controlled signalling pathways.

Metadherin was chosen for further study from the subset of proteins identified in the mass spectrometry screen as it has already been strongly linked to breast cancer progression. It is known to be overexpressed in 40% of breast cancer cases and this overexpression can be correlated to advanced stage of disease and poor patient prognosis (Hu et al. 2009, Cong Li et al. 2011, Li et al. 2008). It also has been shown to mediate breast cancer metastasis to the lung (Hu et al. 2009). This already established link to breast cancer was of great interest to us.

Using immunofluorescence, we identified that RACK1 and Metadherin colocalise in the cell membrane. PP2A and Metadherin were also found to colocalise in the cell membrane of MCF-7 breast cancer cells. We confirmed the interaction between Metadherin and the RACK1/PP2A complex by immunoprecipitating both RACK1 and the PP2A C subunit. In both cases, all three proteins were identified in a complex. We were also able to narrow down the possible binding sites between RACK1 and Metadherin using peptide arrays.
The importance of validating findings in different cohorts of patients is noted in literature (Tokunaga et al. 2014) so we tracked the expression of the Metadherin gene (MTDH) in tissue samples obtained from patients being treated in University Hospital Limerick. Expression of the Metadherin gene (MTDH) was examined in 7 patients with Luminal A breast cancer and found it to be increased in 5 of the patients compared to 1 normal tissue sample (71.4%). We examined the expression of MTDH in 2 patients with TNBC and found expression to be increased in 1 patient (50%) compared to the 1 normal sample. However, it should be noted that in the second TNBC patient (P10), MTDH was also over-expressed and was close to significance. In both TNBC patients, the gene coding for RACK1 (GNB2L1) was down-regulated. It is interesting to note that knocking down RACK1 expression in MCF-7 cells using siRNA saw a subsequent increase in Metadherin expression at both gene and protein level (data not shown). Putting these experiments together, we suggested that RACK1 is playing a regulatory role in sequestering Metadherin. We turned our attention to patient samples and 70% of the patients have been diagnosed with grade 2 disease, with just 3 having grade 3 breast cancer. Of those three patients with grade 3 disease (P1, P3 and P7) they all show up-regulation of Metadherin. However, the sample size is too modest to correlate Metadherin expression with advanced disease but this may become more obvious as the biobank is expanded. However, it does suggest a similar pattern as the three samples that did not have up-regulation of Metadherin were a lower grade of disease (P2, P6 and P8).

It was determined that Metadherin knockdown decreases the invasiveness of the MCF-7 cells in soft agar. This finding is in agreement with previous findings in literature that Metadherin is a driving force behind invasion in cancer (Emdad et al. 2006). Metadherin driven invasion is mediated through its ability to induce epithelial to
mesenchymal transition (Xiaoyan Li et al. 2011) and lipopolysaccharide induced invasion (Zhao et al. 2011). Metadherin also promotes invasion through its activation of the MAPK, Wnt and NFκB signalling pathways (Yoo et al. 2009, Emdad et al. 2006).

In conclusion, we have identified a group of novel proteins interacting with the RACK1/PP2A complex. This diverse group of proteins is from a variety of cellular locations. This work will strengthen the evidence that Metadherin has potential as a prognostic factor in breast cancer. This work also promotes our understanding of RACK1 in cancer and highlights RACK1 involvement with proteins known to be linked to the progression of cancer. It draws attention to the value of considering scaffolding proteins as novel therapeutic targets.
Chapter 6:

General Discussion
6.1 The basis of our study

Screening programmes, earlier diagnosis and targeted treatments have greatly improved the prognosis of patients diagnosed with breast cancer. However, breast cancer remains near the top of the leader board when it comes to cancer related mortality (Comber 2014). The need for continued research into the mechanisms underlying breast cancer progression is essential to develop and identify successful therapeutic strategies in the fight against this disease. My research focus has been to develop models and parameters that will help identify potential therapeutic targets and strategies for breast cancer treatment while focusing particularly on deciphering the role of the RACK1/PP2A complex in breast cancer.

Separately, RACK1 and PP2A have been shown to have roles to play in cancer. RACK1, as a scaffolding protein, mediates substrate specificity of a variety of proteins within tightly regulated signalling pathways, many of which are implicated in cancer (Hermanto et al. 2002, Vomastek et al. 2007, Kiely et al. 2002, Li and Xie 2014, Adams et al. 2011). Aberrant expression of RACK1 has been reported in many cancer types (Shi et al. 2012, Deng et al. 2012, Xi-Xi Cao et al. 2010, Al-Reefy and Mokbel 2010) and the role of RACK1 in cell polarity, migration and invasion has been elegantly described in a number of studies (Serrels et al. 2010, Li et al. 2012, Dave et al. 2013). The role of PP2A in cancer is also well studied. It is most well established as a tumour suppressor (Janssens et al. 2005), however, PP2A inhibition has also been suggested as a therapeutic strategy when an anti-apoptotic function has been described (Schweyer et al. 2007, Duong et al. 2014). Recent work has identified PP2A as a protein that interacts with RACK1. PP2A and β1 Integrin compete for binding to RACK1 on Y302 in an IGF-1 dependant manner to regulate cell migration (Kiely et al. 2006, Kiely et al. 2008). I set out to fully characterise the interaction between these two proteins in breast cancer.
cells and explore the molecular mechanisms by which RACK1 and PP2A regulate the transformed phenotype.

6.2 Using Real Time Cell Analysis as a tool for monitoring cell behaviour in response to novel compounds.

We used the RTCA xCELLigence platform to monitor the effect of VK2 on TNBC cells alone and in combination with a low glucose culture media. This platform proved to be an ideal tool to identify the reduced cell adhesion and viability shown upon treatment of the cells with VK2 with or without low glucose. It provided us with real-time, continuous monitoring of the effects of the treatment, demonstrating for the first time that VK2 has anti-cancer properties on breast cancer cells. The xCELLigence platform also proved extremely useful when correlating data with another laboratory using more traditional cell based assays. We determined the sensitivity of a number of breast cancer cell lines to the small molecule PRIMA1Met and found our results to correlate well with our collaborators in U.C.D. Overall, our results provide a strong basis for further studies involving both VK2 and PRIMA1Met as anti-carcinogenic agents.

6.3 The role of the RACK1/PP2A complex in breast cancer cells- a novel therapeutic target.

Using immobilised peptide arrays we identified potential interaction sites between RACK1 and the PP2A C subunit. Expression of PP2A constructs housing point mutations of these interaction sites confirmed the interaction. It allowed us to disrupt the RACK1/PP2A complex and examine the cellular consequences of this perturbation. Our results indicated that disruption of the RACK1/PP2A complex reduced the phosphatase activity of PP2A. Modelling of the sites suggested a potential dual role for RACK1 with regard to PP2A. Interaction of RACK1 at the F63 and R64 site on the
catalytic subunit of PP2A allows RACK1 to compete with other B subunits for binding to the C subunit of PP2A. This coupled with its ability to stabilize PP2A activity suggests that RACK1 may function as a ‘bone fida’ regulatory subunit of PP2A. Disruption of the complex at this site however, had no effect on the cell adhesion, proliferation, migration or invasion (data not shown).

On the other hand, modelling of amino acids R214 and Y218 suggested that if RACK1 interacted at these sites, it would be possible for other B subunits to still bind to the core PP2A holoenzyme. This indicates the possibility of two RACK1 proteins binding to the PP2A C subunit at the same time. Disruption of the complex by these mutations reduced cellular adhesion, proliferation, migration and invasion of our breast cancer cell model suggesting that disruption of the complex at this site has potential anti-carcinogenic benefit. PP2A is active when bound to RACK1 so we have demonstrated that reduced PP2A activity is of therapeutic value in this breast cancer cell model. Amino acid R214 is part of the active site of the PP2A C subunit and Y218 is in very close proximity to it so disruption at these positions would possibly block substrates trying to interact with the active site. This is important because the anti-tumourigenic results we see as a result of disruption of the RACK1/PP2A complex could be due to a decreased ability of PP2A substrates to bind to the catalytic active site if these substrates had a role to play in the progression of cancer. Our results suggest that inhibition of PP2A has therapeutic benefits in agreement with a number of other studies (Schweyer et al. 2007, Wei Li et al. 2011).

The suggestion that RACK1 could be a regulatory B subunit of PP2A is an interesting possibility. Although no official guidelines have been set down to finalise what constitutes an official B subunit, there have been a number of suggestions to distinguish regulatory subunits from simply, binding partners of PP2A. It has been
proposed that a protein should only be designated as a B subunit if it contains two conserved PP2A A subunit binding domains (ASBD) that have been identified in three out of the four recognised B subunit families (B55, B56 and B48) (Janssens et al. 2008) because these subunits have the ability to compete with the other B subunits for binding to the PP2A A subunit. For example, in the B56α regulatory subunit, ASBD1 is from residues 200 - 303 (FVQQLLELFSEDPRERDFLKTVLHRIVGKFLGLRAFIRKQIN NIFLRFIYTEHFNGVAELLEILGSINGFALPLKAEHKQFLMKVLIPMHTAKGLA LFHAQL) and ASBD2 is from residues 325-383 (FLGEIEILDVIEPTQFKKI EEPLFKQISKCVCSSSFLQQVAFARLYFWNEIYILSLIEE) (Li and Virshup 2002).

Although RACK1 does not have this conserved region (Figure 1.5 (a)), there is other evidence to suggest that it may be a regulatory subunit. Striatin, although not universally accepted, is generally considered to be a regulatory B subunit based on its ability to bind directly to the core PP2A subunit in the absence of any other B subunit and its ability to alter the substrate specificity of PP2A (Gordon et al. 2011). Our findings show that RACK1 is in a complex with both PP2A A and C subunits and it also stabilises PP2A activity as a result of the interaction with the PP2A C subunit. We have identified novel binding partners of the RACK1/PP2A complex suggesting that RACK1, like Striatin, alters the substrate specificity of PP2A. The PP2A B subunits are categorized into 4 diverse families with very low sequence homology. It is interesting to mention that Clustal alignment of RACK1 against many of the regulatory B subunits has shown the RACK1 most resembles the amino acid sequence of Striatin (24.91%) and SG2NA (22.87%) (See Appendix D). Since molecular modelling of the FR site identified in peptide arrays as a potential interacting site between RACK1 and the PP2A C subunit has been suggested to preclude binding of other B subunits to the PP2A core enzyme and modelling of the R214 and Y218 sites suggested that RACK1 binding at
these sites would be to an already formed PP2A holoenzyme, this suggests a dual role for RACK1 as a binding partner of PP2A. We have shown that RACK1 bound directly to PP2A at the R214 and Y218 sites plays a role in the maintenance of the cancer phenotype. However, we have shown that upon binding to the FR site, RACK1 has a role as a potential regulatory B subunit of PP2A through its ability to bind directly to the PP2A C subunit, its ability to stabilize the activity of PP2A and its ability to compete with other B subunits for binding to the PP2A core structure.

6.4 Identification of novel proteins interacting with the RACK1/PP2A complex.

We hypothesised that as RACK1 is a shuttle and a scaffolding protein in signalling pathways, it functions to introduce PP2A to a number of novel substrates. We used 3D cultured spheroids of MCF-7 cells and enriched the protein lysate for the RACK1/PP2A complex by a double IP protocol which had been developed and refined in the laboratory in order to identify proteins that interacted with the complex. A mass spectrometry screen of this complex revealed 66 novel RACK1/PP2A interacting proteins. We set out to characterise a number of these proteins with a specific focus and interest on proteins that are known regulators of the transformed phenotype in cancer.

TTBK-1 was chosen for further study as it has never been identified in breast cancer cells before. It has only been studied for its role in the brain (Sato et al. 2006). Our results show TTBK-1 to be undetectable at a gene level in our non-cancer breast cancer cell model and up-regulated in two breast cancer cell lines, as well as being up-regulated in 70% of patient tissue as analysed by quantitative real-time PCR. This suggests that TTBK-1 has a possible role to play as a prognostic indicator of breast cancer disease. Our subset of patients is low but the patient cohort is being expanded to define the role of TTBK-1 as a marker of disease. Over expression of TTBK-1 has already been linked to Alzheimer ’s disease due to its ability to induce TAU aggregation.
which is implicated in Alzheimer’s Disease onset and progression (Sato et al. 2008)

Down-regulation of PP2A and up-regulation of TTBK-1 have been separately implicated in the pathology of Alzheimer’s disease but no link, until now, has been made between PP2A and TTBK-1. TTBK-1 overexpression in breast cancer could be causing hyper-phosphorylation of its substrates (as in Alzheimer’s disease) which may play a role in maintaining the transformed phenotype in cancer. It would also be very interesting to extend this study into other cancer types to determine if up-regulation of TTBK-1 is a breast cancer specific event. Several new studies are being designed in the lab to address the role of TTBK-1 in breast cancer and determine the dynamics of the RACK1/PP2A and TTBK-1 complex to include, for example, if the complex is mediated by IGF-I.

We also investigated the presence of Metadherin in a complex with RACK1 and PP2A. It was chosen from our RACK1/PP2A complex mass spectrometry screen because of previous connections to the progression of breast cancer and correlation with a more advanced disease as well as a link to the promotion of breast cancer metastasis (Li et al. 2008, Hu et al. 2009). We confirmed that Metadherin interacted with both RACK1 and PP2A in the cell membrane and confirmed that all three proteins were in a complex. We demonstrated that MTDH is overexpressed in MCF-7 cells when compared to MCF10A’s and knockdown of Metadherin in MCF-7 cells reduced the invasiveness of cells. We had also found MTDH (the gene coding for Metadherin) to be up-regulated in 60% of breast cancer biopsies as analysed by quantitative real-time PCR. There have been many studies completed to outline the role Metadherin plays in the progression of breast cancer. The proposed mechanism of action for Metadherin in cancer progression has been linked to a number of specific signalling pathways including the MAPK, NFκB and PI3K/Akt pathways. Our work contributes to this by
adding to the body of knowledge tracking MTDH overexpression in patient breast cancer samples and identifying Metadherin in a complex with the RACK1/PP2A complex.

6.5 Future potential of this study

Our results show that the RACK1/PP2A complex is interacting with proteins known to be involved in the advancement of breast cancer disease and disturbance of the RACK1/PP2A complex shows anti-tumourigenic potential. However, RACK1 is involved in so many diverse and essential biological processes, is it feasible to inhibit this complex without affecting the many relationships that RACK1 has with other proteins in the cell? There is precedent to support this. For example, IQ motif-containing GTPase activating protein 1 (IQGAP1) is a scaffold within the MAPK pathway responsible for assembling the kinases within this pathway to affect signal transmission as well as binding to numerous other proteins including E-cadherin (Jameson et al. 2013, Li et al. 1999). It has been linked to the progression of cancer in numerous studies and is found to drive tumourigenesis in both mouse models and human tissue (Jadeski et al. 2008, Jameson et al. 2013). Up-regulation of the ERK1/2 MAPK cascade is seen in 30% of cancers and IQGAP1 binds to ERK1/2 at a highly conserved 32 amino acid WW domain. A specific YY/AA mutation in that domain disrupted the IQGAP1/ERK1/2 interaction and this was enough to inhibit RAS driven-tumourigenesis and significantly increased the life span of tumour bearing mice without having a negative effect on other proteins it is interacting with (Jameson et al. 2013). Unlike RACK1, deletion of which is embryonically lethal (Volta et al. 2013), IQGAP1 knockout mice are viable so are dispensable for homeostasis (Jameson et al. 2013) however, this study acts as a direct demonstration of how it is possible to block a
scaffold protein at a specific interaction point to gain therapeutic benefit without disruption of its other functions.

With regard to RACK1, Serrels et al disrupted the RACK1/FAK complex by mutating two amino acids in the sequence of FAK to identify that a complex between RACK1, FAK and PDE4D5 is regulating cancer cell polarity, initiation of cancer cell spreading and metastasis (Serrels et al. 2010, Serrels et al. 2011). This was done without any notable disturbance to other vital RACK1 function. Small molecules have also been designed to specifically interrupt the binding between a single protein and a RACK1-like, 7-bladed beta-propeller protein indicating that complexes of beta-propellers and small molecules are possible (Guillermo et al. 2013, Karatas et al. 2012, Orlicky et al. 2010). All the above work serves to suggest that not only is the disruption of a RACK1 scaffolding interaction possible using a small molecule, it can be done without affecting the plethora of essential RACK1 functions and can have therapeutic benefit in cancer.

In this study, I have worked towards characterising the interaction between RACK1 and PP2A. Separately, both proteins been implicated in breast cancer and it was known that they interact with each other in an IGF-1 dependant manner. However, our work has contributed to the knowledge of how these proteins interact by identifying the binding sites between RACK1 and the PP2A A and C subunits. We have determined that disruption of the RACK1/PP2A complex has implications for a wide range of cellular processes involved in maintenance and progression of cancer including migration and invasion. Disruption of the RACK1/PP2A complex results in a slowing down of these processes in breast cancer cells which allows us to suggest the potential therapeutic benefit to be gained in breast cancer by targeting the RACK1/PP2A complex. As we characterised the RACK1/PP2A complex, we identified a novel series
of proteins that interact with the complex in MCF-7 cells. Further research is likely to uncover mechanisms by which the RACK1/PP2A complex can be targeted in cancer.
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Appendix A:

Plasmid Maps
Unless otherwise stated, cDNA was cloned into this vector BamHI/EcoRI and transfected into mammalian cells.
* Inserts must be cloned “in-frame” with the Ha tag.

cDNA encoding PP2A WT was cloned into this vector “in frame” with the HA tag using the BamH I and Apa I restriction sites and transfected into mammalian cells
*Inserts must be cloned “in-frame” with the Ha tag.*

cDNA encoding the PP2A C subunit mutant R214A was cloned into this vector “in frame” with the HA tag using the BamH I and Apa I restriction sites and generated by quick change PCR and transfected into mammalian cells.
cDNA encoding the PP2A C subunit mutant R214A was cloned into this vector “in frame” with the HA tag using the BamH I and ApaI restriction sites and generated by quick change PCR and transfected into mammalian cells

* Inserts must be cloned “in-frame” with the Ha tag.
Inserts must be cloned “in-frame” with the Ha tag.

cDNA encoding the PP2A C subunit mutant Y218F was cloned into this vector “in frame” with the HA tag using the XhoI and ApaI restriction sites and generated by quick change PCR transfected into mammalian cells.
Appendix B:

RNA Quantity and Quality
Appendix B: For all gene analysis work, the concentration and quality of the RNA extracted was determined by reading a sample of the RNA on the NanoDrop. This graph is representative of the graphs obtained by the NanoDrop with the quantity of RNA displayed at the bottom left in ng/µl and the quality determined by a trough in the graph at a wavelength of 230nm and a peak in the graph at a wavelength of 260nm.
Appendix C:

Gene Expression Analysis of PP2A A and C subunits.
Appendix C: Gene expression of PP2A A and C subunits (α and β isoforms) in breast cancer tissue. Core biopsy breast tissue samples were collected from 11 patients undergoing surgery in University Hospital Limerick. Normal tissue from 1 patient was collected approx 3cm from the tumour. RNA was extracted from the tissue using the Qiagen RNeasy Lipid Tissue mini kit. RNA was synthesised into cDNA using the Invitrogen Vilo cDNA synthesis kit. Quantitative real-time PCR was carried out on the cDNA using Taqman® assays. All data was analysed using REST© software. Samples were characterised based on disease subtype. Patient samples with Luminal A disease were normalized using the reference genes; PMM1, PGK1 and ATCB. TNBC samples were normalized using the reference genes; PMM1, PGK1 and PP1A. HER2+ samples normalized using the reference genes; PGK1, ATCB and PP1A (a) Bar graph representing the fold change of PPP2R1A in each cancer tissue sample when compared to the normal tissue sample. (b) Bar graph representing the fold change of PPP2R1B in each cancer tissue sample when compared to the normal tissue sample. *p<0.01. ***p<0.001. N=3.
Appendix C: Gene expression of PP2A A and C subunits (α and β isoforms) in breast cancer tissue. Core biopsy breast tissue samples were collected from 11 patients undergoing surgery in University Hospital Limerick. Normal tissue from 1 patient was collected approx 3cm from the tumour. RNA was extracted from the tissue using the Qiagen RNeasy Lipid Tissue mini kit. RNA was synthesised into cDNA using the Invitrogen Vilo cDNA synthesis kit. Quantitative real-time PCR was carried out on the cDNA using Taqman® assays. All data was analysed using REST© software. Samples were characterised based on disease subtype. Patient samples with Luminal A disease were normalized using the reference genes; PMM1, PGK1 and ATCB. TNBC samples were normalized using the reference genes; PMM1, PGK1 and PP1A. HER2+ samples normalized using the reference genes; PGK1, ATCB and PP1A. (c) Bar graph representing the fold change of PPP2CA in each cancer tissue sample when compared to the normal tissue sample. (d) Bar graph representing the fold change of PPP2CB in each cancer tissue sample when compared to the normal tissue sample. *p<0.01. ***p<0.001. N=3.
Appendix C: Gene expression of PP2A A and C subunits (α and β isoforms) in breast cancer tissue.

PPP2R1A is over-expressed in 6 cancer samples in our cohort (60%) (Appendix C (a)). This corresponds to over-expression of PPP2R1A in 71.4% of Luminal A patients and the one HER2+ sample. PPP2R1B is down-regulated in 80% of the cancer samples including both TNBC samples, the HER2+ sample and 5 out of 7 Luminal A patients (71.4%) (Appendix C (b)). PPP2CA is reported to be overexpressed in 1 Luminal A patient sample (P4) and down-regulated in another Luminal A patient (P6) (Appendix C (c)). No change is reported PPP2CB expression across the patient samples (Appendix C (c)).
Appendix D:

Sequence alignment of RACK1 and the PP2A B regulatory subunits.
Appendix D: Sequence alignment of RACK1 and the PP2A B regulatory subunits.
We used Clustal Omega Multiple Sequence Alignment 1.2.1 to determine the percentage amino acid sequence similarity between RACK1 and a number of known PP2A B regulatory subunits. (a) Table of the percentage difference between RACK1 and a number of known PP2A B regulatory subunits. Striatin showed the highest percentage of similarity at 24.91%.
Appendix D: Sequence alignment of RACK1 and the PP2A B regulatory subunits.

(b) Alignment of the amino acid sequences of RACK1 and Striatin.
Appendix E:

Additional Publications