Identification of a Novel G-protein Interactor, RADIL, and Functional Characterization of its Role in Cancer Cell Motility

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Pharmaceutical Sciences
University of Toronto

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Syed Mukhtar Ahmed
Doctor of Philosophy
Graduate Department of Pharmaceutical Sciences
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2013

Abstract

Cell adhesion and migration play crucial roles in development of multicellular organisms, immune surveillance, wound repair and cancer metastasis. The Gβγ subunits of heterotrimeric G-proteins have been implicated in signalling activities that promote cell adhesion and migration but the molecular mechanisms are unclear. Using a mass-spectrometry based proteomic approach we identified a protein complex between Gβγ and Rap1a that is bridged by a novel Rap1 effector, Radil. Overexpression of constitutively active Rap1a, Gβγ or stimulation of cells with the GPCR ligand fMLP triggers recruitment of Radil to the plasma membrane. Exogenous expression of Radil promotes cell spreading through Rap1-dependent inside-out activation of integrins leading to enhanced cell-matrix adhesion. Structure function experiments demonstrated that the RA and PDZ domains of Radil are required for its ability to promote cell adhesion. Using phage-display and mass-spectrometry we identified the kinesin family protein KIF14 as a novel interacting partner for Radil. Both KIF14 and Radil colocalized on microtubules in a PDZ-dependent manner. Depletion of KIF14 or disruption of microtubules led to accumulation of Radil at the cell membrane. Functionally, KIF14 is a negative regulator of Radil signalling as its depletion increased cell spreading and integrin activation and both phenotypes are rescued by simultaneous knockdown of Radil. Knockdown of KIF14 affects focal adhesion dynamics,
which we determined is due to delayed adhesion disassembly. Depletion of either KIF14 or Radil dramatically decreased breast cancer cell migration and invasion \textit{in vitro}. Additionally, knockdown of Radil compromised the ability of cells to metastasize to the lung and reduced tumor growth in xenograft mouse models. Collectively, these studies describe a functional requirement for the G\(\beta\gamma\)-Rap1a-Radil complex during GPCR signalling for the control of integrin-mediated cell adhesion, cell motility and cancer progression.
I have come loaded with statistics, for I’ve noticed that a man can’t prove anything without statistics. No man can.

- Mark Twain
Acknowledgments

I would like to thank everyone who has helped and inspired me during my doctoral study.

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My deepest gratitude goes to my family for their constant love, support and patience; this dissertation would be meaningless without them. My parents have spared no efforts and sacrifices to provide the best possible environment to foster learning and for that I am forever in debt to them.

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<td>afadin</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>affinity purification</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>3',5'-cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cAR1</td>
<td>cAMP receptor</td>
</tr>
<tr>
<td>CBP</td>
<td>calmodulin binding peptide</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>CID</td>
<td>Collision induced dissociation</td>
</tr>
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<td>CXCR4</td>
<td>CXC chemokine receptor 4</td>
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<tr>
<td>DIL</td>
<td>diluted domain</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>E-coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>EGTA</td>
<td>ethyleneglycoltetraacetic acid</td>
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EMT  epithelial-mesenchymal transition
ESI  Electrospray ionization
FA  focal adhesion
FAK  focal adhesion kinase
FBS  fetal bovine serum
fMLP  formylated methionine-leucine-phenylalanine peptide
FPR  formyl peptide receptor
GAP  GTPase activating protein
GEF  guanine exchange factor
Gβγ  heterotrimeric G proteins beta-gamma
GFP  green fluorescent protein
GPCRs  G protein coupled receptors
GST  glutathione S transferase
HA-tag  human influenza hemagglutinin epitope tag (YPYDVPDYA)
HEK  human embryonic kidney
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC  high performance liquid chromatography
IRM  interference reflection microscopy
K_D  dissociation constant
KIF  Kinesin family proteins
<table>
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<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LPA</td>
<td>lysophospholipid</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption ionization</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal-epithelial transition</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>Mr</td>
<td>molecular weight</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
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<td>tandem mass spectrometry</td>
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<td>sodium fluoride</td>
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<td>NaOVO₃</td>
<td>sodium orthovanadate</td>
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<td>polyacrylamide gel electrophoresis</td>
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<td>phospholipase C</td>
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<td>pleckstein homology</td>
</tr>
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<td>PTX</td>
<td>pertussis toxin</td>
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<td>Description</td>
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<td>-------------</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative PCR</td>
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<td>RA</td>
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<tr>
<td>RBD</td>
<td>Ras/Rap binding domain</td>
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<td>arginine-glycine-aspartic acid epitope</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>real-time PCR</td>
</tr>
<tr>
<td>S1P</td>
<td>sphingosine-1-phosphate</td>
</tr>
<tr>
<td>SBP</td>
<td>streptavidin binding peptide</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
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<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
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<tr>
<td>Strep</td>
<td>streptavidin</td>
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<tr>
<td>TAP</td>
<td>tandem affinity purification</td>
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<tr>
<td>TIRF-M</td>
<td>total internal reflection microscopy</td>
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<tr>
<td>VASP</td>
<td>vasodilator-stimulated phosphoprotein</td>
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<td>western blot</td>
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Chapter 1
General Introduction
1.1 G protein Coupled Receptors

The superfamily of seven trans-membrane G-protein coupled receptors (GPCRs) constitutes the largest family of integral membrane receptors. In early 1980s the first member of the family, rhodopsin, was sequenced and analysis of the primary amino acid sequence predicted that the protein was composed of seven trans-membrane domains. (Hargrave et al., 1984; Nathans and Hogness, 1983, 1984). The first neurotransmitter receptor, the β2-adrenergic receptor, was cloned in 1986 by two independent research groups, and today it is estimated that the human genome encodes for more than 800 GPCRs (Dixon et al., 1986; Dorsam and Gutkind, 2007; Yarden et al., 1986). These receptors are activated by a variety of ligands ranging from peptides, hormones, neurotransmitters, growth factors to odorant molecules and light. Given their wide range of activators these receptors mediate diverse physiological processes including neurotransmission, pain perception, inflammation, immunity, and cardiac functions to name a few and abnormality in their signalling has been linked to several pathological conditions including cancer cell survival, proliferation as well as metastasis (Dorsam and Gutkind, 2007; Marinissen and Gutkind, 2001). For example, the chemokine receptor CXCR4, which has important roles in immune response and development also plays critical roles in various cancers of both hematopoietic and non-hematopoietic origins (Burger and Kipps, 2006). Enhanced expression of this receptor has been linked to increased migration and invasiveness of several cancers (Zlotnik et al., 2011). Likewise, a plethora of other GPCRs (Lappano and Maggiolini, 2011) has been also linked to many aggressive cancers’ progression some of which will be discussed as we go along in this thesis.

Today, as many as 50% of all current therapeutic agents directly or indirectly target GPCRs, thus reflecting the wide-ranging implications of these receptors in diverse cellular pro-
cesses (Allen and Roth, 2011). As suggested by the name of this family of receptors, classically GPCRs are known to interact with heterotrimeric G proteins which are comprised of α-, β- and γ-subunits. In GPCR-mediated signalling pathways, ligand-activated receptors catalyze the exchange of bound GDP for GTP in the α-subunit (Gα). The activated Gα and Gβγ stimulate distinct downstream effectors including enzymes, ion channel and small GTPases, thus regulating multiple signalling pathways (Figure 1-1).

1.2 Heterotrimeric G proteins

Classically, GPCR dependent signals are known to be transmitted via the activation of heterotrimeric G-proteins, which contain α, β and γ subunits. There are 16 different members of Gα, 5 Gβ and 12 Gγ encoded by the human genome (Downes and Gautam, 1999; Hurowitz et al., 2000). The heterotrimeric G-proteins in their inactive state are known to exist as stable complexes of Gα and Gβγ subunits (Figure 1-2). The Gα subunits are GTPases and cycle between inactive (GDP-bound) and active (GTP-bound) states. Whether Gα-GTP dissociates from the βγ dimers upon receptor activation has been debated over the years. Although the concept has been verified in vitro biochemically (Northup et al., 1983; Sternweis et al., 1981b), dissociation of the complex under physiological conditions is not absolutely clear. Resonance energy transfer experiments have suggested heterotrimeric G-protein activation in cells undergo subunit conformational rearrangement rather than dissociation (Gales et al., 2006; Johnston and Siderovski, 2007). The universality of this claim; however, remains ambiguous and investigators using complementary approaches showed that while some G-protein subunits dissociate in living cells, others may remain intact (Arshavsky, 2002; Digby et al., 2006; Janetopoulos et al., 2001). Heterotrimeric G-proteins can also be activated in receptor independent manner by the activators of G-protein signalling (AGS) (Blumer et al., 2005). Regardless of the mode of activation all G-proteins are
GPCRs and heterotrimeric G proteins mediate diverse signalling pathways. A wide variety of ligands bind to their cognate GPCRs to elicit a range of biological responses through heterotrimeric G protein-dependent signalling pathways. There are four major families of Gα subunits. Additionally there are five members of Gβ and fourteen Gγ subunits that form combinations of obligatory dimers and upon receptor activation modulate a cohort of effector as shown. While activation of GPCR-mediated signalling is required for several physiological responses their dysregulation contributes to many pathological conditions.
known to engage effectors and stimulate signalling cascades resulting in myriad biological responses.

1.2.1 Gα subunits

The discovery of the Gα proteins came about between the late 1970 and early 1980 as the regulatory components of adenylate cyclase (Codina et al., 1984; Northup et al., 1980; Sternweis et al., 1981a; Sternweis et al., 1981b). Gα proteins are classified into four major families: Gαs, Gαi/o, Gαq/11 and Gα12/13. Based on sequence homology between the different subunits, Gα proteins can be further subdivided: Gαs (Gαs and Gαolf), Gαi (Gαi1, Gαtc, Gαg, Gαi1-3, Gαo and Gαz), Gαq (Gαq, Gα11, Gα14, and Gα15/16) and Gα12 (Gα12 and Gα13). Gαs has classically been associated with the activation of adenylate cyclase, and production of the second messenger cAMP, whereas Gαi is known to negatively couple to adenylate cyclase activity. Interestingly, the small G-protein Rap1 specific GTPase Activating Protein, Rap1GAP (see section 1.6.4), preferentially interacts with GDP bound Gαo, suggesting that Gαo bound to GDP can sequester Rap1GAP away from Rap1 (Jordan et al., 1999). Another isoform, Rap1GAPII can associate with Gαi and inactivate Rap1 (Jordan et al., 2005; Mochizuki et al., 1999). Activation of Gαq has been associated with the activation of phospholipase C (PLC) and conversion of membrane phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) into Ins(1,4,5)P3 and diacylglycerol, whereas Gα12/13 subtypes modulate the Rho family of small GTPases, which are known to regulate the actin cytoskeleton (Cabrera-Vera et al., 2003) (Figure 1-1).

Gαi and Gαs can be irreversibly uncoupled from receptors by pertussis toxin (PTX) and cholera toxin respectively (Kaslow and Burns, 1992; Milligan et al., 1989). These toxins catalyze ADP-ribosylation of a specific cysteine residue in α subunits thus either preventing GDP to GTP exchange in Gαi or constitutively activate Gαs respectively. Several GPCR ligands have
Figure 1-2. Crystal structure of stable heterotrimeric G-proteins in the inactive state. Crystallographic structure of $G_{\alpha i1}$, $\beta 1$ and $\gamma 2$ in the GDP bound state at 2.3Å showing two non-overlapping regions of contact between $\alpha$ and $\beta$ subunits, an extended contact between nearly all of $\gamma$ and the $\beta$ subunit, and no interface between $\alpha$ and $\gamma$. The major $\alpha/\beta$ interface spans the switch II region of $\alpha$-subunit. Exchange of GTP for GDP leads to a conformational rearrangement of switch II causing subunit dissociation. Red = $G_{\alpha}$, Green = $G_{\beta}$, Blue = $G_{\gamma}$. Structure adapted from: Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) Cell 83, 1047-1058. (PBD ID 1GG2)
been shown to initiate signalling cascades that regulate cell migration (see section 1.3), and signalling through Ga\textsubscript{i/o} subtypes is especially important for the regulation of cell motility (Cotton and Claing, 2009). Stimulation of the bioactive lipids S1P- and LPA- receptors, which couple to Ga\textsubscript{i}, by their ligands has been shown to enhance migration of cells both in physiological and disease settings (Skoura and Hla, 2009; Yoon et al., 2008). Similarly, CXCR4 receptors that bind to the chemokine ligand SDF-1 promote phosphoinositol-3-kinase (PI3K)-mediated cell migration (Dutt et al., 1998). Interestingly, G\textbeta\gamma subunits when released from Ga\textsubscript{i} are thought to also be involved in SDF-1 mediated cell migration via mechanisms that are incompletely understood but may involve activation of Rho family small GTPases (Kirui et al., 2010).

Several non-canonical roles for Ga proteins also exist including interactions with cytoskeletal proteins (Roychowdhury and Rasenick, 2008), role during cell division (Du and Macara, 2004; Schaefer et al., 2000), and signalling in subcellular organelles (Andreeva et al., 2008; Stow et al., 1991). The binding of Ga\textsubscript{i} and Ga\textsubscript{s} to microtubule plus ends has been reported to stimulate tubulin-GTP hydrolysis and destabilize microtubules (Layden et al., 2008; Roychowdhury et al., 1999). This increases the frequency of microtubule catastrophe events that converts long microtubules to greater number of short ones (Dave et al., 2009). Whether Ga interaction with the microtubules is triggered by GPCRs is not known, but the current state of understanding is that these are receptor-independent functions of Ga proteins.

1.2.2 G\textbeta\gamma subunits

1.2.2.1 Structure of \beta\gamma subunits

G\beta subunits of heterotrimeric G-proteins form a \beta-propeller structure with seven WD40 repeats. The G\gamma-subunits make extensive contacts along the N-terminal non-WD repeat region and blades 5, 6 and 7 of G\beta thereby forming an obligatory dimer, hence the name G\beta\gamma subunit
Gβγ binds a hydrophobic pocket within Gα in its GDP bound state (Figure 1-2), which is removed upon GTP exchange, reducing the affinity of Gβγ for Gα (Lambright et al., 1994).

Of the five known Gβ subunits, β1-4 share 80-88% amino-acid sequence identity, whereas β5 has about 50% identity with the rest of the β-subunits (Downes and Gautam, 1999). Gγ genes encode for small proteins ranging between 68 and 75 amino acids. Interestingly, however, the sequence identities between different Gγ is much more diverse compared to Gβs, ranging from anywhere between 31 and 79% (Downes and Gautam, 1999). The carboxy-terminals of Gγ-subunits contain a CAAX motif amenable to prenylation of the molecule that mediates anchorage of the Gβγ subunits in the plasma membrane (Muntz et al., 1992; Simonds et al., 1991).

Given the total number of protein-encoding genes for each subunit, random associations between the different molecules predict 60 different combinations of β and γ partner. While most partners can couple to form a complex, there are a few exceptions that do not (Schmidt et al., 1992). To test the specificity of different Gβ and γ partners, Schmidt and colleagues compared the ability of Gβ1-3 to dimerize with Gγ1 or γ2. The findings indicated that while Gβ1 binds to both γ subunits, the β2 subunit specifically prefers γ2. Gβ3 on the other hand did not bind to either γ1 or γ2. This sort of observed specificity is attributed to the specific amino-acid sequences at the c-terminus of Gγ-subunits (Spring and Neer, 1994). In cells the specific interactions between different subunits of Gβ and γ can be further limited by differential expressions of the different subunits in the cell types under study (Dupre et al., 2009); for example the expression of Gγ1 is restricted to retinal cells (Kisselev and Gautam, 1993). It is possible that different heterodimer combinations are functionally distinct; however, systematically testing this hypothesis has been difficult (Robishaw and Berlot, 2004). Yet, a genetic study showed that knockout of Gγ7 in mice inhibits adenylate cyclase activation in the striatum downstream of the D1 dopamine receptor.
Schwindinger and Robishaw, 2001). Gγ knockout mice also exhibit reduced Gaolf expression while Ga_s expression remains unaffected.

1.2.2.2 Localization of Gβγ

Gβγs are largely localized in the cytoplasmic surface of the plasma membrane by virtue of lipid modifications. This is where they are known to control their conventional signalling pathways. During-cAMP induced chemotaxis of Dictyostelium discoideum, commonly known as slime mold, the Gβγ molecules have been shown to preferentially localize at the leading edge membrane (van Hemert et al., 2010). Although an earlier study suggested that during directional cell migration Gβγ polarizes in a shallow gradient, insufficient to restrict signalling at the leading edge (Jin et al., 2000), a newer study showed that activation of cAMP receptor in Dictyostelium discoideum actually causes activated, receptor-uncoupled Gβγ to immobilize at the leading edge (van Hemert et al., 2010). There they form larger signalling complexes with the local F-actin and associated proteins (van Hemert et al., 2010). This localized subcellular availability of activated Gβγ is proposed to elicit signals that can facilitate polarized pseudopodial activities of the cell. In a different study, use of the PH domain of AKT fused to GFP; known to be recruited to the plasma membrane following Gβγ activation (Meili et al., 1999), demonstrated that stimulation of neutrophil-differentiated HL-60 cells with shallow gradient of fMLP leads to dramatic localization of the probe to the leading edge of polarized cells (Servant et al., 2000). Treatment of cells with pertussis toxin or PI3K inhibitor LY294003 inhibited PHAKT-GFP localization to the plasma membrane suggesting that translocation of the probe at the leading edge is a reflection of localized activation of the heterotrimeric G protein.

In addition to its localization at the plasma membrane, Gβγ can also associate with tubulin along the length of the microtubules and is thought to promote microtubule stability.
(Roychowdhury and Rasenick, 1997). Although the consequences of the dynamics between active Gα and Gβγ in this context remain elusive, it is proposed that the heterotrimeric G-proteins can control cell morphology by directly binding and modulating microtubules in addition to affecting the actin cytoskeleton via regulation of signal transduction. Select Gβγ-subunits such as Gγ5-containing βγ-subunits have been observed to localize with the terminal ends of actin stress fibers and the focal adhesion adapter protein vinculin, at sites of cell adhesion with the surrounding extracellular matrix (Hansen et al., 1994). In separate studies Gγ12-containing βγ-subunits have been reported to promote cell migration by disrupting the F-actin stress fibers (Ueda et al., 1997; Ueda et al., 1999) (see section 1.6.3 for more on actin cytoskeleton). In Hela cells, several Gγ subunits together with Gβ1 induce stress fiber formation and focal adhesion assembly (Ueda et al., 2000). The studies describing the localization of Gβγ with different cytoskeletal components are exciting for the understanding of how GPCR and Gβγ may regulate cell morphology and motility. However, our insight into their physiological significance and mechanism is limited by a lack of follow-up studies.

Other localizations of Gβγ subunits have also been reported. The βγ subunits can dynamically translocate to subcellular organelles and endomembranes such as that of endosomes and Golgi in a GPCR-dependent manner (Garcia-Regalado et al., 2008; Saini et al., 2009). Gβγ-subunits have been recognized to stimulate vesiculation of Golgi through activation of protein kinase D (PKD) and to regulate trans-Golgi network to plasma membrane transport of proteins including insulin (Irannejad and Wedegaertner, 2010; Jamora et al., 1997; Jamora et al., 1999). Recently, several reports have also described nuclear localization of Gβγ where they were shown to regulate transcription in a GPCR-dependent manner (Kino et al., 2005a; Kino et al., 2005b; Park et al., 1999; Robitaille et al., 2010).
Figure 1-3. Crystal structure of the $\beta\gamma$ subunit of the heterotrimeric G-protein transducin. $G\beta\gamma$ subunits dissociate from $G\alpha$ upon nucleotide exchange on $G\alpha$ catalyzed by activation of the receptor. Shown is the structure of the $\beta\gamma$ subunit alone uncoupled from $G\alpha$. The $\beta$ subunit (green) has a $\beta$-propeller structure made up of seven WD40 repeats. The $G\gamma$ subunit (orange) makes extensive contact along the base of $G\beta$. Green = $G\beta$, Orange = $G\gamma$. Structure adapted from: Sondek, J., A. Bohm, et al. (1996). "Crystal structure of a G-protein beta gamma dimer at 2.1Å resolution." Nature 379(6563): 369-374. (PBD ID 1TBG)
1.2.2.3 Gβγ as signalling molecules

βγ subunits were initially thought to contribute to the G protein cycle solely by associating with Ga-GDP and promoting the return of the heterotrimer to the receptor at the plasma membrane (Neer, 1995). In the late 1980s it was discovered that Gβγ could transduce intracellular signals independently of Ga by engaging its own set of effectors. Gβγs were for example shown to directly and selectively activate the G protein-gated inward rectifier channels (GIRKs) upon muscarinic m2 receptors stimulation (Huang et al., 1995; Logothetis et al., 1988; Logothetis et al., 1987; Rebois et al., 2006; Reuveny et al., 1994; Wickman et al., 1994). Gβγs are also known to inhibit other ion-channels including N-, P/Q- and R-type voltage-gated Ca2+ channels (Currie, 2010).

Further evidence substantiating Gβγ as a bona fide signalling molecule come from studies showing Ga_i-derived Gβγ is responsible for the activation of phospholipase C (specifically PLC-β2 and PLC-β3 and to some extent PLC-β1) in neutrophils and human leukemic HL60 cells upon stimulation with the formyl peptide (fMLP). PLC activation causes enzymatic hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) into Ins(1,4,5)P3 (Camps et al., 1992a; Camps et al., 1992b; Illenberger et al., 2003) and diacylglycerol (Berridge and Irvine, 1989), consequently raising cytosolic concentration of Ca2+ and activating PKC. PLCβ interacts with Gβγ via its PH domain and is recruited to the plasma membrane upon Gβγ release induced by receptor activation (Razzini et al., 2000). Similarly, Gβγs have been shown to also stimulate the enzymatic activities of other isoforms of PLC such as, PLCε (Wing et al., 2001) and PLCη2 (Zhou et al., 2008b). Whether direct interactions between the βγ-subunits and these isoforms are required is not well understood as deletion of the PH domains from either PLCε or PLCη2 retains Gβγ-regulated activity. Gβγ can also associate with the p110 β and γ subunits of phospho-
inositide-3-kinase (PI3K) and stimulate the generation of ptdIns(3,4,5)P3 from PtdIns(4,5)P2 (Stoyanov et al., 1995; Suire et al., 2006). The βγ-subunits have been also linked to the activation of MAPK signalling pathways whereby expression of Gβγ in Cos-7 cells was deemed sufficient to increase ERK1/2 activity in a Ras dependent manner (Crespo et al., 1994; Faure et al., 1994). PLCβ as well as PI3K can both activate Ras downstream of Gβγ activity (Camps et al., 1992a; Katz et al., 1992; Kranenburg et al., 1997; Kranenburg et al., 1999; Luttrell et al., 1996; Stephens et al., 1994; Thomason et al., 1994). Since common functional consequences for the aforementioned biochemical pathways are the control of cytoskeletal and cell adhesion dynamics, Gβγ was rapidly implicated in these processes important for cell motility (Afonso and Parent, 2011; Carragher and Frame, 2004; Huang et al., 2004; Illenberger et al., 1998; van Rheenen and Jalink, 2002). This function is evolutionarily conserved since βγ-subunits were shown to be essential for the movements of Dictyostelium discoideum downstream of cell surface cAMP receptors activation (Janetopoulos et al., 2001; Jin et al., 2000).

Today, Gβγ-subunits are known to interact with and activate several effectors including adenylate cyclases (Tang and Gilman, 1991), phosphoinositide 3-kinase (PI3K) (Hawes et al., 1996; Stephens et al., 1994; Tang et al., 1997), components of the mitogen activated protein kinase (MAPK) cascade (Inglese et al., 1995; Luttrell et al., 1996), GPCR receptor kinases (GRKs) (Lodowski et al., 2003; Pitcher et al., 1995; Wu et al., 1998) and many more. The complexity of Gβγ’s functions is thus beyond what was initially recognized (see Table 1-1 and Figure 1-4 for illustration).
Table 1-1. Interactors of Gβγ-subunits.

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<thead>
<tr>
<th><strong>Well characterized Gβγ effectors</strong></th>
<th>References</th>
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<tbody>
<tr>
<td>Inward rectifying K⁺ channels</td>
<td>(Logothetis et al., 1987)</td>
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<tr>
<td>GPCR Kinases 2 and 3</td>
<td>(Pitcher et al., 1995)</td>
</tr>
<tr>
<td>PLC β1, β2 and β3</td>
<td>(Camps et al., 1992a)</td>
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<tr>
<td>Adenylate cyclase (activation),II, IV, VII</td>
<td>(Tang and Gilman, 1991)</td>
</tr>
<tr>
<td>Adenylate cyclase (inhibition),I, III, V, VI</td>
<td>(Diel et al., 2006)</td>
</tr>
<tr>
<td>N-type Ca²⁺ channels</td>
<td>(Ikeda, 1996)</td>
</tr>
<tr>
<td>P/Q type Ca²⁺ channels</td>
<td>(Herlitze et al., 1996)</td>
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<tr>
<td>PI3Kγ</td>
<td>(Stoyanov et al., 1995)</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>(Gerachshenko et al., 2005)</td>
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<td>P-Rex1</td>
<td>(Welch et al., 2002)</td>
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<th><strong>Less characterized Gβγ binding proteins</strong></th>
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<tr>
<td>RACK1</td>
<td>(Dell et al., 2002)</td>
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<td>PLC-ε</td>
<td>(Wing et al., 2001)</td>
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<td>Radil</td>
<td>(Ahmed et al., 2010)</td>
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<td>Group III AGS proteins</td>
<td>(Sato et al., 2006)</td>
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<td>ElmoE</td>
<td>(Yan et al., 2012)</td>
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<tr>
<td>PKD</td>
<td>(Jamora et al., 1999)</td>
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<tr>
<td>Glucocorticoid receptor</td>
<td>(Schmidt et al., 2001)</td>
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<td>AEBP1</td>
<td>(Park et al., 1999)</td>
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<tr>
<td>Fos</td>
<td>(Robillatt et al., 2010)</td>
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<tr>
<td>p21 activated kinase (PAK)</td>
<td>(Menard and Mattingly, 2004)</td>
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<tr>
<td>Raf-1</td>
<td>(Pumiglia et al., 1995)</td>
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<tr>
<td>B-Raf</td>
<td>(Schmitt and Stork, 2002)</td>
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<td>Btk kinase</td>
<td>(Lowry and Huang, 2002)</td>
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<tr>
<td>p114 RhoGEF</td>
<td>(Niu et al., 2003)</td>
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<tr>
<td>Dynamin</td>
<td>(Lin and Gilman, 1996)</td>
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<td>Dbl</td>
<td>(Nishida et al., 1999)</td>
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<tr>
<td>Tubulin/microtubules</td>
<td>(Roychowdhury and Rasenick, 1997)</td>
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<tr>
<td>Phospholipase D1</td>
<td>(Preininger et al., 2006)</td>
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<tr>
<td>IP3 receptor I</td>
<td>(Zeng et al., 2003)</td>
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<tr>
<td>Histone deacetylase 5 (HDAC5)</td>
<td>(Spiegelberg and Hamm, 2005)</td>
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<th><strong>Indirect Gβγ effectors</strong></th>
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<tr>
<td>MAPK</td>
<td>(Crespo et al., 1994)</td>
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<td>Phospholipase A₂</td>
<td>(Katz et al., 1992)</td>
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<th><strong>Gβγ Chaperones</strong></th>
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<tr>
<td>Phosducin like protein-1 (PhLP1)</td>
<td>(Knol et al., 2005)</td>
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<tr>
<td>Tailless complex polypeptide, TCP</td>
<td>(Humrich et al., 2005)</td>
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<tr>
<td>Dopamine receptor interacting protein 78 (DRiP78)</td>
<td>(Dupre et al., 2007)</td>
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Adapted from (Smrcka, 2008)
Figure 1-4. $G_{\beta\gamma}$-mediated signal transduction pathways. A schematic of different signal transduction pathways modulated by $G_{\beta\gamma}$ downstream of GPCR signalling. Solid-lined arrows denote signalling cascades and mechanisms that are well studied. Signalling pathways that have been reported but mechanisms less understood are marked with dash-lined arrows.
1.2.2.4 Implications of Gβγ in cell motility

Chemoattractants bind to their cognate receptors that are often of the GPCR family. Chemoattractants range from chemokines such as SDF-1 that binds to CXCR4 receptors; bioactive lipids LPA and S1P bind to LPARs and EDGs respectively, to $N$-formylated peptides that bind to FPRs. Upon activating their cognate receptors chemoattractants stimulate cell motility, which is essential to perform normal physiological responses during development, immune responses, or wound healing as well as participate in the aberrant migration of cells such as during cancer metastasis (Campbell et al., 1996; Choi et al., 2010; Hobson et al., 2001; Johnston and Butcher, 2002; Su et al., 1999; Tseng et al., 2011; Yao et al., 2011). The process whereby cells are directed to migrate towards a gradient of chemoattractants is known as chemotaxis (Stephens et al., 2008) (also see section 1.3).

By the mid 1990s evidence was accumulating showing that stimulation of pertussis toxin-sensitive chemoattractant receptors rapidly activated integrins in leukocytes and lymphocytes. The regulation of integrin-dependent adhesion by these receptors was then described to be important for the attachment of these cells to the extracellular matrix and for their crawling to sites of infection (Baggiolini et al., 1994; Campbell et al., 1996; D'Apuzzo et al., 1997; Mackay, 1996; Moser et al., 2004; Sham et al., 1993). In the late 1990s and early 2000 a set of key papers using human lymphocytes, human embryonic kidney fibroblasts and *Dictyostelium discoideum* described a key role for βγ-subunits of the heterotrimeric G-proteins in transmitting the chemotactic signals observed upon activation of receptors (Arai et al., 1997; Jin et al., 1998; Jin et al., 2000; Neptune et al., 1999; Zhang et al., 2001). A loss of function study using RNA interference (RNAi) approach in the mouse macrophage cell line, J774A.1, showed that Gβ1 and Gβ2, were indispensable for chemotactic response of these cells to the complement compound C5a (Hwang et al., 2004). Furthermore, the significance of Gβγ-subunits in neutrophil chemotaxis was de-
scribed using βγ-specific small molecule inhibitors M119 and Gallein (Lehmann et al., 2008). Using these inhibitors the authors demonstrated they could dramatically block fMLP-mediated migration of neutrophils. In addition to chemotaxis, Gβγ has been also shown to positively regulate cell adhesion and spreading on extracellular matrix and invasion of artificial 3D matrix called matrigel (Faivre et al., 2001). Overexpression of Ga1 subunits inhibited this function by sequestering Gβγ leading to cell rounding, reduced cell-matrix adhesion and failure to invade the extracellular matrix (Faivre et al., 2001). Although this study positioned Gβγ in the context of cell motility as a possible regulator of cell-matrix adhesion and morphology, the biochemical mechanisms underpinning this process remain unclear. The anticipation of a potential role of Gβγ in the modulation of cell-matrix adhesion was further raised when PLIC-1, which associates with CD47 (an integral membrane glycoprotein that associates with several integrins) (Wu et al., 1999), was shown to associate with βγ subunits (N'Diaye and Brown, 2003). Overexpression of PLIC-1 blocks SDF-1 mediated cell migration in Jurkat T cells and A431 epithelial cells by sequestering Gβγ and preventing it from performing its normal functions (N'Diaye and Brown, 2003). Again, how Gβγ may function in controlling cell adhesion was left unanswered.

Another protein, RACK1 reportedly binds specifically to Gβγ released from the activated heterotrimeric G proteins upon activation of its cognate receptors such as β2-adrenergic receptor and CXCR2 (Chen et al., 2004a; Chen et al., 2004b). Binding of RACK1 to Gβγ was initially shown to inhibit PLCβ2 and adenylate cyclase II, but does not affect other functions of βγ such as activation of MAPK signalling and chemotaxis of HEK293 cells via the chemokine receptor CXCR2 (Chen et al., 2004a). However, in a subsequent paper published by the same authors, they reported that in Jurkat and HL-60 cells RACK1 sequesters Gβγ, preventing it from activating PI3K and thereby negatively regulates directed migration of these cells towards SDF-1. (Chen et al., 2008).
Overexpression of Gβγ in Hela cells has been shown to induce actin stress fiber and focal adhesion formation tantamount to that caused by Ga12 subunit (Ueda et al., 2000) (see sections 1.6.3 for more on actin cytoskeletons and focal adhesion). The actin cytoskeleton remodeling seen was found to be due to Gβγ-mediated activation of RhoA signalling pathway which was independent of Ga. Therefore, it was concluded by the authors that effects mediated by the Gβγ subunits in the context of Hela cells were likely coupled to Ga12 (also see Figure 1-1 for effectors of Ga). Recently the finding that Gβγ associates with ElmoE and DOCK-like proteins in Dictyostelium discoideum has further confirmed βγ-subunits as regulators of actin cytoskeleton remodeling (Yan et al., 2012). The ELMO-DOCK protein complex is a guanine nucleotide exchange factor (GEF) for the small G protein Rac that is known to promote actin rearrangement (Brugnera et al., 2002). Yan et al., in their paper demonstrate that the GPCR cAR1 (cAMP receptor) regulates ELMO function by promoting its binding to Gβγ. ELMO-Gβγ complex further associates with the DOCK-like proteins, RacB and the actin nucleation complex Arp2/3 to enhance pseudopodial activities during chemotaxis (Yan et al., 2012).

The above studies provide compelling evidences suggesting Gβγ may be a potential mediator of integrin-dependent cell adhesion. Interestingly there are plenty of studies also describing GPCR signals modulating integrin-dependent adhesion via activation of the small GTPase Rap1 (see section 1.6.4). (Cha et al., 2010; Gloerich et al., 2010; Li et al., 2007; McLeod et al., 2002; Schmitt and Stork, 2002). Given these evidence a link between Gβγ and Rap1-dependent integrin signalling is possible and would imply a more direct axis by which chemoattractant receptor activation leads to adhesion signals. Chapter 2 of this thesis presents the study of a novel Gβγ-interacting protein called Radil that I identified to play essential roles in mediating cell-matrix adhesion downstream of GPCR signalling.
1.3 GPCRs and Gβγ in Cancer Cell Motility

Recently a number of studies have implicated aberrant GPCR expression and activation in numerous human malignancies. For example, chemokine receptors (e.g., CXCR4), bioactive lipid receptors (LPAR and S1PR), protease-activated receptors, and melanocortin-1 receptors have all been associated with enhanced cell proliferation in different cancers (Benovic and Marchese, 2004; Boire et al., 2005; Darmoul et al., 2004; Fernandis et al., 2004; Mills and Moolenaar, 2003; Visentin et al., 2006). Furthermore, the abnormal expression and stimulation of these receptors control the dissemination of cells to specific organs during tumour progression (see section 1.5). This is exemplified by studies showing that breast cancer cells expressing CXCR4 have increased metastatic properties and preferred tropism to the bone marrow or the lungs due to greater availability of its cognate SDF1 ligand in these environments (Fernandis et al., 2004; Lapteva et al., 2005; Liang et al., 2005; Muller et al., 2001). Another chemokine receptor, CXCR7, which was recently identified as a novel SDF1 and CXCL1 receptor (Burns et al., 2006) is also highly expressed in breast and prostate tumours and enhances growth and metastasis to the lung (Miao et al., 2007; Wang et al., 2008). Similarly, aberrant signalling via LPA has been implicated in tumour cell migration and bone metastasis in breast cancer xenografts (Boucharaba et al., 2004) via signalling pathways implicating nonmuscle myosin II phosphorylation and RhoA GTPase signalling (Kim and Adelstein, 2011). LPA’s role at promoting malignancies has been reported for other tumour types as well including ovarian and lung cancers (Bian et al., 2004; Xu and Prestwich, 2010). Whereas receptor overexpression is at times sufficient, overstimulation of cells by high levels of LPA, S1P, and chemokines ligands also lead to cell transformation, proliferation and metastasis (Fernandis et al., 2004; Mills and Moolenaar, 2003; Pyne and Pyne, 2010).
As mentioned earlier, several GPCRs have been linked to activation of the integrin adhesion receptors in different contexts (see section 1.6.2) (Deevi et al., 2010; Festuccia et al., 2002; Loike et al., 1999; Patcha et al., 2004; Rieken et al., 2006; Short et al., 2000; Tharmalingam et al., 2011; Valenick and Schwarzbauer, 2006). A recent study showed that a range of chemoattractants activating GPCRs can activate the p110γ isoform of PI3K and lead to inside-out activation of α4β1 integrins (Schmid et al., 2011). Given integrins’ pivotal roles during cell adhesion, migration and invasion and their cross talks with GPCR signalling, their collaboration during cancer dissemination is now closely being examined. Now although the Gβγ component of GPCR signalling is recognized as key player during cell migration, its necessity during cancer cell invasion and metastasis is only beginning to emerge. Notably, using \textit{in vivo} mouse models of breast cancer two recent studies revealed that blocking Gβγ interfered with the migration and invasive capacities of aggressive breast cancer cell lines towards chemoattractant gradients of SDF-1, LPA, PAR1 and PAR2 (Kirui et al., 2010; Tang et al., 2011). Gβγ blockade had the same inhibitory effect as pertussis toxin treatment implying that breast cancer cell migration in these contexts is elicited by Gβγ subunits released from Gαi/o coupled receptors. However, the molecular mechanisms by which Gβγ affect the adhesive properties of tumour cells and control integrin activation to facilitate tumour migration are incompletely defined.

1.4 Formyl Peptide Receptors

The human formyl peptide receptor (FPR), first discovered on the surface of neutrophils, binds to the prototypical \textit{N}-formylated bacterial peptides \textit{N}-formyl-methionine-leucine-phenylalanine (fMLP) with high affinity (\(K_D\) 0.1 – 1nM) and provokes chemotactic responses in the cell towards a gradient of this chemoattractant (Le et al., 2002; Wahl et al., 1975). fMLP was initially described to be released from \textit{Escherichia coli} (Keller and Sorkin, 1967; Marasco et al.,
1984; Ward et al., 1968), however, these types of molecules are also found in mitochondria and are released upon cell death to recruit phagocytic leukocytes for engulfment of the dying cell (Carp, 1982).

The first formyl peptide receptor was cloned from the differentiated myeloid leukemia cell line HL-60 (Boulay et al., 1990a, b). Although cellular expression of FPR was first described in phagocytic leukocytes, monocytes and neutrophils, it was later recognized that their expression is not restricted to the blood lineage but also found in several other cell types including fibroblasts (Table 1-2) (Le et al., 2002; Le et al., 2001; VanCompernolle et al., 2003). For example, expression of FPRs in the sarcoma cell line HT1080, which originates from fibroblasts, and their stimulation by fMLP was shown to enhance cell adhesion and migration on the extracellular matrix fibronectin (see section 1.6).

FPR and related FPRL1 and FPRL2 receptors are all coupled to the Gαi family of G-proteins, specifically Gαi1-3, as their response to agonist can be completely blocked upon treatment of the cells with pertussis toxin (Le et al., 2002). The phosphoinositide-3-kinase (PI3K)-Akt pathway, particularly the PI3Kγ isoform that is known to couple to many chemoattractant receptors, has been shown to also be activated (Ferguson et al., 2007; Le et al., 2002; Li et al., 2000) following FPR stimulation. However, whether PI3K activity is absolutely required for fMLP mediated chemotaxis has been recently questioned. Some studies argue that PI3K activity accelerates rather than mediating cell migration (Boulven et al., 2006; Heit et al., 2005). Inhibition of PI3K using pharmacological inhibitors delay fMLP induced cell migration but their overall ability to migrate is unaffected (Heit et al., 2008).

The small G-protein Rap1 (see section 1.6.4), an important modulator of cell adhesion and migration, can also be efficiently activated in neutrophils following fMLP stimulation and
was functionally characterized to be important in the context of neutrophil adhesion (Jenei et al., 2006; M'Rabet et al., 1998). Although PLC and calcium have been reported to potentiate Rap1 activity, their depletion only marginally affects activation of Rap1 following fMLP stimulation. This suggests that there may be alternative mechanisms existing by which Rap1 activity is controlled (M'Rabet et al., 1998). The mode of fMLP mediated signalling in non-hematopoietic cells is however, not well established.

Table 1-2. Distribution of formyl peptide receptor family in human cell types

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPR</td>
<td>Neutrophils</td>
</tr>
<tr>
<td></td>
<td>Monocytes/macrophages</td>
</tr>
<tr>
<td></td>
<td>Immature dendritic cells</td>
</tr>
<tr>
<td></td>
<td>Endothelial cells</td>
</tr>
<tr>
<td></td>
<td>Platelets</td>
</tr>
<tr>
<td></td>
<td>Hepatocytes</td>
</tr>
<tr>
<td></td>
<td>Astrocytes</td>
</tr>
<tr>
<td></td>
<td>Microglial cells</td>
</tr>
<tr>
<td></td>
<td>Fibroblasts</td>
</tr>
<tr>
<td></td>
<td>Vascular smooth muscle cells</td>
</tr>
<tr>
<td></td>
<td>Neutrophils</td>
</tr>
<tr>
<td></td>
<td>Monocytes/macrophages</td>
</tr>
<tr>
<td></td>
<td>Immature dendritic cells</td>
</tr>
<tr>
<td></td>
<td>T cells/B cells</td>
</tr>
<tr>
<td></td>
<td>Endothelial cells</td>
</tr>
<tr>
<td></td>
<td>Epithelial cells</td>
</tr>
<tr>
<td></td>
<td>Hepatocytes</td>
</tr>
<tr>
<td></td>
<td>Astrocytes</td>
</tr>
<tr>
<td></td>
<td>Microglial cells</td>
</tr>
<tr>
<td></td>
<td>Fibroblasts</td>
</tr>
<tr>
<td></td>
<td>Monocytes/macrophages</td>
</tr>
<tr>
<td></td>
<td>Dendritic cells</td>
</tr>
</tbody>
</table>

Table adapted from (Migeotte et al., 2006).

1.5 The Metastatic Spread of Cancers

Metastasis is a serious threat and a challenge in cancer. Even though 90% of the morbidity associated with cancers is caused by metastasis, the mechanistic details underlying this process remain poorly understood (Chaffer and Weinberg, 2011). Metastasis involves the physical migration of a cancer cell from its primary site to distant organs, and its ability thereafter to
grow in these ectopic environments (Figure 1-5). In order to metastasize, tumour cells have to undergo some significant intrinsic changes and overcome several physiological barriers. The first step of the metastatic cascade is the ability of some cancer cells, within the primary tumour, to detach from their neighbours and acquire invasive properties needed to breach the basement membrane via a process known as epithelial-to-mesenchymal transition (EMT). It is thought that advanced primary carcinomas recruit a variety of cell types like fibroblasts, myofibroblasts, granulocytes, macrophages, lymphocytes etc., into the surrounding stroma to foster a micro-environment that promotes EMT (Chaffer and Weinberg, 2011). The EMT process also confers on these cells traits that allow them to survive the harsh journey from the primary to the distant secondary sites. The first among them is the ability of cancer cells to overcome anoikis; a specialized type of apoptosis that is induced upon inappropriate loss of cell adhesion (Grossmann, 2002; Thiery and Sleeman, 2006).

Following detachment from the neighbouring cells, tumour cells degrade or remodel the basement membrane that otherwise impose a physical barrier to their dissemination. It is to be noted though that the extent of matrix degradation must be finely controlled, thus enabling sufficient adhesion to generate the traction required for migration (discussed more in section 1.6). Metastatic cells accomplish matrix degradation by releasing proteases, such as the matrix metalloproteinases, MMP2 and MMP9 (Sternlicht and Werb, 2001). Cell’s ability to degrade matrix is somewhat regulated by GPCRs and integrins as both of these types of membrane proteins can stimulate signalling pathways that promote MMP activity at the leading edge of migrating cells (Chinni et al., 2006; Dang et al., 2006; Kodali et al., 2006; Rupp et al., 2008).

Once the cancer cells pave their way through the surrounding matrix they migrate and penetrate into the blood and lymphatic vessels, via a process known as “intravasation”
In order to achieve this, cancer cells traverse the endothelial layer by degrading their basement membrane as well as disrupting endothelial cell-cell junctions (Weis et al., 2004). Alternatively, lymphatic vessels, which lack continuous basement membrane or tight junction, provide cancer cells a convenient access point to spread to the regional lymph nodes (Witte et al., 2006).

Upon successfully making their way into the circulatory system, cancer cells sometimes adhere to platelets and leukocytes. These provide cancer cells a convenient portal system to access the microcirculation of target organs. Next, as described earlier, tumour cells metastasize to specific organs depending on GPCRs expressed on their surface, and on the release of their ligands from the target tissues (Dorsam and Gutkind, 2007). Currently, it is thought cytokines and inflammatory mediators, secreted by primary tumours can also facilitate the production of chemokines by endothelial cells within the lymph nodes and secondary organs, thereby creating an ideal microenvironment for tumour colonization (Bennewith et al., 2010; Peinado et al., 2011). By sensing a receptive milieu the cancer cells finally exit the bloodstream, and enter the secondary site through a process known as “extravasation”, whereby they again have to alter the basement membranes to migrate into the organ (Valastyan and Weinberg, 2011). Following dissemination the cancer cells undergo a reverse process, mesenchymal-to-epithelial transition (MET), and undertake expansive tumour growth within the secondary site with requirements similar to primary tumours, such as increased nutrient and blood supply (Thiery and Sleeman, 2006).

As seen in the above section, cells need to dynamically interact with the surrounding matrix in order to migrate during metastasis. Some of the key factors that control this process to ensure efficient cell migration and invasion will be discussed in the next section.
Figure 1-5. The metastatic cascade. During early phases of metastatic dissemination of epithelial tumor cells undergo epithelial-mesenchymal transition and gain migratory properties by loss of their epithelial polarity, loss of contacts with the surrounding cells and with the basement membrane. Subsequently these cells invade and remodel the interstitial matrix to make their way towards blood or lymphatic vessels. Once near the blood vessels, cells penetrate the vessel barriers by a process called intravasation. In the circulation, tumor cells can then bind to platelets and leukocytes to form small aggregates. Upon arrival at the microcirculation of target organs, tumor cells exit the bloodstream by a process called extravasation and re-colonize locally.

Adapted from Guo and Giancotti., 2004 Nat Rev Mol Cell Biol
1.6 Cell-Matrix Adhesion and Migration

While the immediate consequence of cell-matrix interaction is attachment of the cell to the extracellular matrix and maintenance of tissue architecture, it is also critical for the regulation of gene transcription, cell shape, polarity and cytoskeletal changes, which lead to cell differentiation, growth, tissue shapes, as well as chemotaxis (migration of cells towards a chemoattractant). Cell migration plays important physiological roles during development, wound healing, immunity as well as in cancer cell metastasis.

Cells adhere to their surrounding extracellular matrix (ECM) via transmembrane integrin adhesion receptors (Barczyk et al., 2010). The extracellular domains of integrins interact with the ECM, while the intracellular domain links the receptors to the cytoskeletal components via multimolecular protein complexes (Campbell and Humphries, 2011; Kim et al., 2011). A lot is now known about the molecular composition of the adhesion complexes. Systematic identification of their components has been made possible by advances in biochemical isolation and proteomic analysis of integrin-associated complexes (Byron et al., 2011; Byron et al., 2010; Humphries et al., 2009; Kuo et al., 2011; Mayhew et al., 2006; Schiller et al., 2011; Zaidel-Bar and Geiger, 2010). Yet, a great deal still remains elusive in terms of knowledge of how integrin-mediated cell adhesion is regulated downstream of GPCR activation.

During directional migration, cells rely on mechanical forces exerted upon interaction with the ECM in order to move towards a gradient of chemoattractants. The migration cycle is initiated by cells first polarizing and extending protrusions towards the extracellular cues. The protrusions are a combination of large, flat lamellipodia at the leading edge as well as long, thin filopodia composed of polymerized actin filaments. Protrusions at the leading edge are then stabilized by adhesion structures that link the cytoskeleton to the extracellular matrices to generate
traction force on the substratum. Combinatorial assembly and disassembly of adhesions at the front and rear together with actomyosin contraction then drive the cell to move forward (Figure 1-6) (Ridley et al., 2003). Tight coordination of these processes is required to allow efficient cell movement.

1.6.1 Role of Extracellular Matrix in Cell Adhesion

There are four primary types of extracellular matrices: collagens, elastins, proteoglycans and glycoproteins. The composition of the ECM is very dynamic depending on cellular differentiation, developmental stages, as well as pathological conditions. Collagen and elastins are fibrous proteins whereas proteoglycans are protein polysaccharides (e.g. heparan sulfate, chondroitin sulphate etc), or pure polysaccharides (hyaluronic acid) that fill the fibrillar mesh formed by collagen or elastin fibers. The structural glycoproteins are also important for the organization of ECM and comprise of fibronectin and laminin. Among them fibronectin has been studied the most. Fibronectin has the propensity to bind to several other ECM proteins including itself and form fibrillar mesh of ECM (Singh et al., 2010). Fibronectin binds to cells through an arginine-glycine-aspartic acid (RGD) epitope that is recognized by the main fibronectin receptor, integrin α5β1 (Danen et al., 1995; Li et al., 2003a; Zhang et al., 1995).

Fibronectin exists in soluble form in plasma and is synthesize by hepatocytes. Soluble fibronectin diffuse into tissues and incorporate into fibrillar matrix. Insoluble fibronectin is also present and are made by fibroblasts, epithelial cell, and other differentiated cell types (Singh et al., 2010). In solution, fibronectin forms a compact dimer and does not readily assemble into fibrils. Upon contact with cells in tissues or in culture, fibronectin binds to integrins and induces their clustering, promoting their activation and interaction with the cytoskeleton. This
Figure 1-6. Cell spreading and directional migration. The schematic shows stepwise processes cells undergo as they adhere, spread, and migrate towards a directional cue on 2D matrix. When cells are plated on top of extracellular matrices integrins are engaged and stimulate adhesion to the matrix substrate. Once adherent the peripheral membranes use the tractions provided by integrin-ECM attachment to crawl and stretch outwards causing cells to spread. Upon spreading the cells elongate to establish front-to-back polarity with a leading and a trailing edge. At the front of the cell, adhesions form, disassemble and signal to Rho GTPases, which lead to actin polymerization. In order to move directionally towards a chemical gradient the adhesion sites go through dynamic assembly and disassembly at the front and rear. This together with actomyosin contractility drives the cell forward.
allows fibronectin to self-assemble by morphing into fibrillar networks (Wierzbicka-Patynowski and Schwarzbauer, 2003). Fibronectin plays an important role in matrix assembly and controls all aspects of cell motility including adhesion, spreading, migration, morphology, and cytoskeletal organization (Singh et al., 2010).

1.6.2 Integrins: Cell-Matrix Adhesion Receptors

The integrin family members play a prominent role in cell adhesion, spreading and migration by adhering to underlying substratum and providing cells the traction for their movements (Huttenlocher et al., 1995). In addition to adhering to the ECMs; such as fibronectin, collagen, vitronectin and laminin, integrins also connect to the extracellular contacts with the cytoskeletal elements inside the cell. While integrins bind to a variety of ECMs via its extracellular domain, it also interacts with many proteins via its much shorter cytoplasmic domains. Proteins that interact with the C-terminal of integrins are crucial for the regulation of integrin activation, linkage to the cytoskeleton, and signalling pathways that are engaged upon adhesion of cells with the-matrix (Campbell and Humphries, 2011; Kim et al., 2011). Integrins are type I trans-membrane proteins and exist as obligate heterodimers consisting of an α and a β subunits. In mammals there are 18 α subunits and 8 β subunits that potentially assemble in 24 different combinations. The heterodimers have selective expression in different tissues (Hynes, 2002). In addition to tissue distribution, each heterodimer selectively recognize different ligands (Table 1-3) (Humphries et al., 2006). Evidence from phenotypes exhibited by knockout mice suggest that integrins have specific and non-redundant function (Hynes, 2002).

Integrin dimers can be dissociated by ionic detergents, which imply that the subunits associate by non-covalent interactions. Determination of the full x-ray structure of integrins has proven challenging due to their size and other difficulties associated with membrane proteins. With much effort, however, the crystal structures of the ectodomains of αvβ3 (Xiong et al.,
2001), αIIbβ3 (Zhu et al., 2008), αxβ2 (Xie et al., 2010) and recently α5β1 (Nagae et al., 2012) have been solved. All of the structures show an overall bent conformation. Co-crystallization of an RGD peptide (derived from fibronectin) with αvβ3 suggests that the ligand makes extensive contact with the major interface between the αv and β3 subunits (Xiong et al., 2002). Furthermore, the study finds that ligand binding elicits an extensive conformational switch in both the tertiary and quaternary structures of the integrin subunits (Xiong et al., 2002). Electron microscopy and biochemical experiments also indicate conformational change (Xiong et al., 2003; Zhu et al., 2008). Use of clever biochemical techniques, where the head-tail interface of α5β1 integrins ectodomain is clamped together by engineered disulfide bonds, showed that the receptor is incapable of binding to fibronectin in this conformation. However, release of this clamp allows

Table 1-3. Different combination of integrins in humans and their ligand specificity

<table>
<thead>
<tr>
<th>Integrin</th>
<th>Prototypic ligand/recognition sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1β1 (VLA-1)</td>
<td>Collagens (collagen IV &gt; collagen I (GFOGER); collagen IX)</td>
</tr>
<tr>
<td>α2β1 (VLA-2)</td>
<td>Collagens (collagen I &gt; collagen IV (GFOGER); collagen IX</td>
</tr>
<tr>
<td>α3β1 (VLA-3)</td>
<td>Laminins</td>
</tr>
<tr>
<td>α4β1 (VLA-4)†</td>
<td>Fibronectin; vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>α5β1 (VLA-5)</td>
<td>Fibronectin (RGD)</td>
</tr>
<tr>
<td>α6β1 (VLA-6)</td>
<td>Laminins</td>
</tr>
<tr>
<td>α7β1</td>
<td>Laminins</td>
</tr>
<tr>
<td>α8β1</td>
<td>Fibronectin, Vitronection, Nerphronectin (RGD)</td>
</tr>
<tr>
<td>α9β1†</td>
<td>Tenascin, vascular endothelial growth factors</td>
</tr>
<tr>
<td>α10β1</td>
<td>Collagens (collagen IV &gt; collagen VI &gt; collagen II (GFOGER); collagen IX</td>
</tr>
<tr>
<td>α11β1</td>
<td>Collagens (collagen I &gt; collagen IV (GFOGER); collagen IX</td>
</tr>
<tr>
<td>αLβ2†</td>
<td>Intracellular adhesion molecule-1, -2, -3, -5</td>
</tr>
<tr>
<td>αMβ2†</td>
<td>IC3b, fibrinogen + more</td>
</tr>
<tr>
<td>αXβ2†</td>
<td>IC3b, fibrinogen + more</td>
</tr>
<tr>
<td>αDβ2†</td>
<td>Intracellular adhesion molecule-3, vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>αIIbβ3</td>
<td>Fibrinogen, fibronectin (RGD)</td>
</tr>
<tr>
<td>αδβ4</td>
<td>Laminins</td>
</tr>
<tr>
<td>αvβ1</td>
<td>Fibronectin, vitronection (RGD)</td>
</tr>
<tr>
<td>αvβ3</td>
<td>Vitronection, fibronectin, fibrinogen (RGD)</td>
</tr>
<tr>
<td>αvβ5</td>
<td>Vitronection (RGD)</td>
</tr>
<tr>
<td>αvβ6</td>
<td>Fibrinectin, TGFβ-latency associated peptide (RGD)</td>
</tr>
<tr>
<td>αvβ8</td>
<td>Vitronection, TGFβ-latency associated peptide (RGD)</td>
</tr>
<tr>
<td>αEβ8†</td>
<td>E-cadherin</td>
</tr>
<tr>
<td>α4β7†</td>
<td>Fibronectin, vascular cell adhesion molecule-1</td>
</tr>
</tbody>
</table>

†Leukocyte specific receptors
Adapted from (Barczyk et al., 2010)
separation of the leg, as well as a conformational conversion from bent to straight allowing ligand binding to the head (Askari et al., 2010; Takagi et al., 2001). Similar observations were also made with αvβ3 and αIIbβ3 integrins suggesting that the highly bent conformation has low affinity for the ECM, but the addition of high affinity ligand peptide mimetic or Mn$^{2+}$ results in an open conformation of the receptors (Takagi et al., 2002). Furthermore a structural study of the region encompassing the β I-like domain (which is the key regulatory extracellular domain of β subunit) and hybrid region (which connects the β I-like domain to the leg region) of β3-integrin explicitly showed that transition from closed to open conformation happens due to motion of the α7-helix within the β I-like-domain, which causes the hybrid region to swing-out by 62° (Xiao et al., 2004). Now the conformational switch model is well accepted and considered responsible for the unmasking of epitopes that are recognized by activation specific β1-integrin antibodies such as 9EG7, HUTS4 and 12G10 (Askari et al., 2010; Bazzoni et al., 1995; Humphries et al., 2005; Mould et al., 2005). Figure 1-7 highlights the different regions of integrin subunits and where the monoclonal antibodies bind to. These antibodies have been extensively used with different cell lines (Martel et al., 2000; Pellinen et al., 2012; Wu et al., 2004) including the ones described in this thesis; fibrosarcoma cell-line HT1080 and breast cancer cell line MDA-MB-231, to monitor integrin activation (Wei et al., 2005).

Integrin α subunits that bind to RGD sequences present in fibronectin and laminin lack the extra α-I-domain that is inserted between the second and the third blades of the N-terminal 7-bladed β-propeller, leaving just the β-propeller domain sitting on the top (Pierschbacher and Ruoslahti, 1984). During ligand binding, the carboxylate side-chain of the aspartate residue of RGD binds to the β I-like domain, while the arginine side chain directly interacts with β-propeller (Xiong et al., 2002). Binding of ligands to the β-propeller and β I-like domain are
Figure 1-7. Schematic of α5β1 Integrin activation. Integrins in their bent conformation are inactive. When activated integrins take an upright conformation and legs separated, which allows for ligand (fibronectin) binding to the head. Once active conformation is stabilized integrins mediate downstream cellular signalling such as focal adhesion and stress fiber formations, activation of Rho GTPases, gene transcription etc. Integrins can transduce bidirectional signaling. The different regions within α and β subunits are highlighted. The conformation specific antibodies towards active β1-integrins bind to the regions shown once the epitopes are exposed upon receptor activation. α-9EG7 antibody binds to the I-EGF region at position D522 in β1 integrin, which is crucial for the antibody’s recognition.
dependent on divalent cations like Mg$^{2+}$, Ca$^{2+}$ and Mn$^{2+}$ (Gailit and Ruoslahti, 1988). The divalent cations themselves have been shown to cause marked conformational changes to integrins resulting in a shift from inactive to active state (Bazzoni et al., 1995; Mould et al., 2005). While Mn$^{2+}$ supports conformational change the strongest and Mg$^{2+}$ to a lesser extent; Ca$^{2+}$ on the contrary has inhibitory effects (Gailit and Ruoslahti, 1988). The conformation change caused by Mn$^{2+}$ has been also captured by crystallography and shows drastic movement of the $\alpha 7$ helix (Lee et al., 1995).

1.6.2.1 Bidirectional Signalling of Integrins

The overall cellular adhesiveness is governed by the affinity of integrin for their ligands. In order to maintain an optimal level of adhesion to their surrounding, cells regulate integrins’ affinity for their ligands. A good example is the case of continuously flowing leukocytes and platelets in blood. Integrins, if constitutively active would trigger spontaneous binding of cells to their surrounding vascular endothelial walls or aggregate to each other leading to unwanted consequences, such as thrombosis or autoimmunity (McMurray, 1996; Ugarova and Yakubenko, 2001). Yet, cells in blood flow freely and only respond locally at sites of infection or wound as a result of extracellular signalling cues that cause integrin activation. In non-hematopoietic cells, the balance of integrin activation is also crucial and is dynamically regulated in order to enable cell migration during wound healing, development and to allow proper cell divisions (Bokel and Brown, 2002; Margadant and Sonnenberg, 2010; Midwood et al., 2006; Reverte et al., 2006). Aberrant expression and activation of integrins have been linked to diseases including angiogenesis and cancers (Desgroisellier and Cheresh, 2010; Stupack and Cheresh, 2004).

In cells integrins relay signalling bi-directionally. Intracellular signals elicited by other extracellular cues such as chemoattractants, growth factors or cytokines can lead to recruitment
of components like talin to the cytoplasmic tail of integrins. This leads to conformational switch on the outside; therefore, conferring high affinity ligand binding state. This relay of intracellular signal to the outside of cells to regulate adhesive capacity of integrins is known as “inside-out signalling” (Abram and Lowell, 2009; Hynes, 2002; Kim et al., 2011). Activated integrins can then cluster by homotypic oligomerization of transmembrane domains (Abram and Lowell, 2009; Li et al., 2003b). Ligand occupancy and receptor clustering (valency) initiates signalling events that links the extracellular matrix to the cytoskeletal system of the cells leading to reinforced cell adhesion (avidity). This transmission of signals from the outside to the inside of cells is known as “outside-in signalling” (Abram and Lowell, 2009; Hynes, 2002; Kim et al., 2011).

1.6.2.1.1 Inside-out signalling

Studies where the cytoplasmic domains of integrins were mutated indicated that this region is crucial for their activity. Point mutations in α or β subunit cytoplasmic tails or deletion of the region from either subunits resulted in constitutive activation of integrins (Hughes et al., 1995; O'Toole et al., 1994; O'Toole et al., 1991). The α and β subunits when in the inactive state rest in close association with each other (Haas and Plow, 1996). This interaction between the two subunits is disrupted when the cytoskeletal protein talin’s head domain binds to the cytoplasmic tail of the β subunit causing integrin activation (Calderwood et al., 1999; Kim et al., 2003; Tadokoro et al., 2003; Vinogradova et al., 2002). The head region of talin contains a FERM domain and interacts with the membrane-proximal NPXY/F motif found in the cytoplasmic tails of β1, β2 and β3 integrins (Garcia-Alvarez et al., 2003). Further confirmation of a pivotal role of talin in integrin inside-out signalling came from in vivo studies using knock-in mice expressing mutant β3 integrins deficient for talin binding (Petrich et al., 2007). The authors of this study demonstrated that platelets from animals homozygous for this mutation showed decreased agonist induced fibrinogen binding, platelet aggregation and integrin αIIbβ3 activation.
The small GTPase Rap1 has emerged as an important signalling molecule regulating integrin’s inside-out signalling functions (Bos, 2005; Bos et al., 2003). Of the several Rap1 effectors that have been shown to regulate integrin signalling, RapL and RIAM thus far have been described to modulate integrins directly (Raaijmakers and Bos, 2009). In leukocytes and platelets, RIAM upon associating with Rap1 activates and recruits talin to integrins (Han et al., 2006; Lafuente and Boussiotis, 2006; Lafuente et al., 2004; Watanabe et al., 2008). On the other hand, in lymphocytes, RAPL associates with the α-subunit tail of αLβ2 (LFA-1; a family of leukocyte integrins) and causes destabilization of the interface between α and β subunits tail leading to integrin activation (Katagiri et al., 2003). The role of Rap1 in cell-matrix adhesion will be discussed in details in section 1.6.4.

The kindlin family proteins are another important protein family involved in integrin inside-out signalling. Kindlins have FERM domains and can bind to the cytoplasmic tail of β-subunits of integrin and affect their inside-out activation. Kindlins interact with the membrane distal NXXY motif (distinct from talin interaction site) of β1 and β3 integrins. However, unlike talin, kindlin alone is incapable of activating the receptor (Harburger et al., 2009; Ma et al., 2008). Instead kindlin acts as a coactivator of integrins together with talin and coexpression of the FERM domains of talin and kindlin-2 have synergistic effect on integrin activation (Montanez et al., 2008).

Several proteins such as filamin, Dok1 and ICAP1 can inhibit integrin activation by competing with talin for binding to the β-subunit tail and thus regulate integrin activation (Anthis et al., 2009; Kiema et al., 2006; Millon-Fremillon et al., 2008). Recently, a new member, sharpin, was added to this list and was described as an inhibitor of integrin activation. Sharpin behaves
similar to filamin whereby it binds to the α-subunit c-tail and inhibits the recruitment of talin and kindlin to integrins (Rantala et al., 2011).

1.6.2.1.2 Outside-in signalling

Ligand occupancy and clustering of integrins evoke outside-in signalling resulting in various intracellular signalling cascades. During adhesion, for cells to hold on to the extracellular matrix firmly they need to generate tension stress. This is achieved by anchorage of integrin cytoplasmic tail to the cytoskeletal elements (Parsons et al., 2010). Once integrins are activated, they cluster with other integrin molecules and assemble with highly organized intracellular protein complexes to form structures known as focal adhesions (see section 1.6.3.2) (Amano et al., 1997; Askari et al., 2010; Geiger et al., 2009). These structures contain cytoskeletal proteins (e.g. α-actinin, paxillin, vinculin, zyxin etc.) as well as an extensive array of signalling molecules such as focal adhesion kinase (FAK) and Src, which can trigger intracellular signals affecting cell shape, movement, differentiation as well as survival (Aplin and Juliano, 1999; Gardel et al., 2010; Parsons et al., 2010; Vachon, 2011). The extent of adhesion signals conveyed from given contact sites subsequently determine various aspects of actin polymerization and stress fiber formations, which in turn dictates adhesion strength and migration capacity of cells. The cytoskeleton and components of focal adhesion will be further discussed in section 1.6.3.

1.6.2.2 Role of Integrins in Cell Migration

Integrins play essential roles during cell migration in many biological contexts such as, leukocyte trafficking, immune surveillance, tissue wound repair and embryonic morphogenesis. Alterations in cellular adhesion and migration caused by integrin deregulations have been associated with several pathologies including cancer metastasis (Danen et al., 2005; Mierke et al., 2011). For efficient cell migration, dynamic and polarized assembly as well as disassembly of
integrin-mediated adhesion is of paramount importance to give cells directionality and speed (Kaverina et al., 2002b; Small et al., 2002). Although adhesion is a pre-requisite for mesenchymal cell movements there is a biphasic response in migratory speed with respect to the strength of cell-matrix adhesion. At low levels of adhesions the weakly attached cells cannot generate sufficient traction to move forward; high adhesiveness on the other hand causes cells to stall by making them incapable of breaking away from the matrix contact. Therefore to achieve maximal speed during migration it is crucial that cells maintain intermediate levels of cell-matrix adhesion and ensure efficient attachment and release from the substratum (DiMilla et al., 1993). This is highlighted by observations that optimal migratory response is achieved upon adequate expression of α5β1 and α2β1 integrins together with intermediary levels of ECM density and compliance (stiffness/softness) (Duband et al., 1991; Lo et al., 2000; Palecek et al., 1997). Similarly, there is an inverse correlation of integrin-mediated adhesions size and organization with cell migration speed (Wu et al., 2008). Hence, balanced regulation of factors such as integrin concentration and affinity, their association with the cytoskeleton are all cardinal determinants to favor effective cell migration (Huttenlocher et al., 1996; Palecek et al., 1997; Wolf et al., 2003).

One of the key functions of integrin-mediated adhesion during migration is generation of traction by connecting the extracellular matrix with the actomyosin filaments (see section 1.6.3) (Gardel et al., 2010; Gupton and Waterman-Storer, 2006; Wolfenson et al., 2011). Currently, the “molecular clutch” model proposes a model by which integrins mediate traction forces. According to this model, ‘the rate of cell protrusion is inversely correlated with the rate at which F-actin networks move away from the leading edge towards the center of the cell’; known as “retrograde flow” (Jurado et al., 2005; Mallavarapu and Mitchison, 1999; Ponti et al., 2004). Formation of integrin-mediated adhesions acts as a mechanical clutch, which impinges on F-actin retrograde flow speed. Thus newly polymerized actins are constrained to the cell periphery and contribute
to increased protrusive activities and generate traction forces on the ECM to drive the cell forward (Alexandrova et al., 2008; Gardel et al., 2008). Integral to the ability of integrins to link ECM with the cytoskeletal elements, integrin-mediated adhesion structures; i.e., focal adhesions (see section 1.6.3.2), function as signalling centers where they coordinate a highly complex network of proteins and signalling pathways that mediate cell migration. Signalling by these adhesion sites is highly regulated both spatially and temporally, enabling cell polarization and directed migration (Huttenlocher and Horwitz, 2011).

1.6.2.3 Integrins and Cancer Progression

In cancers, integrins are best known for their role in cell migration and tissue invasion during the complex process of metastasis in response to growth factors, chemokines and cytokines (Goodman and Picard, 2012; Guo and Giancotti, 2004; Hood and Cheresh, 2002). Even though integrins are not considered as bona fide oncogenes or tumour suppressors their expression levels have been demonstrated to be altered during transformation (Pignatelli et al., 1992; Plantefaber and Hynes, 1989). Indeed, numerous integrins have been reported to be upregulated in several classes of tumours including that of breast carcinoma, melanomas, prostate, pancreatic and lung cancers and their nearby vasculatures, whereas some other integrins are downregulated (Desgrosellier and Cheresh, 2010). The overexpressed levels of α5β1, α6β4, αvβ3, αvβ5 in tumour cells is correlated with metastatic progression in breast carcinomas, melanomas, prostate, and lung cancers (Feldman et al., 1991; Friedrichs et al., 1995; McCabe et al., 2007; Mierke et al., 2011; Nip et al., 1992; Shah et al., 2012). In addition to their role in cell motility, integrin binding to the extracellular matrix also activates pro-survival signals to prevent induction of apoptosis. As mentioned earlier, induction of apoptosis in cells that fail to properly interact with the ECM is a normal cellular program to prevent aberrant cell growth and migration (Matter and Ruoslahti, 2001; Schwartz, 1997). However, malignant tumour cells have the ability to survive
in these conditions. The upregulation of certain ligation-independent integrins and their association with downstream pro-survival signalling pathways involving FAK, PI3K, Ras, Rac, Erk pathways are thought to mediate this anchorage-independent cell growth (Cho and Klemke, 2000; Desgrosellier et al., 2009; Frisch et al., 1996; Guo and Giancotti, 2004; Khwaja et al., 1997).

Even though integrins inherently do not have the capacity to transform cells directly, in concert with oncogenes and tyrosine kinases they can enhance tumourigenesis (Desgrosellier et al., 2009; Huveneers et al., 2007; White et al., 2004). In a transgenic breast cancer mouse model expressing the polyoma middle T (PyVmT) oncoprotein it was shown that deletion of β1 integrin severely impairs tumourigenesis suggesting that this integrin subtype is critical for PyVmT mediated cell transformation and tumour initiation (White et al., 2004). In another study, β3 integrin was reported to synergize with the mutated Src oncogene and enhance tumourigenesis (Huveneers et al., 2007). β3 subunit containing integrins have been also demonstrated to associate with Src and trigger EMT of mammary epithelial cells (Galliher and Schiemann, 2006).

During metastasis, tumour cells have to circumvent the ECM barrier and invade through the surrounding environment (Valastyan and Weinberg, 2011). Cells overcome this in part by virtue of integrin’s ability to interact and remodel the ECM. Alongside the dynamic hold and release wave at the front and rear of the cell described above, during invasion integrins can also interact and enhance the activation of matrix metalloproteinases (MMPs); enriched within specialized structures known as invadopodia (Jia et al., 2004; Weaver, 2006; Yilmaz and Christofori, 2009). Activation of MMPs allow degradation and remodeling of the ECM thus making path for the tumour cells to move forward (Overall and Lopez-Otin, 2002). Association of integrins with urokinase plasminogen activator receptor (uPAR) is yet another mechanism by
which they can modulate ECM degradation to facilitate tumour invasion (Smith and Marshall, 2010). The importance of integrins in cancer progression was convincingly demonstrated in a recent paper where the authors developed β1 integrin knockout transgenic mice expressing activated erbB2 under the control of mouse mammary tumour virus (Huck et al., 2010). In their model, β1 integrin-deficient mice manifested delayed onset and decreased tumour burden compared to control erbB2 mice. Moreover, when tumour cells from β1 integrin null mice were injected into recipient immunodeficient mice they exhibited dramatically reduced ability to metastasize compared to controls, suggesting that β1 integrins play critical roles in tumour progression. Although several integrins including the fibronectin receptor α5β1 are upregulated in many cancers, among them breast cancers is one (Mierke et al., 2011), the mechanism by which their activation is regulated to prevent hyperactivation is not yet well understood. As noted earlier, intermediate levels of adhesion to the substratum is essential to permit optimal movement of cells. In spite of this requirement, the fact that integrins expression levels are retained or even increased during oncogenesis suggests that alternative regulatory mechanisms are in place to fine tune adhesion dynamics to benefit cancer progression. Some of the mechanics such as focal adhesion assembly, their turnover and factors regulating them are currently hot topics of investigations in cancer research (Gardel et al., 2010; Kaverina et al., 2002b). As discussed later and important for this thesis, the small G protein Rap1 and its effectors are emerging as important regulators of integrin-mediated processes by controlling the avidity of integrins for their substrates, a process already described earlier, inside-out integrin signalling (Boettner and Van Aelst, 2009; Bos, 2005; Kim et al., 2011; Zhang and Wang, 2012).

Activation and ligation of integrins to ECM, and subsequent generation of traction force are translated into forces applied to the actin cytoskeleton. The following section of this chapter will highlight the close interactions between integrin-mediated adhesions and the cytoskeleton.
1.6.3 Interplay of Adhesion and Cytoskeletal Dynamics Governs Cell Motility

Cell migration is initiated by extension of actin-based protrusions at the leading edge. These protrusions called pseudopods are comprised of broad, flat lamellipodia and/or finger-like structures such as filopodia that are driven by polymerization of monomeric actins into structured filaments (Figure 1-8a) (Parsons et al., 2010). Protrusions are stabilized by adhesions that link actin cytoskeleton to the ECM via integrin engagements. Involvement of myosin II with the adhesion and actin complexes then generates contractions causing traction stress on the matrix, and detachment at the rear of the cell thus propelling the cell forward (Ridley et al., 2003). The relationship between adhesion and the cytoskeleton is bidirectional. Newly formed as well as more stable adhesion sites influence cytoskeletal organizations while cytoskeletal structures reciprocally influence adhesion formation and disassembly (Parsons et al., 2010). It should also be noted that during migration the formation of adhesion sites, their maturation as well as their disassembly is a continuous process with no apparent stable intermediates or endpoint (Huttenlocher and Horwitz, 2011; Webb et al., 2002). The sort of single-cell movements described above is often exhibited by mesenchymal cells and therefore referred to as mesenchymal cell migration. The relationship between the cytoskeleton and integrin-mediated adhesion structures that together impact cell adhesion and migration is described below.

1.6.3.1 Assembly and Dynamics of Actin Filaments at Cell Protrusions

As mentioned above, cells extend several protrusions during their movements to gain traction or sense directional cues. Although they are related, each protrusive structure has distinct actin filament organizations. The first two types of protrusive structures evident during cell spreading and/or crawling are lamellipodia and lamella (Figure 1-8a). They are thin sheets of membrane-enclosed cytoplasm that form near the bottom edges of cells (Chhabra and Higgs, 2007). The lamellipodium (100-160nm thick) starts at the distal leading edge and spreads sever-
al micrometers back (Abercrombie et al., 1971). Lamellum (> 200nm in thickness) on the other hand follows behind and extends up to the cell body (Abercrombie et al., 1971). Lamellipodia are weakly adherent and dynamically cycle newly formed adhesion sites. Strong adhesion begins at the boundary between lamellipodia and lamella (Bailly et al., 1998; Gupton and Waterman-Storer, 2006).

In two elegant studies using fish and *Xenopus laevis* keratocytes and fibroblasts, Svitkina and colleagues demonstrated that dendritically branched actin filaments dominate the leading edge of lamellipodia (Svitkina and Borisy, 1999; Svitkina et al., 1997). The Y-junctions of the dendritic networks co-localize with Arp2/3 complex suggesting its role in the nucleation of dendritically branched filaments (Svitkina and Borisy, 1999). These studies were undertaken by complementary usage of fluorescence imaging and electron microscopy. The observations of Svitkina and colleagues were taken a step further by a seminal paper showing that two distinct populations of filaments exist at the leading edge (Ponti et al., 2004). Using fluorescence speckle microscopy (Waterman-Storer and Danuser, 2002), where speckle movements of cellular structures can be observed due to low level incorporation of fluorescently tagged proteins, it was shown that actin filaments in epithelial cells have two distinct speckle populations at the protrusions. The first population, lamellipodial network, exhibits rapid retrograde flow that extends from the leading edge plasma membrane to about 1-3μm back and disassembled abruptly at the lamellipodia-lamella transition region. The second population, lamellar speckles, is characterized by slow retrograde flow of actin that increases in frequency with distance away from the leading edge continuing into the lamellum (Figure 1-8b). Taken together these studies suggest that the two populations of actin nucleate and disassemble independently at the protrusions.
Figure 1-8. The actin cytoskeletal system of migrating cells. (A) Cells frequently send out various protrusions (lamellodia and filopodia) at the leading edge. Nascent adhesions form at the lamellipodium and these adhesion frequencies are coupled to the rate of protrusions. Nascent adhesions either disassemble within the lamellipodium of mature further to become stable adhesions at the lamellipodia-lamella transition zone. As adhesions mature they become smaller focal complexes or larger focal adhesions. These adhesions are linked to bundled actin-filaments that cross-bridge two focal adhesions. Actomyosin contractions further stabilize adhesions and increase their size. (B) (Left) Shown is schematic of the top view of lamellipodia and lamella where the blue branches at the front denote dendritically branched actin filaments in the lamellipodia. These structures assemble at the leading edge and disassemble within 1-3 µm from the edge at the lamellipodia-lamella transition. The red lamellar actin filaments are present throughout the lamellipodia and lamella but becomes more prominent as it moves away from the lamellipodia and their disassembly can happen anywhere in this region. (Right) Shown is the side view of lamellipodia and lamella. Initially the lamellipodial filaments lie above the lamellar actin filaments at the leading edge. Lamellipodium maintains weak adhesion to the matrix substrate as shown in the upper panel. Myosin II contraction stabilizes the adhesion and due to the retrograde flow of the lamellipodial actin filaments brings mature adhesions at the lamellipodium-lamellum transition zone. At the transition zone, depending of the cytoskeletal engagement and stress exerted on them, the adhesion will either remain as focal complexes and disassemble quickly or mature to become focal adhesions and last longer. If the weak nascent adhesions at the distal end of lamellipodium are broken the membrane sheet moves rearward as a peripheral ruffle.
Another related structure at the plasma membrane is “ruffles”, which are also sheet-like membrane protrusions but do not adhere to the substratum. Peripheral ruffles assemble at the leading edge, and move rearward (Abercrombie et al., 1970). Ruffles are created at the periphery of many cell types and are potentiated upon chemotactic responses. These structures, like lamellipodia, are enriched with meshwork of newly synthesized dendritically branched filaments and integrally linked to cell motility and spreading (Figure 1-8) (Ridley, 1994; Svitkina, 2007; Wurtzel et al., 2012).

Filopodia unlike lamellipodia are finger-like protrusions and contain bundles of long parallel filaments that are thought to function as directional sensors (Ridley, 2011; Zheng et al., 1996). One proposed mechanism of filopodia assembly is reorganization of a subset of lamellipodial actin filaments nucleated by Arp2/3 complex through the binding of proteins such as fascin, and VASP (Gupton and Gertler, 2007). This model is however conflicted by reported observations that filopodia generation is intact in Arp2/3 depleted cells (Steffen et al., 2006).

Visualization of cytoskeletal organization at the protrusions was instrumental in understanding how actin-based cellular structures mediate cell movements. The current model suggests that the weakly adherent lamellipodia, with loose actin networks, propel forward with ease to find new substrate attachment points. Upon ligation of lamellipodial integrins to the interstitial matrix, some go on to stabilize at the lamellipodia-lamella boundary and conjugate to the actin cytoskeleton (Gardel et al., 2008; Gardel et al., 2010; Parsons et al., 2010). By virtue of actin-binding properties, myosin-II couples to large bundles of polymerized acts called stress fibers - that extend from near the front to the sides, center or rear of the cell (Figure 1-8a) (Vicente-Manzanares et al., 2009). The myosin-II based contractile complexes are assembled at sites of stable adhesions behind the lamellipodia (Giannone et al., 2007). The tension generated
upon contraction of actin filaments mediated by myosin-II is translated to traction force on the ECM to move the cell forward (Aratyn-Schaus and Gardel, 2010; Gardel et al., 2008; Oakes et al., 2012). Actomyosin contraction is also responsible for the rearward pull of lamellipodial actin network, edge retraction (peripheral ruffles) and initiation of new adhesion sites (Giannone et al., 2007). A schematic of the organization of actin cytoskeleton with respect to cellular protrusions and adhesion sites and the way they function is further illustrated and explained in Figure 1-8.

1.6.3.2 Assembly of Adhesion Structures

So far I have described the adhesion sites as a bridge between the ECM and the actin cytoskeleton linked by integrins. By linking to the ECM these adhesion sites also exert cell shape changes such as induction of spreading, and provide traction stress to confer cell movements. Integrin-dependent ECM attachment and cytoskeletal coupling is mediated by highly complex structures and processes. In the last 40 years, since their initial visualization, the apparent molecular complexity of integrin-mediated adhesion has increased and is now deemed to be regulated by an array of over 150 protein-protein interactions (Abercrombie et al., 1971; Zaidel-Bar and Geiger, 2010; Zaidel-Bar et al., 2007). Depending on cell types and the environment they are in, adhesion structures can greatly vary in size, location, and morphologies. Generally, adhesion sites are referred to as focal adhesions; however, to be precise they can be further divided into different subclasses: nascent adhesions, focal complexes, focal adhesions and fibrillar adhesions. While nascent adhesions are very small and the earliest sites of adhesion contacts, fibrillar adhesions are large and very stable form of adhesion usually absent in migrating cells (Geiger and Yamada, 2011). The sizes, organization and strength of focal adhesion typically have an inverse relationship with the migration speed and rapidly motile cells tend to have more nascent adhesion and focal complexes while maintaining fewer mature focal adhesions (Beningo et al., 2001;
Lauffenburger and Horwitz, 1996; Palecek et al., 1997). Each of these adhesion types is further defined in the subsequent paragraphs.

Nascent adhesions are early adhesion structures that form in the distal leading edge of lamellipodia. These are small structures in size (≤0.25μm), and below the resolution to be observed with regular light microscopes. However, they can be optimally visualized using total internal reflection fluorescence (TIRF) microscopy (Choi et al., 2008; Gardel et al., 2010). In TIRF microscopy, fluorescently labeled proteins that are localized at the plasma membrane are excited by evanescence waves created at the interface of glass and aqueous media upon total internal reflection of incidence light waves. Nascent adhesion sites are usually short lived (~1 min) and undergo rapid turnover as they move towards the lamellipodia-lamella boundary unless they are captured and stimulated by myosin-II complex (Alexandrova et al., 2008; Choi et al., 2008). Once connected to the myosin-II complex these adhesions are linked to bundled actin-filaments, which cross bridge two adhesion sites, and the pulling force allow these adhesions to mature into larger focal adhesions (Alexandrova et al., 2008; Choi et al., 2008). It is currently thought that nascent adhesion sites initiate upon integrin clustering that is induced by their binding to the ECM (Parsons et al., 2010). Development of these initial adhesion sites leads to recruitment of several proteins on their clustered cytoplasmic tails (Figure 1-9). Interaction of integrin c-tails with the actin bundling protein α-actinin (Choi et al., 2008), talin and another actin crosslinking protein, vinculin (Ziegler et al., 2006), is thought to be responsible for the link between ECM bound integrins and the actin cytoskeleton (Chen et al., 2006). There is also evidence that vinculin and focal adhesion kinase (FAK) can directly bind to the actin nucleating Arp2/3 complex prior to adhesion formation and can supposedly, to some extent, have pre-arranged integrin adhesion complexes prior to integrin-ECM attachment (Alexandrova et al., 2008; Choi et al., 2008; DeMali et al., 2002; Serrels et al., 2007). The exact sequence of events
Figure 1-9. Formation of adhesions. Integrin molecules present at the lamellipodia are activated by binding of talin to its c-terminal tail and initiates nucleation of initial adhesion to ECM. Subsequent recruitment of other adhesion protein complexes and cytoskeleton adaptor proteins such as vinculin, α-actinin etc., tether nascent adhesions to the actin cytoskeleton. Tension mediated on adhesions by the cytoskeleton promotes their maturation.
leading to nascent adhesion activation is still debateable and currently an active field of investigation.

Focal complexes are slightly larger (~0.5-1μm) in size compared to nascent adhesion. They lie at the lamellipodia-lamella interface and are likely stabilized form of nascent adhesions (Gardel et al., 2010). Unlike nascent adhesions, focal complexes are stable for several minutes. As cell spreading or migration progress, focal complexes either disassemble or elongate centripetally to mature into larger focal adhesions, which are typically between 1-5μm in size. Focal adhesions were first observed using interference reflection microscopy (IRM), a technique that applies the properties of polarized light reflection to visualize objects that are at the interface between two different refractive indices (Curtis, 1964; Verschueren, 1985). In IRM, part of the polarized light is reflected on the glass surface, which to a certain extent is reflected by the cell membrane. If the cell membrane is close to the glass (adherent regions) the two reflected light waves are out of phase and undergo interference resulting in dark spots on the final image. However, if the membrane is further away the light waves will have varying phases causing less interference and thus results in a brighter image. Based on this principle, adhesion sites at the cell membrane were observed as dark spots. These observations were later confirmed by electron microscopy as electron dense plaques associated with actin filaments or stress fibers (Abercrombie et al., 1971; Heath and Dunn, 1978).

Focal adhesions accumulate a large number of proteins in an organized interaction network. Although many components have now been identified the exact sequence in which they assemble, and their inter-relationship at modulating adhesion signals still remains to be fully defined. This proves challenging since focal adhesions assembly and disassembly are highly dynamic processes. Focal adhesions function both as structural, ECM to stress fibers anchors and
signalling centers. Their maturation and elongation differs from that of nascent adhesions by way of protein complex compositions. Soon after integrin engagement with the ECM, the adapter protein paxillin is recruited to integrin clusters via some yet unknown mechanisms (Pasapera et al., 2010). Activation of β1 integrin and its binding to fibronectin also recruits, phosphorylates and activates a non receptor tyrosine kinase “focal adhesion kinase” (FAK) (Burridge et al., 1992; Friedland et al., 2009; Parsons, 2003; Schaller et al., 1992; Shi and Boettiger, 2003). Activation of FAK subsequently phosphorylates paxillin at residues Y31 and Y118, which in turn recruits the cytoskeletal adaptor proteins vinculin and zyxin to focal adhesions to direct their maturation (Ballestrem et al., 2006; Bellis et al., 1997; Burridge et al., 1992; Choi et al., 2008).

A visual understanding of the molecular architecture of focal adhesions was recently made possible using super-resolution microscope techniques (Kanchanawong et al., 2010). In this study the authors employed interferometric photoactivated localization microscope, iPALM (Shtengel et al., 2009), to determine the spatial localization of photoactivateable fluorescent protein tagged focal adhesion proteins with respect to the surface membrane as well as each other, with an axial resolution of 10-15nm (Kanchanawong et al., 2010). In doing so they revealed that integrins and actin cytoskeleton are vertically separated by ~ 40nm, which is connected by focal adhesion core proteins. Remarkably, these protein complexes were organized in rather stratified layers instead of all mixed together. The “membrane-apposed integrin layer” contained integrin cytoplasmic tails, FAK and paxillin whereas vinculin was stacked just above in a region defined as “intermediate force transduction layer”. The “upper most layer”, which is closest to the actin cytoskeleton, was resided by zyxin, VASP and α-actinin. Interestingly, talin was observed to have polarized orientation with its N-terminus (containing the head domain) close to the plasma membrane and its C-terminus extending into the actin-regulatory layer. Having a picture of the spatial residence of the different components of focal adhesion proteins provided a blueprint for
understanding how focal adhesion functions (Kanchanawong et al., 2010). However, since these experiments were done using fixed cells, the organizational dynamics of focal adhesion remained unknown. In a very recent paper, Rossier and colleagues used super-resolution microscopy to investigate the dynamics of fibronectin receptors α5β1 and αvβ3 in live cells (Rossier et al., 2012). They showed that integrins are not constantly active in focal adhesion, but rather undergo dynamic cycle of binding or unbinding to both ECM and actin thereby facilitating tuneable adhesion to its surrounding. Using single protein tracking and super-resolution microscopy, they showed that integrin in the plasma membrane freely diffuse in its inactive state, whereas integrin activation immobilizes it within focal adhesions. Inhibition of integrin-fibronectin interaction or integrin-talin interaction reduced receptor immobilization, showing that binding to these extracellular and intracellular substrates to integrins are necessary for their immobilization. Strikingly however, talin did not show similar free diffusion with integrins at the plasma membrane; something one would expect if the hypothesis that talin is required for integrin activation and its recruitment to focal adhesion is accurate. Instead, talin is recruited to focal adhesion only after integrin immobilization, suggesting talin binds after integrin activation. Nevertheless, interaction of integrins with talin within focal adhesions was important to maintain integrin immobilization and sustained activation.

As mentioned earlier, myosin-II dependent contraction and force generation is thought to be an important step towards focal adhesion development. To understand tension-mediated focal adhesion maturation, Dr. Clare Waterman’s group recently set-out to identify the temporal recruitment of proteins to focal adhesion in a myosin-II dependent manner. Using pharmacological inhibitors like blebbistatin (a myosin-II specific ATPase inhibitor) and Rho kinase inhibitor Y27632, or by varying ECM rigidity to modulate myosin-II mediated cellular tension, they demonstrated that the interaction between phosphorylated paxillin and vinculin is promoted by
myosin II contraction-mediated signalling (Pasapera et al., 2010). Pasapera and colleagues proposed that paxillin in nascent adhesion is inadequately phosphorylated to recruit vinculin, which may be required for adhesion maturation. However, contraction of actomyosin networks increases FAK activity, which in turn amplifies paxillin phosphorylation leading to enhanced vinculin recruitment to adhesions and causing development of focal complexes and focal adhesions. The study also demonstrated that temporal recruitment of β1-integrins, paxillin and talin to adhesions are independent of myosin II activity. However, interaction of the above protein complexes with FAK, vinculin, α-actinin and zyxin requires myosin II-mediated signalling. As such, it is conceivable that the myosin II-independent complex is likely present as early as during nascent adhesion formation but the rest of the components assemble later as adhesions mature.

While the findings presented above, especially that of talin and FAK, are in agreement with contemporary perceptions in the field (Chen et al., 1995; Zhang et al., 2008), somewhat surprising and contradictory evidence has been also coined by a recent study (Lawson et al., 2012). Lawson and colleagues showed that in mouse embryonic fibroblasts (MEFs), SKOV3 carcinoma cells and human umbilical vein endothelial cells (HUVEC), FAK is required for the recruitment of talin to nascent adhesions within the first 60 minutes after plating cell on fibronectin rather than the other way around (Lawson et al., 2012). Likewise, another counterintuitive finding was reported in a different study designed to study the function of talin using talin-1 -/- cells together with siRNA-mediated depletion of talin-2 (Zhang et al., 2008). Contrary to understanding in the field, talin loss-of-function was found to be dispensable for initial cell spreading. However, talin-depleted cells exhibited impaired recruitment of vinculin and paxillin to focal adhesions at the protrusions of fully spread cells, consequently unable to sustain cell spreading, adhesion and the ability to generate traction force. In light of the conflicting evidence one can perhaps deduce that localization of the different components of adhesion proteins are very dy-
namic and the mechanisms directing their assembly are temporally distinct during different steps of adhesion maturation. It appears that while early on FAK is required for talin recruitment to nascent adhesions, as adhesions mature talin persists to maintain or even reenroll FAK to these sites. Careful analysis of the temporal dynamics of FAK activity over the lifetime of adhesions and how it interacts with and phosphorylates other molecules will help better decipher these mechanisms. As is evident from the Rossier et al. and Zhang et al., studies talin may not be absolutely required for initial integrin activation, but is definitely required to maintain its activation.

The significance of FAK-mediated focal adhesion dynamics control has been also described in the context of GPCRs. Stimulation of ET-B receptors with Endothelin-1 can induce activation of the small GTPase of the ADP-riboylation factor family, Arf6 (D'Souza-Schorey et al., 1997; Radhakrishna and Donaldson, 1997). This has been linked to promotion of endothelial cell migration, while depletion of Arf6 impairs this phenotype (Daher et al., 2008). In this study, it was shown that activation of the hormone receptor cause FAK phosphorylation and its association with Src as well as GIT1. GIT1 is an ArfGAP that can also bind to the RhoGEFs α-PIX, and β-PIX and regulate focal adhesions (Turner et al., 1999; Zhao et al., 2000). Furthermore, in the context of Endothelin-1 stimulation, knockdown of Arf6 blocked agonist promoted binding of FAK with GIT1, thereby preventing focal adhesion disassembly and endothelial cell migration (Daher et al., 2008).

The importance of focal adhesions in vivo, especially during development has been also highlighted by experiments conducted using knockout mice models. Mice deficient in paxillin (Hagel et al., 2002), FAK (Ilic et al., 1995) or vinculin (Xu et al., 1998) are embryonically lethal. MEFs derived from mice deficient in paxillin show de-structured, thick focal adhesions with de-
layed cell spreading and migration on fibronectin (Hagel et al., 2002). Vinculin in the same cells; however, was able to properly localize to focal adhesions arguing that paxillin is not absolutely required for vinculin recruitment to focal adhesions. However, it may be that paxillin’s role is more to accelerate, maintain or turnover focal adhesions (Hagel et al., 2002). Contrary to paxillin, MEFs derived from vinculin deficient embryos display smaller focal adhesions and decreased adhesion strength on multitude of ECMs including fibronectin, collagen, vitronectin and laminin (Xu et al., 1998). Yet, unlike paxillin depletion, vinculin knockout cells tend to migrate faster on all of the above ECMs suggesting vinculin acts as a negative regulator of cell motility most possibly attributable to it ability to stabilize adhesions.

Another means of adhesion strengthening is possible by virtue of increased integrin-ECM bond lifetime; a concept known as catch bond behavior (Thomas, 2008). Catch bond behavior has been observed for several different receptor-ligand pairs including α5β1 integrins and fibronectin (Kong et al., 2009). Force applied to cells confers increased lifetime and strength of single integrin molecule’s ligation to the ECM. Addition of monoclonal antibodies that induce active conformation tend to reduce the forces required for catch bond, suggesting that this phenomenon is driven by force-assisted activation of the integrin headpiece rather than direct conformational change in the integrin molecule (Kong et al., 2009).

While several mechanisms of integrin activation and adhesion assemblies have been proposed in the literature, one can imagine that these events likely happen in an orchestrated fashion. Initial integrin inside-out activation tethers the ECM, causing formation of focal contacts and adhesion assembly. Strapping of integrins to the actin cytoskeleton and subsequent forces generated by actomyosin contractility may further increase catch bonds to strengthen adhesion. These evidences therefore highlight the complexity of integrin-based adhesion and suggest that
fine regulation of adhesion strength is crucial during different steps of adhesion maturation and disassembly to cell motility.

1.6.3.2.1 Vinculin

Vinculin is an actin-binding scaffold protein that is ubiquitously expressed in cells and known to localize in focal adhesions. As such vinculin is a well-used marker for such structures (Hansen et al., 1994; Hu et al., 2007; Pasapera et al., 2010; Wolfenson et al., 2011). Vinculin has a globular head and a tail domain which during inactive state are folded in such a way that masks regions important to bind ligand partners (Johnson and Craig, 1994; Ziegler et al., 2006). Upon activation, vinculin undergoes conformational changes and localizes to focal adhesions (Chen et al., 2005). It is believed that vinculin exists in a dynamic equilibrium between inactive and active states, and the active state is stabilized upon binding to a subset of partners during integrin-mediated adhesion signalling. Consistent with studies using vinculin knockout MEF cells mentioned above, overexpression of vinculin in SV-40-transformed Balb/c 3T3 fibroblast and rat adenocarcinoma BSp73ASML cells impairs their tumourigenic growth abilities and motility (Rodriguez Fernandez et al., 1992). Conversely, downregulation of vinculin using antisense oligos enhances their migration (Rodriguez Fernandez et al., 1993). Given these results, vinculin has therefore been implicated as a tumour suppressor (Rodriguez Fernandez et al., 1992).

Several biophysical experiments using fluorescence recovery after photobleaching techniques (FRAP) have been carried to look at the exchange rate of GFP-tagged vinculin in focal adhesions (Chandrasekar et al., 2005; Cohen et al., 2006; Lele et al., 2006). All of these studies concluded that the half-life of vinculin residency in focal adhesions is much quicker (between 10 seconds and 1 minute) than the kinetics observed for other cytoskeletal proteins such as paxillin, zyxin and α-actinin (10 minutes to 20 minutes). This is likely because longer duration of vincu-
lin in focal adhesion would lead to enhanced adhesion strength and impair cell motility. This hypothesis was somewhat verified in a study that measured the fluid shear force required to detach single cells from fibronectin-coated micropatterned surface (Gallant et al., 2005). To perform these experiments the investigators took advantage of the fact that starving NIH3T3 cells from serum dramatically reduces focal adhesion formation. Subsequent stimulation with LPA results in rapid formation of focal adhesion and adhesion strengthening. Remarkably, this phenotype was accompanied by approximately 300% increase in vinculin localization at adhesions with no changes in integrin levels (Gallant et al., 2005). Similar trends were also observed in the FRAP study performed by Cohen et al., indicating that full-length constitutively active vinculin promotes focal adhesion growths. However, in their observations the decreased mobility of vinculin was also accompanied by reduced turnover rate of talin and integrin in focal adhesions (Cohen et al., 2006).

Collectively, it is now well established that vinculin has an effect on increased adhesion strength while possibly negatively regulating focal adhesion dynamics and cell motility. Exactly how vinculin is released to allow focal adhesion disassembly during cell migration remains unclear.

1.6.3.3 Disassembly of Adhesions in Migrating Cells

In the preceding sections it was emphasized that enhanced adhesion to the ECM is unfavorable for locomotion of cells. Therefore, disassembly (turnover) of these adhesions is critical for optimal cell migration. While the majority of nascent adhesions at the lamellipodial front undergo rapid turnover, the mature adhesions in a motile cell are also disassembled. The molecular events that lead to focal adhesion disassembly are still poorly understood; but recent advances have started to shed light on the mechanisms. Firstly, FAK has come to be recognized as
a crucial regulator of focal adhesion turnover. Fibroblasts or cancer cells deficient of FAK show reduced motility and enlarged vinculin, talin as well as hyperactivated integrin and associated actin stress fiber staining that are defective for disassembly (Chan et al., 2009; Ilic et al., 1995). Several mechanisms have been proposed for how FAK regulates adhesion turnover. FAK can phosphorylate and activate p190-RhoGAP to decrease RhoA and Rho kinase (ROCK) activity and subsequently reduce myosin II mediated contractions (Arthur and Burridge, 2001; Schober et al., 2007). Alternatively, FAK can also resist tension-mediated focal adhesion maturation by activating Rac1 at adhesions, which is known to antagonize RhoA activity (Arthur and Burridge, 2001; Sander et al., 1999). FAK has been also implicated in dynamin-mediated integrin endocytosis resulting in adhesion dissolution independent of Rho and Rac (Ezratty et al., 2005). More recently the Rac GEF Sif and Tiam1-like exchange factor (STEF) was identified to be responsible for Rac activation during microtubule outgrowth (Rooney et al., 2010). Rac activation was concomitant with focal adhesion disassembly and knockdown of STEF reversed this process and stalled cell migration. Taken together it is becoming clear that for efficient cell motility there is a requirement for tight regulation of adhesion and cytoskeletal dynamics. Another important factor that plays a pivotal role in focal adhesion disassembly are the microtubules (Gail and Boone, 1971; Liao et al., 1995; Schutze et al., 1991; Vasiliev et al., 1970). Microtubules have been long known to extend into sites of focal adhesions and to induce their disassembly (Kaverina et al., 1999). Disruption of microtubules using nocodazole on the other hand leads to stabilization of focal adhesions by preventing their disassembly, consequently enhancing cell adhesions to ECM (Ezratty et al., 2009; Ezratty et al., 2005; Krylyshkina et al., 2002). In the next section I will introduce some of the aspects of focal adhesion turnover mechanisms that are attributed to microtubules.
1.6.3.4 Role of Microtubules in Cell Migration

Over the years, microtubules have emerged as an important component of the cytoskeleton for precise modulation of focal adhesions and cell migration (Kaverina et al., 1998; Rinnerthaler et al., 1988; Vasiliev et al., 1970), amongst its other functions in cell division (Inoue, 2008) and vesicle transports (Sheetz et al., 1987). Microtubules are 25nm diameter hollow structures made of 13 head-to-tail protofilaments of α and β tubulin (Carrington et al., 1995). Microtubules nucleate at the minus end usually rooted in the microtubule organization center (MTOC) and grow outwards towards the plus end direction in a GTP-dependent fashion (Kirschner and Mitchison, 1986). GTP capping of tubulin at the growing end stabilizes microtubules, whereas its hydrolysis leads to its depolymerization (Mandelkow et al., 1991). As such the plus end of microtubules undergoes periods of growth and shrinkage, a state termed “dynamic instability”. Microtubules that reside for prolonged period at the leading edge are often post-translationally modified by acetylation or detyrosination (Gundersen and Bulinski, 1988; Piperno et al., 1987). Acetylation of tubulin has been linked to increased microtubule stability, and shown to have a higher propensity to bind to certain molecular motors such as kinesin-1 (Hammond et al., 2010; Nakata et al., 2011; Reed et al., 2006) and KIF17 (Jaulin and Kreitzer, 2010) (see section 1.8).

During cell migration microtubules selectively stabilize and polarize towards the leading edge, and help propagate lamellipodial protrusions by locally activating the Rho GTPase, Rac1 to drive actin polymerization at cell protrusions (Waterman-Storer et al., 1999). This study showed that cells exhibit increased Rac1 activity and membrane ruffling along with microtubule extensions toward the lamellipodia during recovery phase of microtubule depolymerization experiment upon removal of nocodazole. Conversely, the authors demonstrated that treatment with the microtubule stabilizer, taxol, induced forward extension of microtubules with associated ele-
vation of Rac1 activity. Later in a separate study the same group reported that constitutively active Rac1 could also promote microtubule growth and turnover at the cell edges (Wittmann et al., 2003). Taken together these results are suggestive of a positive feedback loop in which microtubule growth activates Rac1 and where Rac1 activation, in turn supports further microtubule extensions to induce lamellipodial spreading. Several studies now show that microtubule plus-end binding proteins (+TIPs) such as CLIP170 (Fukata et al., 2002), APC (Watanabe et al., 2004) and CLASPs (Watanabe et al., 2009) interact with the Rac1 and Cdc42 effector, IQGAP1 that can coordinate Arp2/3-dependent actin nucleation at cell protrusions. One of the +TIP proteins, APC has been linked to focal adhesion turnover via its interaction with paxillin at leading edges (Matsumoto et al., 2010). Localization of APC near the distal ends of microtubules is thought to be dependent on kinesin motors proteins, specifically KIF3B (Jimbo et al., 2002) (for kinesins see section 1.8).

Research in this field in the past several years has made it clear that microtubules have a crucial role in adhesion disassembly. Initial live cell microscopy studies using goldfish fibroblast reported that microtubules persistently target focal adhesions and lead to their dissolution (Kaverina et al., 1999; Kaverina et al., 1998; Kaverina et al., 1997; Krylyshkina et al., 2002; Small and Kaverina, 2003). Detailed visualizations of their targets showed that in multiple occasions microtubules grew to the same or different focal adhesions where they paused and underwent shortening (Kaverina et al., 1998). Curiously, the transition of microtubules from growth to shrinkage occurred seven times more frequently at sites of focal adhesions compared to anywhere else in the cytoplasm and this catastrophic event was paxillin dependent (Efimov et al., 2008). Both depolymerization and eliminating microtubule dynamics with low concentrations of nocodazole or taxol respectively interfere with cell motility (Liao et al., 1995). Disruption of the
microtubule structures with nocodazole leads to enriched aggregation of large focal adhesions accompanied by enhanced stress fibers formation (Bershadsky et al., 1996; Liu et al., 1998). This phenomenon was shown to be reversible upon either nocodazole washout or microtubule stabilization with taxol following nocodazole treatment (Ezratty et al., 2005; Hamadi et al., 2005; Waterman-Storer et al., 1999). While microtubule’s engagement in focal adhesion disassembly has become obvious, the exact mechanism by which microtubules are guided to these sites has only begun to come to light. Recently, an actin-microtubule bridging molecule, ACF7, was unveiled to have an essential role at capturing actin and microtubules in parallel at sites of focal adhesions (Wu et al., 2008; Wu et al., 2011). Depletion of ACF7 resulted in the failure of microtubules to co-align with actin stress fibers. Furthermore, ACF7 null endodermal and keratinocyte cells display disorganized microtubule landscape around the cell as opposed to radially projecting microtubules to the cell periphery in wild-type cells (Kodama et al., 2003; Wu et al., 2008). Consistent with these observations ACF7 null cells bore large, stable focal adhesions and were significantly slower in their movement. The Abl-related gene (Arg) is another molecule, which seems to function similar to ACF7 by cross-linking actin and microtubules. Fibroblasts deficient of Arg have reduced lamellipodial dynamics (Miller et al., 2004). Lately, microtubules have been reported to also mediate focal adhesion turnover by recycling integrins and other adhesion components (Ezratty et al., 2009; Ezratty et al., 2005; Pellinen and Ivaska, 2006).

While most literature point towards the role of microtubules in focal adhesion disassembly, scattered evidence indicates that there may be more to microtubule’s function in adhesion regulation than just their turnover. In the next section I shall point out some effectors of the Rap1 small G protein that localize on microtubules under resting state. Upon activation of Rap1, which is known to enhance cell adhesion, these effectors are released from microtubules and translocate to sites where they promote Rap1-mediated adhesion (Beraud-Dufour et al., 2007;
Fujita et al., 2005; Liu et al., 2011). Interestingly a Rap specific GTPase exchange factor, Epac1 is also localized on microtubules where it likely activates Rap1 and enables the release of its effectors from microtubules (Sehrawat et al., 2008). Hints that microtubules could sequester adhesion-promoting components to regulate the strength of integrin activation, cell adhesion and migration exist in the literature. Chapter 3 of this thesis describes a novel Rap1 effector, Radil as a kinesin-associated protein localized on microtubules where its activation is controlled in order to promote breast cancer cell migration.

1.6.4 Rap1: A Small G Protein with a Big Role

Rap (Ras-proximate) proteins are members of the Ras superfamily of small G-proteins. In mammals there are two Rap1 (Rap1a and Rap1b) and three Rap2 (Rap2A, Rap2B and Rap2C) proteins. Rap1 and Rap2 proteins share 60% sequence homology but can signal through distinct downstream signalling pathways (Fu et al., 2007; Miertzschke et al., 2007). For the purpose of this thesis we will focus on Rap1 in this introduction. Rap1 was originally characterized as an antagonist of Ki-Ras-induced transformation (Kitayama et al., 1989). This function of Rap1 to block transformation is attributed to its ability to competitively bind to Ras effectors such as Raf-1 without stimulating their activities (Zwartkruis et al., 1998). More recently the control of cell-matrix and cell-cell adhesions has been described as the more predominant function for Rap1 (Bos, 2005; Bos et al., 2001). The two isoforms of Rap1 share 95% sequence identity, and are ubiquitously expressed in many tissues. Despite this remarkable sequence homology the two isoforms appear to function differently as revealed by knockout experiments in mice. Knockout of Rap1a resulted in 40% embryonic lethality and the surviving Rap1a^{-/-} mice exhibited cardiac hypertrophy in vivo and defects in T and B cells adhesion in vitro (Chrzanowska-Wodnicka et al., 2008; Li et al., 2007). By contrast, Rap1b^{-/-} cells exhibit severe phenotypes with up to 85% late embryonic and perinatal lethality due to embryonic hemorrhage (Chrzanowska-Wodnicka et
al., 2005). Mice that survive are smaller in size, exhibit prolonged tail bleeding and defective platelet spreading. These phenotypes were attributed to reduced αIibβ3 integrin-mediated cell adhesion and platelet aggregation. Even though there are stark phenotypic differences in knock-out mice, absence of either Rap1a or Rap1b appears to exhibit reduced cell adhesions in both T and B cells (Chrzanowska-Wodnicka et al., 2005; Chu et al., 2008). In platelets however, Rap1b is the predominant Rap1 protein (Wang et al., 2009). Hence the phenotypic differences seen in mice may be at least partly due to differential expression of the Rap1 proteins.

Rap1 like all G proteins exist in an inactive GDP-bound state and activated GTP state. Guanine-nucleotide-exchange factors (GEFs) proteins that catalyze GDP-GTP exchange have been identified for Rap1 proteins. GEFs catalyze the release of GDP, which is then replaced by the more abundant GTP (Bos et al., 2007). Some of the known Rap specific GEFs are: C3G, Epac1, Epac2, CalDAG-GEF1, CalDAG-GEFIII, PDZ-GEF1, PDZ-GEF2, PLC-ε etc., which themselves are activated by diverse set of activators (Gloerich and Bos, 2011; Stork and Dillon, 2005). The exchange of guanine nucleotides results in an allosteric change in the Switch-I and Switch-II regions of G proteins (Boriack-Sjodin et al., 1998). The Switch-I configuration exposes the effector loop, which binds to a variety of different effector proteins when Rap1 is in GTP-bound state (van den Berghe et al., 1999). Various GTPase-activating proteins (GAPs) bind to GTP-bound Rap1 to negatively regulate or terminate its activity by enhancing the low intrinsic GTPase activity of the Rap proteins (Gloerich and Bos, 2011; Jordan et al., 1999). This results in the hydrolysis of GTP to GDP causing an allosteric shift to the inactive state. There are two major groups of Rap1-specific GAPs that have been identified. The first type, Rap1GAP and its alternative splice variant Rap1GAPII are expressed in several tissues (Kurachi et al., 1997; Rubinfeld et al., 1991; Tsygankova et al., 2004). The second group of structurally related GAPs is SPA-1 and SPAR. SPA-1 is lymphoid-specific (Kurachi et al., 1997), whereas SPAR are
widely expressed (Gao et al., 1999). Mutation of Rap1 at position 12 from glycine to valine (Rap1G12V), or glutamine at position 63 to glutamic acid (Rap1Q63E) impairs its intrinsic GTPase activity and provides them resistance to the GAPs (Brinkmann et al., 2002; Cook et al., 1993; Scheffzek et al., 1997; Sot et al., 2010) therefore locking Rap1 in the constitutively activated state and enhancing downstream signalling (Arthur et al., 2004; Lafuente et al., 2007; Lafuente et al., 2004).

Upon activation, Rap1 interacts with several downstream effectors and affects a variety of biological processes including modulation of integrins, cadherins and cytoskeletal remodeling etc. Rap1 typically interacts with its effectors via structurally similar RBD (Ras/Rap-binding domain) or RA (Ras/Rap1 association) domains. By associating with their effectors Rap1 eventually functions to regulate cell-matrix adhesions, cell-cell adhesion (Bos, 2005), cell proliferation (Zheng et al., 2009) and establishment of cell-polarity (Itoh et al., 2007). This thesis will focus on Rap1 and its effectors’ roles in tuning cell adhesion, emphasizing on adhesion to ECM.

1.6.4.1 Subcellular localization of Rap1

Biochemical and fluorescence-based visualization approaches revealed that Rap1 is localized in multiple membrane subcellular compartments such as the plasma membrane (Bivona et al., 2004; Jeon et al., 2007b; Nagata and Nozawa, 1995), the perinuclear golgi body (Beranger et al., 1991), endocytic vesicles (Pizon et al., 1994) as well as the nuclear envelope (Mitra et al., 2003). Rap1 is targeted to membranes by geranylgeranyl lipid modification at its carboxy-terminal (Bivona et al., 2004). Despite Rap1’s propensity to localize in varied subcellular compartments its distribution is dynamically influenced by signalling pathways to promote its availability to execute specific functions. Indeed during integrin-mediated cell adhesions Rap1 is localized to the plasma membrane, in part due to recruitment by membrane anchored protein com-
plexes such as activated Ezrin, Radixin and Moesin (ERM) proteins in complex with Epac1 (Bivona et al., 2004; Gloerich and Bos, 2011; Gloerich et al., 2010). Recruitment of Rap1 to the ERM-Epac1 complex is augmented upon GPCR activation leading to enhanced Rap-mediated cell adhesion (Gloerich et al., 2010). Similarly, stimuli from mechanical forces generated at the membrane, or receptor activation by platelet-derived-growth-factor (PDGF) can induce C3G interaction with an adaptor protein of the Crk family. This complex is then recruited to tyrosine-phosphorylated proteins at the plasma membrane. Once at the plasma membrane C3G can locally activate Rap1 and induce cell adhesion, spreading and migration (Ohba et al., 2001; Takahashi et al., 2008; Tamada et al., 2004). Additional signalling mechanisms to locally activate Rap1 at the plasma membrane or elsewhere have been also described. Diverse but specific molecular networks thus exist to ensure fine spatiotemporal activation and localization of Rap1 (Gloerich and Bos, 2011).

1.6.4.2 Rap1 Activates Integrin-Mediated Cell Adhesion and Spreading

Research in the last 10 years positioned Rap1 as a master regulator of integrin-mediated inside-out signalling (Bos, 2005; Bos et al., 2003). It activates integrins that are linked to the cytoskeleton, including integrins of the β1, β2 and β3 family (Bos, 2005; Caron, 2003). One of the first evidence for this came from transient overexpression experiments where Rap1 led to strong integrin αLβ2-mediated T cell and mouse pre-B-cell leukemia adhesion in response to CD31 stimulation (Katagiri et al., 2000; Reedquist et al., 2000). Conversely, expression of the dominant negative Rap1 mutant Rap1S17N, Rap1GAP or overexpression of RBD of RalGDS blocked CD31-mediated T cell adhesion (Reedquist et al., 2000). These initial experiments were later confirmed in vivo with the use of mice harboring Rap1G12V expression in their T-cells (Sebzda et al., 2002). Both thymocytes and mature T-cells in these mice displayed increased
LFA-1 mediated cell adhesion. Additionally, lymphocytes from these mice displayed enhanced adhesion to ECM due to increased avidity of α4β1 and α5β1 integrins.

In CHO cells, phorbol 12-myristate 13-acetate (PMA)-induced αIIbβ3 integrin activation can be reconstructed by expressing PKCα and talin at levels analogous to that in platelets (Han et al., 2006). Without expressing these proteins, PMA fails to activate αIIbβ3 integrin in CHO cells. This was the landmark study implicating the involvement of talin in Rap1-mediated inside-out activation of integrins. The study also found that the requirement for PKCα to activate talin could be bypassed by expressing Rap1G12V. This observation suggests Rap1 is acting downstream of PKCα. Several other studies have now also confirmed these findings (Lee et al., 2009; Lim et al., 2010). Talin is also known to attach integrins to the actin filaments (Burridge and Connell, 1983; Calderwood, 2004), and to facilitate force-induced maturation of adhesion sites as well as stress fiber formation, which eventually support cell spreading (Gallant et al., 2005; Giannone et al., 2003; Zhang et al., 2008). Surprisingly in a recent observation by Zhang et al., talin was not required for initial spreading, including primary adhesion and early cell-edge extensions but crucial for further reorganization of actin filaments and formation of focal adhesions (Zhang et al., 2008). This implies that Rap1 may also cooperate with factors other than talin to support early spreading of cells. The fact that Rap1, especially Rap1a is required for basal spreading has been previously reported (Arthur et al., 2004; Enserink et al., 2004; Jeon et al., 2007b; Ohba et al., 2001; Rebstein et al., 1997; Ross et al., 2011).

PLCγ can also activate Rap1 in Jurkat T-cells when activated by T-cell receptor or the chemokine SDF1 (Katagiri et al., 2004b). PLCγ mediated increase in intracellular Ca2+ and DAG modulates the activity of CalDAG-GEF1, which in turn activates Rap1 and the LFA-1 integrin upon T-cell receptor activation (Katagiri et al., 2004b). Likewise cAMP can directly bind
and activate Epac1, leading to Rap1 and integrin activation (Carmona et al., 2008; de Rooij et al., 1998). Today Rap1 is unambiguously accepted to play a major role at controlling inside-out activation of integrins to mediate adhesion of not only the hematopoietic cells and platelets but a range of other cell types downstream of various extracellular cues including signals relayed via GPCRs (Enserink et al., 2004; Gloerich et al., 2010; Katagiri et al., 2000; Lafuente et al., 2004; Peak et al., 2008; Rangarajan et al., 2003; Reedquist et al., 2000; Sakkab et al., 2000; Shimonaka et al., 2003; Tsukamoto et al., 1999). The exact mechanisms in place for Rap1 to mediate integrin activation in different cell types in various contexts is not yet fully understood and currently actively researched. Some insights of how Rap1 does this by interacting with its various effectors will be discussed in the next section (see section 1.6.4.3).

While it is known that Rap1 can induce cell spreading, the mechanism by which it does so is not fully understood. However, a previous study showed that following integrin-mediated adhesion to fibronectin Rap1 activates the Rho family GTPase Rac1. It does so by directly binding to Rac1 GEFs VAV2 and Tiam1 and facilitates Rac1 activation at the plasma membrane (Arthur et al., 2004). Rac1 induces formation of membrane protrusions that associate with the contiguous fibronectin matrix and therefore promote cell spreading. The authors of this study also demonstrated that while overexpression of activated form of the GEFs induced spreading, blocking Rap1 activity compromised this effect. The expression of constitutively active Rac1 was able to restore cell spreading otherwise hindered if Rap1 activity was impaired. RacGEFs such as Cool-1 and SWAP70 which do not bind to Rap1 can also induce cell spreading. It was therefore inferred that Rap1 interacts with VAV2 and Tiam1 to spatially activate Rac1 and induce cell spreading (Arthur et al., 2004).
1.6.4.3 Rap1 Effectors

Once activated Rap1 mediates its downstream signalling and integrin inside-out activation by associating with effector proteins. Despite the many effectors of Rap1 that have been discovered in the last few years, their functions mostly converge towards the control of inside-out signalling and integrin activation. The various effectors are thus likely to participate in integrin regulation in a context-dependent manner (Ross et al., 2011). The precise molecular mechanisms underlying the activity of Rap effectors need to be better defined to fully understand the cellular effects mediated by Rap1.

1.6.4.3.1 RIAM

RIAM is a member of the MRL (Mig-10/RIAM/Lamellipodin) family of proteins that can interact with Profilin and Ena/VASP proteins to affect actin remodeling (Lafuente et al., 2004). RIAM contains an RA domain, a PH domain and several proline-rich motifs that can bind to the EVH1 domain of Ena/VASP proteins. Both the RA and PH domains of RIAM are required for its interaction with activated Rap1 and its recruitment to the plasma membrane (Lafuente et al., 2004). Overexpression of RIAM in Jurkat T-cells induces cell spreading, lamellipodia formation as well as β1 and β2 integrin activation to levels obtained with expression of constitutively active Rap1Q63E (Lafuente et al., 2004). Additionally, in the same study siRNA-mediated knockdown of RIAM in Jurkat T-cells eliminated Rap1-mediated integrin activation, suggesting RIAM as a bona fide effector of Rap1 required for T-cell integrin activation. Knockdown of RIAM also reduces F-actin content in Jurkat T-cells, suggestive of a possible role in actin dynamics via its association with Profilin and Ena/VASP proteins (Lafuente et al., 2004).

Further insights into the mechanism by which RIAM links Rap1 to integrin activation came from αIIbβ3 integrin activation reconstruction studies in CHO cells described earlier (Han...
et al., 2006; Lee et al., 2009). While Rap1G12V-induced integrin inside-out activation required talin, this was blocked upon depletion of RIAM. RIAM also forms a strong complex with talin and Rap1-GTP. Upon interaction this complex co-localizes in talin positive clusters at the plasma membrane in a Rap1G12V or protease-activated receptor (PAR) activity dependent manner (Han et al., 2006; Watanabe et al., 2008). The intermolecular dependence of Rap1-RIAM-talin was addressed in a study expressing fusion constructs between the membrane targeting sequence of Rap1 and the talin binding region of RIAM in CHO cells expressing αIIbβ3. Expression of this construct was sufficient to promote RIAM and talin recruitment to the plasma membrane and to activate αIIbβ3 integrins (Lee et al., 2009). This notion that activated Rap1-RIAM complex recruits talin to integrins at the plasma membrane was also shown in platelets and megakaryocytes using bimolecular fluorescence complementation experiments (Watanabe et al., 2008).

In T-cells RIAM has been also found to enter into a complex with the adapter proteins ADAP and SKAP-55 (Menasche et al., 2007). The ADAP-SKAP-55 complex helps relocalize RIAM and Rap1-GTP to the plasma membrane following TCR activation and facilitate inside-out activation of integrin-mediated adhesion to fibronectin and ICAM (Kliche et al., 2006; Menasche et al., 2007). In fact the ADAP-SKAP-55 module was essential for localization of RIAM-Rap1 as disruption of this module strongly interfered with RIAM and Rap1 delivery to the plasma membrane (Menasche et al., 2007). Collectively based on current knowledge it is thought that RIAM functions as a scaffold protein whose association with Rap1-GTP enables its recruitment to the plasma membrane by virtue of the membrane targeting sequence of Rap1. At the membrane, RIAM recruits talin to integrins to promote their activation. Given RIAM’s possible role in modulating filamentous F-actin, it is further possible that it connects talin to the local polymerized actins following integrin activation as a way to stabilize adhesions.
1.6.4.3.2 RAPL

RAPL also known as Nore1B is another regulator of Rap1-induced integrin activation that was first identified in a yeast-two hybrid screen with RapG12V as the bait (Katagiri et al., 2003). RAPL is a small protein with a central RA domain that binds active Rap1, and a C-terminal coiled-coil SARAH domain. This Rap1 effector is highly expressed in lymphocytes where it can interact with activated Rap1, possibly downstream of PLCγ1 and CalDAG-GEF1 activation (Katagiri et al., 2004b), upon T-cell receptor activation or stimulation by chemokines such as SDF-1, CXCL13, CCL21 and CCL25 (Kanemitsu et al., 2005; Katagiri et al., 2006; Katagiri et al., 2003; Parmo-Cabanas et al., 2007). Lymphocytes from RAPL-deficient mice manifest defective chemokine-induced adhesion (Katagiri et al., 2004a). Overexpression of RAPL or its activation by chemokines and other GPCRs in lymphocytes cause increased membrane ruffling, cell polarization and promote LFA-1 and α4β1 integrins’ affinity and avidity. Activation of these integrins subsequently enhances adhesion to ICAM-1 and fibronectin matrices (Ebisuno et al., 2010; Hitchcock and Kaushansky, 2007; Kanemitsu et al., 2005; Katagiri et al., 2003; Katagiri et al., 2004a; Parmo-Cabanas et al., 2007).

RAPL is indispensable for integrin-mediated adhesion and migration of lymphocytes and dendritic cells (Katagiri et al., 2004a). Indeed knockout of Rap1 in mice blocks migration of these lymphocytes to peripheral lymph nodes and spleen (Katagiri et al., 2004a). RAPL associates with the cytoplasmic tail of the α-subunit of LFA-1 in a Rap1-dependent manner and promote their clustering at the leading edge thereby resulting in chemokine-dependent polarization and chemotaxis of lymphocytes (Katagiri et al., 2003). The kinase MstI was identified as a crucial effector of RAPL through its binding to the SARAH domain. Upon activation of Rap1, RAPL and MstI translocates with LFA-1 to the leading edge of T-cells (Katagiri et al., 2006; Zhou et al., 2008a). The polarized localization of RAPL and LFA-1 induced by Rap1 activation
requires MstI as knockdown of the kinase dramatically blocks this phenotype leading to decreased cell adhesion. Although overexpression of MstI enhances LFA-1 clustering and adhesion, it has no effect on integrin affinity suggesting integrin avidity may have been responsible for Rap1-dependent adhesion (Katagiri et al., 2006).

Recently, a better understanding of the mechanisms describing the activation of LFA-1 by T-cell receptors was revealed. The formation of RAPL-Rap1 complex absolutely requires binding of the adaptor protein SKAP-55 to RAPL (Raab et al., 2011; Raab et al., 2010). Upon T-cell receptor ligation SKAP-55 binds to the coiled-coil domain of RAPL via its PH domain and promotes translocation of RAPL to the plasma membrane. There, RAPL interacts with Rap1-GTP and LFA-1, and induces T-cell adhesion to ICAM-1 in vitro. The requirement of RAPL for adhesion in a more physiological setting was also demonstrated by assessing the contact times of T-cells or mature dendritic cells with organotypic cultures of lymph nodes (Raab et al., 2010). Under wild type RAPL overexpression conditions both T-cells and dendritic cells manifest increased dwell time on lymph nodes ex vivo. This effect was lost when a mutant of RAPL that does not bind to SKAP-55 was transfected (Raab et al., 2010). Interaction of SKAP55 with RAPL does not interfere with MstI binding suggesting that MstI directs the complex to LFA-1.

Although RAPL function has been mostly studied in the context of lymphocytes, it was also linked to endothelial cell migration (Fujita et al., 2005). In human aortic endothelial cells RAPL was shown to associate and localize on microtubules where the interaction is mediated via its RA domain in a Rap1 independent manner. Activation of Rap1 dislocates RAPL from the microtubule networks and targets it to the plasma membrane. To observe localized Rap1 activity during directional migration the chemoattractant S1P was locally released from a micropipette,
which oriented the cells towards the source of the chemoattractant. Using FRET-based biosensors to monitor Rap1 activity, the authors showed that stimulation with S1P from a micropipette strongly activates and localizes Rap1 at the leading edge membrane ruffles. Concurrently, microtubules marked with GFP labeled RAPL also grew towards the protrusive regions at the leading edge co-localizing with focal adhesion sites. Because RAPL-labeled microtubules extended towards the front of migrating cells the authors inferred that they are captured by activated Rap1 and utilized to facilitate directional migration (Fujita et al., 2005). Inactivation of Rap1 by Rap1GAP or deletion of RAPL RA domain perturbed directional cell movement in this context. While RAPL’s ability to interact with both Rap1 and the microtubules seems essential for directional migration, the exact role of microtubules needs further elucidation.

1.6.4.3.3 PKD1

In T-cells PKC has been shown to activate and re-localize Rap1 to the plasma membrane by phosphorylating PKD1 (Medeiros et al., 2005). PKD1 binds to Rap1 via its PH domain, activates it and results in their plasma membrane translocation. At the plasma membrane PKD1 and Rap1 associate with the C-terminal tail of β1-integrin causing increased adhesion to fibronectin in response to phorbol esters (PMA) or T-cell receptor stimulation (Medeiros et al., 2005; Woods et al., 2004). Expression of a PH domain-deleted PKD1 construct failed to promote PMA-mediated integrin activation and clustering even though the translocation to the plasma membrane and interaction with β1-integrin were intact (Medeiros et al., 2005). Furthermore, Rap1’s interaction with PKD1 was essential for the activation of Rap1 by PMA or CD3 stimulation. Interestingly, the study also found that expression of β1-integrin in Jurkat cells was absolutely required for PKD1 localization to the plasma membrane and activation of Rap1. Expression of a truncated version of β1-integrin missing the cytoplasmic domain could not restore Rap1 activation upon PMA or CD3 stimulation. Given the current understanding that Rap1 activation is up-
stream of integrin engagement the observations made in the paper by Medeiros et al., that β1-integrin was absolutely required for Rap1 activation is puzzling. How PKD1 and Rap1 interact with the other Rap1 effectors in T-cells to modulate integrin activation is currently unknown and requires further investigation.

1.6.4.3.4  ARAP3

ARAP3, a GTPase activating protein for Arf and Rho G proteins and a PI3K effector has been described as another Rap1 effector (Krugmann et al., 2004). ARAP3 interacts with activated Rap1 via the RA domain. ARAP3 also contains ArfGAP and RhoGAP domains and several PH domains one of which can bind to phosphatidylinositol-3,4,5-triphosphate (PIP3) (Krugmann et al., 2002). Interestingly, Rap1 and PIP3 binding to ARAP3 was shown to be required for its RhoGAP activity and its ability to block platelet-derived-growth-factor-induced lamellipodia formation in pig aortic endothelial cells (Krugmann et al., 2004).

1.6.4.3.5  PLCε

PLCε is dually regulated by small G proteins of the Ras family as well as by the heterotrimeric G proteins. While PLCε, like other PLC isozymes, contain a pleckstrin homology (PH) domain, the catalytic X and Y boxes and a Ca²⁺/lipid binding (C2) domain, it has additional regions like the GEF domain on the N-terminus and two RA domains at the C-terminal. As mentioned earlier, Gβγ-subunits have been shown to modulate PLCε activity (Wing et al., 2001). Additionally, PLCε can be activated by GTP-bound Ras and Rap proteins via their interaction with the second RA domain (Kelley et al., 2001; Song et al., 2001; Song et al., 2002). Interestingly though, unlike the other Rap1 effectors described here, PLCε’s interaction with activated Rap1 leads to its localization to the perinuclear Golgi, while activated Ras can translocate PLCε to the plasma membrane (Song et al., 2001). PLCε reportedly also increases the rate of GDP re-
lease from Rap1 (Song et al., 2002) and Ras proteins (Lopez et al., 2001) independently of its phospholipase activity and results in downstream MAPK signalling. Particularly, its GEF activity on Rap is critical for the enzyme’s prolonged self activation, and is important for cell survival and proliferation (Song et al., 2002). By virtue of its ability to engage different G proteins, PLCε can be differentially modulated upon stimulation by diverse GPCRs or receptor tyrosine kinases (Kelley et al., 2004). Although, PLCε acts as a GEF and an effector of Rap1, whether it participates in Rap1-mediated cell adhesion or migration is unknown.

1.6.4.3.6 KRIT1

KRIT1 also known as CCM1 is found mutated in the disease cerebral cavernous malformation (Laberge-le Couteulx et al., 1999). This condition mostly affecting the central nervous system is characterized by abnormally enlarged capillary cavities without intervening brain parenchyma. KRIT1 was first discovered in a two-hybrid screen of HeLa cell cDNA library when searching for novel interactors of Rap1 (Serebriiskii et al., 1997). It has several ankyrin repeats and a FERM domain that resembles the RA and RBD domains; therefore, interacts with Rap1-GTP (Wohlgemuth et al., 2005). KRIT1 is implicated as a major effector of Rap1 to maintain and regulate the integrity of endothelial cell-cell junctions (Glading et al., 2007; Lampugnani et al., 2010; Liu et al., 2011). Although KRIT1 is generally sequestered in the microtubules (Beraud-Dufour et al., 2007), binding to activated Rap1 re-locates it to the cell-cell junction where it is known to stabilize the adhesion sites in vitro and support cardiovascular development in zebrafish (Liu et al., 2011).

1.6.4.3.7 AF-6

AF-6 otherwise known as Afadin is a multidomain adaptor protein initially characterized as a junctional protein interacting with various proteins localized at the junctions including ZO-1
(Zhadanov et al., 1999). AF-6 contains two N-terminal RA domains followed by a forkhead (FHA), dilute (DIL) and a C-terminal PSD-95-Dlg-ZO1 (PDZ) domains. The FHA domain is a small protein module that is known to recognize phosphoserine epitopes on proteins (Durocher and Jackson, 2002). No precise function of the DIL domain is known yet; however, it is a domain that is also found in the C-terminal of myosin V (Sattarzadeh et al., 2011). PDZ domains are modular protein-protein interaction modules and described in details in section 1.7. The first RA domain of AF-6 is responsible for the binding to Rap1, preferentially over other Ras family proteins. Rap1 and AF-6 colocalize together at the plasma membrane especially at cell-cell adhesion sites (Boettner et al., 2000). The functional relevance of this interaction has been nicely characterized in *Drosophila* where their ability to interact was shown to be essential for dorsal closure during embryonic development. This phenotype is similar to *Rap1* loss-of-function in *Drosophila*, thus implicating AF-6 and Rap1 as important regulators of cell-cell junction (Boettner et al., 2003). Although the actin-binding protein Profilin also interacts with AF-6 via the C-terminus, whether this interaction is functionally required for actin remodelling proximal to adhesion complexes at cell-cell junctions remains to be addressed (Boettner et al., 2000).

While AF-6 has been implicated in cell-cell adhesion, its role in cell-matrix adhesion is less well established. In one study AF-6 was suggested to sequester activated Rap1 and influence its activity during cell adhesion (Zhang et al., 2005). Overexpression of AF-6, despite the associated increase in Rap1 activity, caused decreased cell adhesion whereas its depletion in HB6 cells (a mouse myeloma cell line) enhanced their adhesion on fibronectin. The PDZ domain of AF-6 recruits the Rap GAP, SPA-1 to sites of Rap1 activation allowing AF-6 to regulate β1-integrin dependent cell adhesion (Su et al., 2003).
Clinically loss of AF-6 has been associated with poor prognosis in breast cancer patients (Letessier et al., 2007). While more studies are needed to elucidate the mechanisms some clues are beginning to surface. AF-6 was recently found in complex with the adhesion protein Junctional Adhesion Molecule-A (JAM-A) and the Rap1 activator PDZ-GEF2 in primary breast tumours (McSherry et al., 2011). Knockdown of JAM-A in breast cancer cells reduces their matrix adhesion and migration due to concomitant decrease in β1-as well as αV and α5 integrins expression.

1.6.4.3.8 Radil: A Novel Rap1 Effector

Ras-associating and diluted domain-containing protein (Radil) is a novel Rap1 effector recently identified as a gene induced by an oncogenic translocation in a pediatric sarcoma. Radil has been also implicated in neural crest cell migration during zebrafish development and for the control of cell-matrix adhesion in different cell types (Smolen et al., 2007). Abrogation of Radil in zebrafish using antisense morpholinos manifested defects in craniofacial cartilage, enteric neurons, cranial ganglia, glial cells of the lateral line, phenotypes that are consistent with defects in early neural crest development (Smolen et al., 2007). Knockdown of Radil did not affect initial induction of neural crest at the six somites stage, but subsequent migration of these cells was profoundly impaired. The molecular mechanism describing Radil function was not studied in this early study. As will be described in the main body of this thesis, when this original paper describing Radil’s function was published we had simultaneously discovered Radil in mass spectrometry experiments as a novel interactor of Gβγ subunits of heterotrimeric G proteins. We provided evidence that Radil modulates cancer cell adhesion, spreading and migration and attributed these functions to Radil’s ability to support Rap1-mediated inside-out activation of β1-integrins downstream of GPCR signalling. Following our initial description of Radil’s function in cell spreading, a study employing a small scale siRNA screen, looking at different Rap1
effectors found Radil as the only Rap1 effector in the human lung adenocarcinoma cell line, A549 cells essential for cell spreading downstream of EPAC activation (Ross et al., 2011).

The \textit{RADIL} gene resides in the locus 7p22.1 (Hillier et al., 2003) and encodes for a protein of 1075 amino acids. Radil has a domain architecture similar to AF-6 and has a single RA domain in the N-terminus, center FHA and DIL domains and a PDZ domain at the C-terminus (Figure 1-10A). The paralog of Radil in human is the protein Rasip1, which is a vascular-specific regulator of GTPase signalling and critical for tissue architecture and adhesion (Xu et al., 2011). Orthologs of Radil have been preserved from \textit{Danio rerio} to \textit{Homo sapiens}. In humans, mRNA for Radil appears to be ubiquitously expressed in several tissues and organs (see Figure 1-10B) (BioGPS; http://biogps.gnf.org). Although Radil has a domain arrangement similar to AF-6, the two proteins have been suggested to have different molecular functions (Zhang et al., 2005). Recently two different types of mutations in the \textit{Radil} gene have been identified in the exome sequence of liver fluke-associated cholangiocarcinoma patients. One of these causes a missense mutation in the RA domain while the other mutation falls in a splice site (Ong et al., 2012). The functional significance of these mutations remains unknown.
Figure 1-10. Radil, a novel interactor of Rap1 and heterotrimeric G-protein Gβγ. (A) Shown is a schematic of the domain architecture of Radil. Human Radil is a protein encoded by 1075 amino acids and contains an N-terminal RA domain, a FHA, and DIL domain in the middle and a PDZ domain at the C-terminal. (B) Expression pattern of Radil in different tissues according to BioGPS (http://biogps.gnf.org).
1.6.4.4 Role of Rap1 in Cell Migration and Cancer Metastasis

As described above it is now clear that Rap1 plays paramount roles in mediating cell adhesion and spreading by regulating both integrin affinity and avidity. By doing so it also controls cell movements that require dynamic changes in adhesion site assembly and disassembly, control of precise strength of the tether between the cell and the matrix and accompanying directional traction force to drive the cell forward. Direct evidence supporting a function for Rap1 in cell migration came from studies in leukocytes, where chemokine-induced integrin activation causes leukocyte arrest on the endothelium, followed by endothelial transmigration (Ebisuno et al., 2010; Hyun et al., 2009; McLeod et al., 2002; Shimonaka et al., 2003). There are also accumulating evidences asserting Rap1 and its effectors in the regulation of migration and invasion of a number of non-hematopoietic and cancer cells (Bailey et al., 2009; Freeman et al., 2010; Fujita et al., 2005; Hernandez-Varas et al., 2011; Lyle et al., 2008; Miyata et al., 2009; Severson et al., 2009).

Discussed earlier (in section 1.6.4.3.2), the localized activity of Rap1-RAPL complex at the leading edge of vascular endothelial cells hinted an essential role of Rap1 during directional migration (Fujita et al., 2005). A more direct participation of Rap1 during migration was confirmed by loss of function experiments where knockdown of Rap1 hindered prostate cancer cell migration towards the CXCR4 receptor ligand SDF-1 (Bailey et al., 2009). In chemotaxing *Dictyostelium discoideum* Rap1 preferentially localized at the leading edge to induce adhesion (Jeon et al., 2007b). Interestingly however, overexpression of Rap1G12V enhanced adhesion but dramatically reduced cell migration. Furthermore, it appears RapGAP1 in these primitive cells localizes at the leading edge upon chemoattractant stimulation, and by negatively regulating Rap1 activity, promotes cell motility (Jeon et al., 2007a). Hyperactivation of Rap1 in epithelial cells was also shown to cause dramatic cell adhesion and spreading and reduced migration velocity.
(Lyle et al., 2008). This was suggested not to be solely due to increased integrin activity but rather to the spurious stabilization of focal adhesions that form after integrins crosslinking to the actin cytoskeleton (Lyle et al., 2008). It should be noted though that the study was performed by uniformly activating the RapGEF Epac with a small molecule activator. Doing so would inadvertently stimulate Rap1 activity throughout the cell causing unpolarized and constitutive adhesions signals. Nevertheless, taken together current evidence suggests that the Rap1 signalling module likely functions in a dynamic and spatiotemporal manner to regulate integrins. Rap1 activity thus needs to be controlled optimally to ensure optimal adhesion and de-adhesion during cell migration. Conforming to this hypothesis, a recent study by Freeman and colleagues established that precluding either activation or cycling of Rap1 via expression of Rap1GAP or Rap1G12V respectively block breast as well as melanoma cancer cell migration and invasion (Freeman et al., 2010).

The importance of Rap1 in migration is further exemplified in the context of cancer invasiveness. Rap1GAP has been reported to be downregulated in several invasive cancers including those of thyroid, melanomas, and colorectal carcinomas (Tsygankova et al., 2010; Tsygankova et al., 2007; Zheng et al., 2009). In thyroid cancers Rap1GAP is downregulated in a subset that exhibited mesenchymal morphology and invasive properties (Tsygankova et al., 2007). Restoring Rap1GAP expression in these tumours effectively inhibited cell migration and invasion, effects that correlated with decreased Rap1 activity. Likewise, elevated Rap1 activation in melanoma cells endows them with increased capacity for migration and invasion (Gao et al., 2006). This phenotypic trait was suggested to be at least partly due to Rap1GAP downregulation found in many human melanoma tumours and cell lines via promoter hypermethylation that inhibits gene transcription (Zheng et al., 2009). A bona fide role of Rap1 in cancer cell invasion and metastasis was confirmed in human prostate cancers showing that knockdown of Rap1 blocked dissemi-
nation of these cells to the bone in an *in vivo* metastasis mouse model. Requirement of Rap1 for
tumour migration, invasion and metastasis has now been also described in melanomas, B-cell
lymphomas and breast cancers (Hernandez-Varas et al., 2011; Lin et al., 2010; McSherry et al.,
2011).

Given the importance of Rap1 in the control of integrin-mediated cell adhesion, migra-
tion and tumour metastasis more work is clearly needed to understand its dynamic and complex
roles. As it has become apparent from previous sections of this chapter, adhesion structures
downstream of integrin activation such as focal complexes and focal adhesion are crucial entities
regulating cell adhesion and motility. In light of the fact that Rap1 appears to induce and regu-
late focal adhesion complexes (Lyle et al., 2008) and the requirement for fine regulation of Rap1
activity for efficient cell migration, chapter 3 will address the significance of balanced Rap1-
Radil activity in regulating aggressive breast cancer motility and progression.

1.7 PDZ Domains

PDZ domains are modular protein-protein interaction modules that are abundantly repre-
sented in the genome of a wide variety of species ranging from bacteria, plants, flies to humans.
The domain was named after the proteins where it was first discovered: post synaptic density-95
(PSD-95), discs large (Dlg), and zonula occludens-1 (ZO-1) (Kennedy, 1995). The human ge-
nome encodes for over 250 PDZ domains spanning over 152 proteins either as single or multiple
modules (te Velthuis et al., 2011; Tonikian et al., 2008). A complete list of PDZ-containing pro-
teins is available on the SMART website (http://smart.embl-heidelberg.de/). PDZ domains are
~90 amino acids long and play important roles in the assembly of large protein complexes in-
volved in signalling or subcellular transport. PDZ domain containing proteins are frequently
found in complexes associated with specialized subcellular structures and connect cortical pro-
tein networks with the cytoskeleton as well as the plasma membrane. Given the abundance of PDZ domains in the proteome and their ability to guide assembly of supramolecular complexes, defining the specific interactions with their ligands is crucial to understanding their functional importance in physiology and diseases.

1.7.1 Specificity of PDZ domain interactions

The PDZ domain was first recognized as a protein-protein interaction module after the finding that the first and second PDZ domains of PSD-95 bind to the c-terminal peptide sequence of Shaker type \( K^+ \) channels, and NMDA receptor subunit NR2 (Kim et al., 1995; Kornau et al., 1995; Niethammer et al., 1996). Soon it became apparent that PDZ domains have specific preferences for binding to c-terminal peptide sequences in proteins. In a seminal study Songyang and colleagues used a degenerate peptide library and described two major classes of PDZ domains on the basis of binding specificities (Table 1-4) (Songyang et al., 1997). These early findings showed that PDZ bind to four amino acid sequences at the extreme c-terminus of proteins. To date, twenty structures of PDZ domains in complex with their ligands validate these claims (Laskowski et al., 2005). PDZ domains were first classified into three broad classes based on their binding specificities (Table 1-4). However, recently two large scale studies using phage displayed c-terminal peptide libraries (see Section.1.10) and protein microarrays have suggested the existence of as much as 16 classes. Binding preferences to PDZ domains can be determined by up to six amino acids in the peptide ligand (Stiffler et al., 2007; Tonikian et al., 2008). These investigations have addressed an important difference between the standard view of PDZ domain selectivity and a much broader apparent selectivity adapted by nature to optimize and avoid cross reactivity.
For class I PDZ, which is the most studied, there is a strong requirement for an aliphatic hydrophobic amino acid (such as valine, leucine or isoleucine) at the extreme c-terminus, or 0 position (Hung and Sheng, 2002; Skelton et al., 2003; Songyang et al., 1997; Tonikian et al., 2008). Mutation of the end residue to alanine has shown to obliterate the interaction of the ligand with its cognate PDZ domain. In contrast another study employing phage-displayed peptide libraries and x-ray crystallography to understand ligand binding to HtrA2 PDZ domain showed that this PDZ domain has preference for highly hydrophobic c-terminal sequences, although no single hydrophobic residue was preferred exclusively over others at any particular position. Of significance, although valine was most prevalent at the 0 position, mutation from valine to alanine at this position only affected its affinity by threefold. Additionally, in their screen larger hydrophobic amino acids (like tryptophan and phenylalanine) dominated positions -1, -2, and -3, tryptophan being prevalent at -1 position, and mutation of tryptophan to alanine at this position caused decrease in affinity by greater than three-hundred folds (Zhang et al., 2007).

Most PDZ domains interact with their partners constitutively with binding affinity of 1-10μM; however, some interactions can be promoted in an agonist-dependent fashion. AF-6 and NHERF interaction with EphB3 tyrosine kinase receptor and β2-adrenergic receptor respectively are promoted by receptor activation (Hall et al., 1998; Hock et al., 1998). PDZ domain interactions can also be disrupted by phosphorylation of PDZ binding sites by serine-threonine kinases like in the case of the GIRK channel. GIRK channel interaction with PSD-95 can be disrupted by PKA dependent phosphorylation of the serine residue at -2 position within the GIRK c-terminus. Although rare, PDZ domains have also been reported to interact with internal peptide sequences like nNOS interaction with PDZ domains of PSD-95 and syntrophin (Brenman et al., 1996a; Brenman et al., 1996b). While most PDZ domains are monomers, some PDZ domains
such as that of Shank-1 can form dimers. However, dimerization does not seem to affect the binding of partners as the ligand-binding pockets of the PDZ domains remain available (Im et al., 2003).

**Table 1-4. Common classifications of PDZ domains based on specificity for C-terminal peptide ligands**

<table>
<thead>
<tr>
<th>Class</th>
<th>C-terminal sequence</th>
<th>Ligand protein</th>
<th>PDZ protein</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Class I</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-X-S/T-X-Φ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-ETDV</td>
<td>Shaker K⁺ channel</td>
<td>PSD-95 (PDZ2)</td>
<td></td>
</tr>
<tr>
<td>-ESDV</td>
<td>NMDA receptor NR2A/B subunits</td>
<td>PSD-95 (PDZ2)</td>
<td></td>
</tr>
<tr>
<td>-TTRV</td>
<td>Neuroligin</td>
<td>PSD-95 (PDZ3)</td>
<td></td>
</tr>
<tr>
<td>-ESLV</td>
<td>Voltage gated Na⁺ channel</td>
<td>Syntriphin</td>
<td></td>
</tr>
<tr>
<td>-QSAV</td>
<td>Protein Kinase C α</td>
<td>PICK1 (PDZ1)</td>
<td></td>
</tr>
<tr>
<td>-DSSL</td>
<td>β2-adrenergic receptor</td>
<td>NHERF (PDZ1)</td>
<td></td>
</tr>
<tr>
<td>-DTRL</td>
<td>CFTR</td>
<td>NHERF (PDZ1)</td>
<td></td>
</tr>
<tr>
<td>-QTRL</td>
<td>GKAP</td>
<td>Shank</td>
<td></td>
</tr>
<tr>
<td>-SSTL</td>
<td>mGluR5</td>
<td>Shank</td>
<td></td>
</tr>
<tr>
<td>-DSWV</td>
<td>P0071</td>
<td>Erbin</td>
<td></td>
</tr>
<tr>
<td><strong>Class II</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-X-Φ-X-Φ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-SVKI</td>
<td>AMPA receptor subunit</td>
<td>PICK1</td>
<td></td>
</tr>
<tr>
<td>-EYFI</td>
<td>GluR2</td>
<td>Erythrocyte p55</td>
<td></td>
</tr>
<tr>
<td>-EYYV</td>
<td>Neurexin</td>
<td>CASK</td>
<td></td>
</tr>
<tr>
<td>-EFYA</td>
<td>Syndecan-2</td>
<td>CASK</td>
<td></td>
</tr>
<tr>
<td>-YYKV</td>
<td>EphB1</td>
<td>PICK1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GRIP (PDZ6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Syntenin (PDZ2)</td>
<td></td>
</tr>
<tr>
<td><strong>Class III</strong></td>
<td>-X-D/E-X-Φ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-SVEV</td>
<td>EphB2</td>
<td>PICK1</td>
<td></td>
</tr>
<tr>
<td>-DVPV</td>
<td>ErbB2</td>
<td>Erbin</td>
<td></td>
</tr>
<tr>
<td>-VDSV</td>
<td>Melatonin receptor</td>
<td>mLIN10/Mint1/X11</td>
<td></td>
</tr>
<tr>
<td>-GEPL</td>
<td>KIF17</td>
<td></td>
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</table>

Adapted from (Nourry et al., 2003) and (Hung and Sheng, 2002)

### 1.7.2 PDZ domain structure

Structural insights of the PDZ domain emerged when the X-ray crystal structure was solved for PSD-95-PDZ3 (Doyle et al., 1996; Morais Cabral et al., 1996). Numerous crystal structures then followed describing highly similar domain fold (Daniels et al., 1998; Tochio et al., 2000). These structures were important to understand how PDZ ligands interact with PDZ domains. Today, there are several structures of PDZ domains in complex with free C-terminal binding peptides (Doyle et al., 1996; Tochio et al., 1999; Zhang et al., 2007; Zhou et al., 2005).
From these structures it is apparent that PDZ domains generally comprise six β-strands (βA to βF) with two or three α-helices (Figure 1-11). The canonical PDZ domains have a highly conserved fold; however, the secondary structure varies in length. All PDZ domains have a conserved R/K-XXX-G-Φ-G-Φ motif; where Φ is a hydrophobic residue and X is any amino acid, found within the βA-βB connecting loop (Doyle et al., 1996; Fanning and Anderson, 1999; Morais Cabral et al., 1996). The c-terminal peptide binds to a groove between the βB strand and αB helix which extends from a conserved arginine or lysine residue. The peptide ligand binds to the groove as an antiparallel β-strand (Doyle et al., 1996). The three amide protons of the G-Φ-G-Φ motif form hydrogen bonds with the terminal carboxylate group of the peptide. Furthermore, the guanidinium group of arginine can interact with the free carboxylate group via a water molecule and stabilize the interaction, hence the preference for free c-terminus for PDZ binding.

Despite highly similar overall structures PDZ domains bind to very specific ligands with very little promiscuity (Tonikian et al., 2008). For example, in the case of the interaction of PSD-95-PDZ3 with its peptide ligand, the side chain of the residue at position -1 points away from the interaction surface and is not too important to dictate the specificity of binding (Doyle et al., 1996). Rather in this case the residues at positions 0 and -2 are important to determine binding specificity. In constrast, the guanidinium group of arginine at position -1 of the cystic fibrosis transmembrane regulator (CFTR) protein is essential for binding to NHERF-PDZ1 (Karthikeyan et al., 2001). More recently x-ray crystal structures of the Erbin-PDZ, ZO-1-PDZ1 and HtrA2-PDZ domain showed that a tryptophan at the -1 position is crucial for the interaction of their ligands with the amino acids of the conserved PDZ domain fold. Differential selectivity of ligands for respective PDZ domains are further determined by residues between the 0 and -6 sites and the way they interact with residues connecting the region between βB and βC (Zhang et al., 2007).
**Figure 1-11. PDZ domain structures.** Ribbon diagram of (A) PSD-95 PDZ3 (PDB 1BE9), (B) AF-6 (PDB 1XZ9) and (C) RADIL (PDB 1UM1). All three structures were aligned using Pymol Molecular Graphics System. The different β-barrels and α-helices are marked from N-terminus to C-terminus for PSD-95. The figure shows similar basic overall fold for three different PDZ domains. (D) C-terminal peptides bind as an antiparallel β-strand in the groove between βA and βB. The image shows crystal structure of PSD-95 with ligand KQTSV. The groove is depicted by vacuum electrostatic profile. Ligand shown as sticks. (E) Shown is the crystal structure of HtrA2 PDZ domain bound to a peptide ligand WTMFWV (PDB 2PZD). Left panel shows the ligand superimposed on the ribbon and right panel shows the ligand sitting in the groove created by the PDZ binding pocket. Images recreated using Pymol.
1.7.3 Cellular functions mediated by PDZ interactions

PDZ domains have historically been considered as protein scaffolding module or involved in receptor aggregation at the plasma membrane. Now they are known to direct supramolecular complexes to specific subcellular compartments, contributing to signalling specificity and participate in a number of cellular processes including, cell polarity, synaptic density, cytoskeletal remodeling, adhesion, migration as well as invasion of cancer cells (Nourry et al., 2003; Subbaiah et al., 2011). PDZ domain containing proteins are often localized at specific subcellular sites near the plasma membrane of polarized cells. The prototypical PDZ protein, PSD-95 is localized at the synaptic terminals of neurons and collect proteins and receptors which are involved in neurotransmission (Kornau et al., 1995; Xia et al., 2003). Two evolutionarily conserved protein complexes that involve PDZ domains: Crumbs-PALS1-PATJ (Straight et al., 2004) and Cdc42-Par6-Par3-aPKC (McCaffrey and Macara, 2011) play important roles in the assembly of tight junctions at the apical surface of epithelial cells and establish polarity. Similarly localization of Dlg-Lgl-Scribble protein complex resides at the base of cells (Bilder et al., 2000). The asymmetrical localization of the above complexes is required for the development of apico-basal polarity of epithelial cells (Humbert et al., 2008; Nelson, 2009). The Crumbs, Scribble and Par3 complexes have been also implicated in polarity establishment of T-cell, astrocytes and fibroblasts and affect their morphology and migration (Nelson, 2009; Pike et al., 2011).

Besides regulating synaptic density and cell polarity PDZ proteins and their partners are being increasingly associated with functions related to cell adhesion and motility. There is vast amount of literature available on this topic. A few cases are exemplified below highlighting the different functions executed by PDZ-domain or PDZ-motif containing proteins. Cell surface molecules like Claudin, Occludin, and Junctional Adhesion Molecules (JAM) bind to PDZ proteins MUPP1, ZO-1 and AF-6 and affect their localization at the tight junctions (Ebnet et al.,
2000; Furuse et al., 1994; Hamazaki et al., 2002). Evidence from studies of AF-6 knockout mice revealed a critical role of AF-6 in the regulation of cell-cell junctions, and loss of neuroepithelial polarity was evident in mutant embryos that died 10 day post coitum (Zhadanov et al., 1999). AF-6 also associates with JAM-A and the Rap1 activator PDZ-GEF2 to control cell-cell adhesion in breast cancer cells (McSherry et al., 2011). Although in this study the investigators showed inhibition of JAM-A and downstream signalling pathways control cell migration, how exactly AF-6 fits in the mechanism is not clear (McSherry et al., 2011). AF-6 can also negatively regulate Ras activity at cell junctions by interacting with the protein Bcr. This inhibition is lost in several human leukemia cancers when Bcr fuses with Abl kinase and block the free c-terminal binding site, preventing its binding to AF-6 and contributing to Ras mediated malignancy (Radziwill et al., 2003). In another example, the kinase c-Src, which is an important component in cell-matrix and cell-cell contact sites, was demonstrated to mediate epithelial cell migration and invasion by interacting with AF-6 in a PDZ dependent manner (Baumgartner et al., 2008; Radziwill et al., 2007). Syntenin is another PDZ protein that is frequently overexpressed in diverse cancers and is associated with increased migration and metastasis (Boukerche et al., 2008; Das et al., 2012; Koo et al., 2002; Sarkar et al., 2008). Recently, syntenin was characterized as a signalling component downstream of fibronectin-mediated focal adhesions (Hwangbo et al., 2010). In this study syntenin was described to be required for the formation of integrin-β1-FAK-c-Src complexes in human breast cancer and melanoma cells upon their binding to fibronectin and to mediate cell migration and invasion.

The above examples underscore the wide range of functions served by PDZ domain containing proteins and how their dysregulation can lead to diseases. In chapter 3, I will present my findings showing that the kinesin family protein, KIF14, is a novel PDZ-dependent interactor of
Radil. KIF14 functions by sequestering Radil away from its site of activation to promote and regulates cell adhesion and motility, notably during breast cancer metastasis.

1.8 Kinesin Superfamily Proteins (KIFs)

Intracellular transports of newly synthesized proteins, protein complexes, and organelle are essential for cellular functions. Several of these functions are performed by molecular motors such as kinesins, dyneins, and myosins. Kinesin superfamily proteins (also referred to as KIFs) use microtubules as rails in order to transport cargo in mostly anterograde direction (Hirokawa et al., 2009). Dyneins are the retrograde transport machinery, which also use microtubules (Kardon and Vale, 2009). Myosins on the other hand move along actin filaments and are generally known to perform short range transports; however, new evidence suggest they may also be able to perform long range transports of vesicle as well (Schuh, 2011; Sweeney and Houdusse, 2010). For the purpose of the thesis this section will focus on KIFs.

KIFs were first discovered by observation under electron microscopes as short cross-bridges between organelles and filamentous structures like microtubules (Hirokawa, 1982). The first five KIFs were cloned almost ten years after these observations from mouse brain tissues (Aizawa et al., 1992). KIFs exhibit high amino acid sequences conservation within the motor domain; however, there is little similarity outside of this region (Miki et al., 2001). The motor domain contains an ATP-binding and a microtubule-binding sequence. Most KIFs use their “stalk” regions to dimerize with each other, but some have a short coiled-coil region and exist as monomers (Hirokawa and Noda, 2008). Their interaction with cargo molecules occurs outside the motor domain, generally via the variable tail region (Hirokawa, 1998). To generate the motile force required for progressive movement along microtubules KIFs utilize the chemical ener-
gy of ATP to drive conformational changes in the motor domain (Schief and Howard, 2001; Vale et al., 1985).

1.8.1 Classifications

An extensive systematic identification of KIFs in human and mouse genome by the Hirokawa laboratory reports a total of 45 KIFs (Miki et al., 2001). Alternative splice variants also exist for each kinesin giving rise to different tail domains that can bind different cargoes. KIFs are broadly classified into three types based on the position of the motor domain. The N-kinesins, M-kinesins and C-kinesins groups depict the presence of the motor domain at the N-terminus, middle or C-terminus of the proteins. Of the total 45 kinesins 39 of them belong to N-kinesin family, 3 in M- and 3 C-kinesins. The N-kinesins move along the microtubules in anterograde fashion whereas C-kinesins go the retrograde direction (Hirokawa and Noda, 2008). The N-kinesins are further subdivided into 11 classes (N1-11), comprising of 16 families based on phylogenic analysis (Miki et al., 2001). The KIF16 family, which belongs to N-3 kinesins consist of three proteins whose C-terminal sequence and expression pattern differs considerably. This suggests they may perform separate functions (Miki et al., 2001). KIF14, which is described as a novel interactor of Radil in this thesis belongs to this family of kinesins.

1.8.2 Functions of KIFs

A major role of KIFs is intracellular transport of specific cargoes ranging from protein complexes, mRNA-protein complexes to larger organelles (Hirokawa et al., 2009). Although kinesins typically bind to cargoes via scaffold or adaptor proteins, they can also directly interact. Several kinesins also play critical roles in various steps of cell division including bipolar spindle assembly, chromosome alignment, chromosome segregation and cytokinesis (Glotzer, 2003; Moore and Wordeman, 2004; Tanenbaum and Medema, 2010). Because of their importance in
cell division during cancer progression, kinesins have recently attracted attention as a new class of drug target for cancer therapeutics (Huszar et al., 2009).

In addition to their function in cell division kinesins have been also implicated in the control of cell-matrix adhesion, shape and motility (Kaverina et al., 1997; Kopp et al., 2006; Krylyshkina et al., 2002; Rodionov et al., 1993). Blocking kinesin-1 activity with antibody or overexpression of a dominant negative kinesin-1 heavy chain induces a dramatic increase in focal adhesion size while decreasing the overall number of focal contacts (Krylyshkina et al., 2002). Impairment of kinesin-1 activity however does not prevent targeting of adhesions by microtubules (Krylyshkina et al., 2002) but suppresses pseudopodial activity at the leading edge of migrating fibroblasts (Rodionov et al., 1993). Another kinesin family protein, KIF1C in association with non-muscle myosin IIA has been linked to promotion of podosome turnover (podosomes are invasive ventral integrin-associated actin structures, analogous to focal adhesions, found at the base of macrophages) (Kopp et al., 2006). Since myosins directly bind to actin filaments the authors interpreted that the interaction between KIF1C and myosin-IIA is an interface via which microtubules are targeted to these actin rich structures where they can then induce podosome turnover. Given KIFs’ role in trafficking, the above studies allude that the regulation of focal adhesion dynamics is likely mediated by components shuttled as cargoes by the kinesins, which may be responsible for focal adhesion turnover. Shunting the activity of these molecular motors in cells thus hampers adhesion disassembly. However, the identity of the factors delivered and specifically required for these functions were not addressed directly in any of these studies.

Alternative modes of adhesion regulation by KIFs have been also observed. KIF26b was recently shown to be important for adhesion of embryonic mesenchymal cells to the adjacent
ureteric bud during mice kidney development (Uchiyama et al., 2010). KIF26b interacts with non-muscle myosin II and was deemed responsible for augmenting cell adhesion by regulating actin filaments. Additionally, integrin α8 in KIF26b knockout mice failed to localize at the ureteric bud-mesenchymal junctions suggesting KIF26b may be required for the coordination of polarized distribution of α8-integrin; the mechanism however remains unknown. Kinesins have been recently also implicated in the regulation of cancer cell motility. A mitotic kinesin, KIF18A is overexpressed in human breast cancers and associated with increased metastasis (Zhang et al., 2010). Depletion of this kinesin in breast cancer cells delayed mitosis and reduced their migratory potential by stabilizing the microtubules at the leading edge (Zhang et al., 2010).

1.8.3 The Oncogene KIF14

The regions spanning 1q31-1q32 in the long arm of chromosome 1 is frequently gained or amplified in many cancers including that of breast (Corson et al., 2005). According to Progenetix database 43% of 414 breast cancers have either gain or amplification of this region making this region of the chromosome a hotspot for genomic alterations linked with breast cancer etiology (Baudis and Cleary, 2001). The gene encoding for the protein KIF14 resides in this region of the chromosome and is highly expressed in a variety of cancers including breast cancers, retinoblastoma, lung cancers, ovarian cancers etc.(Corson and Gallie, 2006; Corson et al., 2005; Corson et al., 2007; Theriault et al., 2012). High levels of KIF14 expression in cancers have been linked to decreased patient survival (Corson et al., 2005). Moreover, clinical studies using breast tumour samples revealed KIF14 expression is tumour-specific and significantly elevated in more aggressive and invasive tumour cells (Corson and Gallie, 2006). KIF14 was first identified and characterized as an interactor of Protein Regulating Cytokinesis-1 (PRC1) and Citron Kinase in Hela cells (Carleton et al., 2006; Gruneberg et al., 2006). By binding to PRC1 and Citron kinase to the N-terminus and C-terminus respectively KIF14 localizes to the central spindle
midbody of dividing cells where it is required for successful completion of cytokinesis (Gruneberg et al., 2006). Although overexpression of KIF14 has been correlated with increased tumour cell proliferation the mechanism by which these cells may acquire the propensity to migrate and invade more aggressively is not understood.

1.9 Mass Spectrometry Based Proteomics

1.9.1 Proteomics

Proteomics deal with qualitative or quantitative identification of proteins in cells, tissues or biological fluids at a given condition. Proteins in a cell seldom act alone but rather in larger complexes in concert with other proteins in order to perform intricate biological functions. The study of functional proteomics involves the identification of the proteins interacting with a candidate protein when they together perform a given cellular task. This can be performed under normal or pathological conditions thereby understanding the normal cellular functions of these proteins and their dysfunctions in diseases.

One of the technologies widely used today to study proteomics is mass spectrometry, which enables inquiry and characterization of proteins in an unbiased manner providing important insights into cellular processes. The use of mass spectrometry in proteomics has flourished since the development of two “soft” ionization methods: electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) (see below) (Fenn et al., 1989; Tanaka et al., 1988). There are two categories of methods in mass spectrometry based proteomics: “bottom-up” and “top-down” approaches (Chait, 2006). The bottom-up approach involves peptide-level analysis of a proteolytically digested protein mixture, followed by chromatographic separation and tandem mass spectrometry (MS/MS) analysis. The use of liquid chromatography (LC) followed by MS/MS enhance resolving power for complex proteomic samples and enables the
detection of low abundant species. On the other hand, top-down approach uses entire protein samples, which are difficult to separate by LC-MS/MS making their analysis quite challenging. Multiply charged product ions from intact proteins generate very complex spectra introducing ambiguity for data interpretation. Also bioinformatics tools to analyze top-down proteomics data are still primitive compared to those available for bottom-up approaches.

The proper functioning of cells depends largely on dynamic interplay of millions of protein molecules in very specific manners. Hence, analysis of protein complexes is of great importance to interpret protein-protein interactions and to understand their roles in biological systems. To study protein complexes using mass spectrometry-based methods, target proteins and their complexes are isolated by affinity purification protocols such as the tandem affinity purification or immuno-purification and complexes subsequently analyzed by liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) (Gingras et al., 2005).

1.9.2 Tandem Affinity Purification

Tandem Affinity Purification (TAP) schemes optimized to isolate protein complexes were pioneered using yeast, but have since been extended to mammalian cells (Gingras et al., 2005; Rigaut et al., 1999). The original TAP protocol established in Dr. Séraphin’s laboratory used an affinity purification cassette with the TEV protease cleavage site flanked by protein A IgG binding motif and the Calmodulin-Binding Peptide (CBP) affinity tags genetically fused to proteins of interest to efficiently enable their purification and the interacting proteins from cell lysates (Rigaut et al., 1999). Extracts from these cells are purified in a two-step process that minimizes the carryover of abundant or intrinsically sticky proteins that may have low affinity either to the tags or to chromatography matrices.
The TAP method was subsequently adapted for isolation of mammalian protein complexes using Streptavidin Binding Peptide (SBP) and CBP as the affinity tags (Angers et al., 2006a; Angers et al., 2006b). The SBP tag has high affinity for streptavidin, and bound proteins can be efficiently eluted in the presence of biotin (Keefe et al., 2001). Eluted proteins are then taken through a second round of purification using calmodulin beads through binding to the CBP tag (Klevit et al., 1985). Bound protein complexes can be eluted in the presence of EGTA as binding of CBP to calmodulin requires Ca\(^{2+}\) ions (Stofko-Hahn et al., 1992). Eluted proteins can then be run on SDS-PAGE and bands cut and subjected to proteolytic digestion, commonly with trypsin, before analysis of the peptides by mass spectrometry (Rigaut et al., 1999). Alternatively, the eluted proteins can be directly trypsinized followed by LC-MS/MS to ultimately identify interacting proteins (Ahmed et al., 2011; Angers et al., 2006a; Angers et al., 2006b).

1.9.3 Mass Spectrometry

Mass spectrometry is widely used and a powerful analytical tool for measuring the molecular weight of ions based on their mass-to-charge (m/z) ratios. The basic mass spectrometry scheme has three fundamental components: (a) ionization source, (b) mass analyzer and (c) detector. To enhance the resolving power of mass-spectrometry an added level of separation of highly complex samples can be obtained through liquid chromatography (LC) before the ionization. A common LC method used in proteomics is reversed-phase liquid chromatography (RP-LC), which separates peptides on the basis of hydrophobicity. The use of reversed phase column leads to adsorption of hydrophobic molecules onto the hydrophobic solid support in a polar mobile phase. As the polarity of the mobile phase is progressively reduced by using organic solvents, hydrophobic interactions between the solute and the support decrease resulting in desorption. The avidity of adsorption is dictated by the degree of hydrophobicity of the peptides. Higher concentration of organic solvent is required to elute highly hydrophobic peptides. Sever-
al factors including the type of stationary phase used (C4, C8, C18, phenyl, cyano, etc), carbon load, organic solvents, pore size and support material contribute to the selectivity of the RPLC separation (Baczek and Kaliszan, 2009).

Ionization source is the component of mass spectrometer where target materials are ionized. Early on, due to lack of proper ionization methods samples were ionized by electron impact, which was harsh as it could easily break the covalent bonds in molecules producing severely fragmented molecules. This is why mass spectrometry could not be used for bio-molecules until the introduction of soft ionization methods like electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), for which John B. Fenn and Koichi Tanaka won the Nobel Prize in Chemistry in 2002. ESI is considered the softest ionization method where the energy retained by the ions are less than that of MALDI and can be easily coupled to nano-scale LC, thus making it a choice of ionization source for studying protein-protein interactions (Feng et al., 2008). In MALDI, the targets analyzed are embedded together with a matrix. The sample is then pulsed with UV laser thus vaporizing the matrix together with the analytes as gaseous ions, which can then be analyzed by the mass spectrometer. MALDI-coupling time-of-flight (MALDI-TOF) is also used extensively for protein identification (Feng et al., 2008). The mass spectrometer that we used for the studies described in this thesis uses nano-RPLC-ESI, and C18 was the choice of RP column.

After the ionization process, ionized molecules are separated and resolved in mass analyzers according to their $m/z$ ratio. Commonly used mass analyzers are quadrupole, quadrupole ion trap, linear ion trap, time-of-flight, and orbitrap. Quadrupole mass analyzer uses quadrupole radio frequency (RF) field to selectively filter ions of interest based on their $m/z$ ratio. This is closely related to quadrupole ion trap where ions are created and trapped in a 3D quadrupole RF
field and then sequentially ejected for separation. Linear ion trap also works similar to quadrupole ion trap, except ions are trapped in a 2D quadrupole field instead of a 3D field. This gives linear ion traps the advantage of higher injection efficiencies and ion storage capacity than 3D quadrupole ion trap. This is important in a system with continuous ionization source like ESI. Time-of-flight uses electric field to accelerate ionized molecules and measures the time required for them to reach the detector. Since the instrument used to perform the mass spectrometry experiments in this thesis used a linear ion trap, the introduction is restricted to explaining the mode of operation of this particular type of mass analyzer.

The linear quadrupole was originally developed in 1958 by Paul and colleagues for mass spectrometry, and since widely used as mass filters, ion guides and linear ion trap (Douglas, 2009). The electric potential of the linear quadrupole is produced by four parallel precision-machined and precision aligned hyperbolic electrodes (Figure 1-12). Each rod is cut into three sections of 12, 37 and 13mm length, with two of the center rods, called exit rods, having 0.25x30mm slot through which ions are ejected during scan out. The application of the radiofrequency (RF) voltage to the rod pairs create 2D-quadrupole field and when the amplitude of the RF voltage is maintained low, all ions above the minimum $m/z$ ratio are trapped in the radial direction (Douglas, 2009; Douglas et al., 2005). The threshold of minimum $m/z$ ratio is chosen to be greater than that associated with air, water and solvent ions. In 2D quadrupoles the ion motions in the $x$ direction are independent of the motions in $y$ direction and vice versa. During scan out, the RF voltage is increased, leading to ions of increasing $m/z$ ratio becoming successively unstable in the radial direction and ejected from the mass analyzer in order of increasing $m/z$ ratio (Douglas, 2009; Douglas et al., 2005; March, 2009). The ejected ions are then detected upon striking the conversion dynodes on each side of the ion trap (Figure 1-12).
Although mass spectrometry has high separation power to attain high resolution it can be further enhanced by tandem mass spectrometry analysis. Tandem mass spectrometry is achieved by employing two mass analyzers “in space” or “in time” separated by collision cell to provoke peptide fragmentations. Target molecules fragmentations can be achieved through methods like collision-induced dissociation (CID), electron capture dissociation (ECD) or electron transfer dissociation (ETD). CID is the classic and commonly used method for fragmentation of ions and involves the use of neutral gas such as helium or argon for collision (Feng et al., 2008). For “in space” configurations, ions are analyzed sequentially as they travel through the instrument. However, linear ion traps can also be used as standalone tandem MS instruments to set up “in time” configurations. This is the kind that is used in our instrument. Here tandem MS is performed within one analyzer where ions are trapped and consecutively ejected for analysis. In tandem mass spectrometry, the first MS performs a mass scan, followed by selecting ions with given m/z to be fragmented by CID. Upon collision with inert gas the peptide can theoretically break anywhere along the amino acid backbone or in the side chain; however, mass spectrometers are calibrated to induce voltage that excites the peptide ions to the point where they fragment along their backbone (Figure 1-13). A second MS scan can then analyze the m/z of product ions decomposed from specified parent ions. Each collision generates a pair of m/z output; each representing a part of the whole peptide (Figure 1-13). The different peptide fragments produce different m/z peaks creating a fragmentation pattern that is unique to that peptide ion.

The LTQ-XL instrument that we used is programmed for data dependent MS/MS acquisition. The machine is asked to perform one survey scan which records all the different ion species within the ion trap at a given time, followed by further MS/MS analysis of the five most abundant peaks (Figure 1-14). The acquisition software is also programmed so when the same ion (m/z ± 3) is sequentially detected three times it is put in an “exclusion list” for 3 minutes.
During this time no more data is collected for the same ion. This helps prevent highly abundant ions from masking all the other ones.

Once mass spectra are generated from a given run they are subsequently subjected to human NCBI protein sequences containing database search through computer algorithms such as MASCOT, Sonar, or SEQUEST, and the matches are used to assign peptides and proteins. For mass spectrometry analysis in this thesis the accuracy of SEQUEST assignments of peptide and protein sequences from the given MS/MS spectra were estimated by the PeptideProphet™ and ProteinPhophet™ softwares (Keller et al., 2002).
**Figure 1-12. Linear ion trap mass spectrometers.** (A) A general workflow of LC coupled tandem mass spectrometry. (B) Internal components of linear quadrupole ion trap mass spectrometer LTQ-XL (Thermo Scientific). Ionized molecules are collected at the ion sweep cone, and guided along the instrument to the linear quadrupole mass analyzer by the various “ion guides” operated by application of RF voltages. (C) Schematic of a linear ion trap quadrupole rods. The interior face of the four rods form hyperbolic shapes. (D) Schematic of mass analyzer operation. Ions of various masses enter the linear quadrupole ion trap and during scan out ions are ejected from the ion trap through slots in the exit rod according to increasing m/z ratio. Ions then strike the conversion dynodes placed on either sides of the mass analyzer where they are detected.

Adapted from LTX-XL Hardware Manual (Thermo Scientific) and Douglas et al. 2005.
Initial peptide detected in MS1: L-M-S-V-K-Y-V-A-R (m/z)

Possible product ions subsequently detected by MS2 following fragmentation of initial peptide:

<table>
<thead>
<tr>
<th>B-ion series</th>
<th>Y-ion series</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-M-S-V-K</td>
<td>Y-V-A-R</td>
</tr>
<tr>
<td>L-M-S-V-K-Y</td>
<td>V-A-R</td>
</tr>
<tr>
<td>L-M-S-V-K-Y-V</td>
<td>A-R</td>
</tr>
<tr>
<td>L-M-S-V-K-Y-V-A</td>
<td>R</td>
</tr>
</tbody>
</table>

Figure 1-13. Peptide fragmentation and detection by tandem mass spectrometry. (A) Shown is a schematic of potential breaking points in a peptide during collision induced fragmentation in tandem mass spectrometry. (B) An example of a peptide detected by first MS, and the ion species detected by second MS following fragmentation. Mass scan by the second mass spectrometry then create peaks which is unique to each peptide.
A. MS scan graph over time showing peptides eluted.

Sample peptides present at this peak shown below.

B. MS1 scan of peptide ions from a particular peak at 24.49 minutes shown above.

Major peaks circled

C. MS2 scan of one of the five most abundant peptide ions following fragmentation from MS1 scan.

Figure 1-14. Sample MS scan graphs. (A) Shows peptides eluted and scanned by MS1 over time. (B) Different peptides present at a sample peak shown in A when scanned by first MS. Five most abundant peptides were chosen for further analysis after collision induced fragmentation. (C) Peptide fragmentation profile of one of the most abundant peaks in B after fragmentation as scanned by second MS in tandem.
1.9.4 Workflow of MS data analysis

To investigate novel interactors of a target protein, constructs expressing affinity tags are expressed in cells, followed by cell lysate extraction and purification of the tagged protein together with its associated proteins. The purified samples are treated with proteolytic enzymes; commonly trypsin, and the resultant peptides analyzed by LC-MS/MS. Once the sample is analyzed by the mass spectrometer, thousands of spectra are searched against theoretical tryptic databases to assign peptides and ultimately the proteins.

The mass spectra generated are analyzed by a series of three computer algorithms: SEQUEST, PeptideProphet™ and ProteinProphet™ integrated into Sorcerer™2 data analysis system. First, SEQUEST takes the mass spectra generated from the samples analyzed and compares them to theoretical spectra database generated by in silico tryptic digestion of proteins (Yates et al., 1995). The comparison between the observed and theoretical spectra yields a set of scores that is then used by PeptideProphet™ to designate each spectrum a peptide sequence and its probability of being the correct sequence using a statistical model (Keller et al., 2002). Once peptides probability scores have been calculated for the assigned peptides the data is passed on to the next algorithm, such as ProteinProphet™. ProteinProphet™ then substantiates the peptides by assigning them to proteins or protein groups based on statistical models to provide a probability score that denotes the likelihood of the given protein being correct or incorrect (Nesvizhskii et al., 2003). A flowchart of different steps in our mass spectrometry analysis workflow is shown in Figure 1-15.
Tandem affinity purification

Trypsin digestion

LC-MS/MS

Mass spectrometry data analysis

SEQUEST performs cross-correlation of sample spectra to theoretical spectra based on tryptic peptide database

Outputs cross correlation scores

PeptideProphet™ performs statistical analysis to assign probability scores for the likelihood that assigned peptides for MS/MS spectra are correct.

Using statistical models ProteinProphet™ assign the peptides determined by PeptideProphet™ to proteins or protein groups and calculates the probability of a correct match

**Figure 1-15. Workflow of MS data analysis.** The flowchart above shows schematic of logical steps taken to compute the data acquired by the mass spectrometer.
1.10 Phage Display to Explore Protein-Protein Interactions

The revolutionary potential in monoclonal antibody production for human therapeutics was the motivation behind development of phage display methods. In the mid-1980s Dr. George Smith made the discovery that certain bacteriophage linked their genotype directly with phenotype by expressing the gene encoded by the encapsulated nucleic acid as capsid proteins (Smith, 1985). In M13 filamentous bacteriophage when a foreign nucleic acid sequence is inserted immediately upstream of the gene that codes for a coat protein (pIII), they express the amino acids corresponding to the nucleic acid sequence in the amino terminus of the capsid protein (Figure 1-16a). Phage display technology have been subsequently adapted to create peptide libraries and used to search for potential binding sequences for proteins or protein domains, identify receptor agonists and antagonist, enzyme substrates etc. Phage displayed peptide libraries comprise libraries of random peptide sequences (~$10^{10}$ random peptides) of defined length, typically between 6 and 12 amino acids, which are expressed as fusion to pIII.

The phage display methods have been also used to study the peptide recognition domains of proteins such as PDZ and Src-homology 3 (SH3) domains, which have helped unravel the diverse protein-protein interactions otherwise difficult to define using other complementary methods. The C-terminally displayed peptide libraries generated with M13 and lambda phage have been successfully used to explore a range of potential PDZ domain binders for INADL, MAGI-3, Erbin, PSD-95 and many more (Fuh et al., 2000; Sidhu et al., 2003a; Sidhu et al., 2003b; Skelton et al., 2003; Tonikian et al., 2008). An improved version of the technology now displays the C-terminal peptides in lambda phage that results in high-valency display on the caspids, thus enabling the selection of low affinity binders (Skelton et al., 2003).
In the phage display method selection of ligands for the target protein requires enrichment of specific binding phages from the large excess of non-specific or non-binders. This is achieved by multiple rounds of “panning”. Panning is the process where phages are incubated with the target, followed by washing to remove non-specific or non-binders, elution of specific binders, and re-infection of *Escherichia coli* (*E-coli*) for propagation of the binding phages. The process is repeat several times to enrich for specific binders, upon which bacterial colonies can be sequenced to reveal the DNA sequence corresponding to the peptides that bound specifically to the bait (Fuh et al., 2000) (Figure 1-16b). Once the sequences are known for all the binders they are aligned to generate a “consensus” sequence using computer algorithms.
A library of phage ($10^{10}$), each displaying a different peptide sequence, is exposed to a plate coated with the target. Unbound phage are washed away, specifically bound phage are eluted by lowering pH. Eluted pool of phage is amplified and the process repeated for a total of 3-4 times to enrich for specific binders. After 3-4 rounds individual clones are isolated and sequenced.

Figure 1-16. Phage Display. (A) Fusion of DNA sequence upstream of sequence encoding pIII coat protein of bacteriophage is expressed as a fusion protein and displayed on the outside of the phage. (B) Shown is a workflow of a typical phage display experiment. The bait is immobilized on assay plate and incubated with a library of phage displaying ~ 10 billion combinations 6 to 12 long amino acids peptides. Upon phage binding, the plate is washed, and bound phage eluted by either lowering the pH of the buffer or increasing salt concentrations. Eluted phage are amplified in bacteria and the panning process repeated to enrich for positive binders. After 3-4 round of panning clones are isolated and sequenced to reveal the peptide sequence that bound to the bait.
1.11 Rationale and Objectives

While Gβγ subunits of heterotrimeric G-protein have been implicated in cell motility downstream of GPCR activation our understanding of the mechanism by which they function in this context is poor. One attractive hypothesis is Gβγ can somehow regulate integrin-mediated adhesions of cells to the extracellular matrix. In the first chapter of this thesis I took functional proteomic approaches to identify novel interactors of Gβγ. By doing so we identified a novel Gβγ and Rap1 interacting protein, Radil, and characterized the function of the protein complex in cell adhesion and migration.
Chapter 2:
G Protein βγ Subunits Regulate Cell Adhesion through Rap1α and its Effector Radil

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All experiments and data analysis was performed by Syed M. Ahmed except for tandem affinity purification and mass spectrometry of Gγ subunit which was done by Dr. Avais M. Daulat. Alexandre Meunier carried out some of the replicates for mass spectrometry analysis of Gβ and Radil.
2.1 Abstract

The activation of several G protein coupled receptors is known to regulate the adhesive properties of cells in different contexts. Here, we reveal that Gβγ subunits of heterotrimeric G proteins regulate cell-matrix adhesiveness by activating Rap1a-dependent inside-out signals and integrins activation. We show that Gβγ subunits enter in a protein complex with activated Rap1a and its effector Radil and establish that this complex is required downstream of receptor stimulation for the activation of integrins and the positive modulation of cell-matrix adhesiveness. Moreover, we demonstrate that Gβγ and activated Rap1a promote the translocation of Radil to the plasma membrane at sites of cell-matrix contacts. These results add to the molecular understanding of how GPCRs impinge on cell adhesion and suggest that the Gβγ-Rap1-Radil complex plays important roles in this process.

2.2 Introduction

G Protein Coupled Receptors (GPCRs) form the largest family of cell surface signal transducing molecules in vertebrates. The regulation of cell migration as well as cell-cell and cell-matrix adhesion by GPCRs is essential for normal embryonic development (Carmona et al., 2008; Doitsidou et al., 2002; Scott et al., 2007) and is important for several cellular processes in adults such as immune response (Hopken et al., 1996; Servant et al., 2000; Snyderman and Goetzl, 1981), hemostasis (Kahn et al., 1998; Sambrano et al., 2001) and vascular integrity (Carmona et al., 2008; Komarova et al., 2007). The activation of chemokine receptors, for example, has been well demonstrated to regulate directional cell migration and cell-matrix adhesion in the context of various immune functions (Baggiolini, 1998) but also during several other instances of cell motility (Askari et al., 2009; Tiveron and Cremer, 2008). The activation of platelets at sites of vascular wall injury is another context where GPCR signalling is involved in
regulating cell adhesion with the extracellular matrix (Offermanns, 2006). Revealing the importance of these processes, defects in GPCR signalling downstream of pro-migratory or adhesive signals have been linked with different human diseases including inflammation (Druey, 2009; Fraser, 2008; Lattin et al., 2007; Rozengurt, 2007) and cancer metastasis (Dankort et al., 2001; Mills and Moolenaar, 2003; Muller et al., 2001).

Because pertussis toxin-sensitive $G_{\alpha i}$-activated pathways are thought to play important roles downstream of GPCRs during migration and adhesion, the $G_{\beta\gamma}$ subunits of the heterotrimeric G proteins can be predicted to play integral signalling functions during these processes. Supporting this, $G_{\beta\gamma}$ subunits are known to associate and potentiate the activity of the p110$\beta$ and $\gamma$ isotypes of phosphoinositide-3 kinase (PI3K) that regulate directed cell migration (Guillermet-Guibert et al., 2008; Stoyanov et al., 1995) by facilitating the polarized recruitment of downstream signalling proteins which lead to cytoskeleton remodeling. The discovery that PLIC-1 interferes with chemokine receptors-mediated chemotaxis by sequestering $G_{\beta\gamma}$ and hindering its normal cellular functions as well as evidence implicating $G_{\beta\gamma}$ in cell invasion and spreading further highlight the importance of $G_{\beta\gamma}$ in cell movement (Faivre et al., 2001; N'Diaye and Brown, 2003). However, the precise molecular mechanisms by which GPCRs and $G_{\beta\gamma}$ impinge on cell motility remain incompletely understood especially with respect to the dynamic control of cell adhesiveness.

In all instances of cell motility, cells constantly need to alter their interactive associations with the extracellular matrix (ECM) in response to environmental cues. Central to this process is the regulation of integrin avidity and affinity for matrix protein components (Askari et al., 2009; Ginsberg et al., 2005; Takagi et al., 2002). The small GTP binding protein Rap1 is implicated in the “inside-out” signalling that rapidly leads to integrin activation. For example, overexpression...
of the constitutively active mutant Rap1aQ63E leads to increased cell adhesion (Lafuente et al., 2007) and integrin activation (Lafuente et al., 2004) by inducing a change in the conformation of the extracellular region of integrins. This unclasping of the inhibitory interaction between the α and β integrins switches their affinity for the ECM to a high affinity state. Although Rap1-induced inside-out signalling has been deemed important in the context of GPCR signalling during platelet and leukocyte activation (Katagiri et al., 2003; Laudanna et al., 2002; Ley et al., 2007; Offermanns, 2006; Watanabe et al., 2008; Zarbock et al., 2007), how this signalling and integrin activation are regulated by GPCRs and whether it extends to other cellular contexts is not well understood.

Despite significant indirect evidence positioning Gβγ subunits as functional regulators of cell motility and adhesion downstream of GPCR activation, our understanding of the molecular mechanisms underlying their roles in these processes is incomplete. In the present study we thus employed a functional proteomic approach to further interrogate how Gβγ subunits mediate their actions. We identify Rap1a and its effector Radil as novel Gβγ-associated proteins and present evidence functionally implicating this protein complex downstream of GPCR activation for the control of cell-matrix adhesion.

2.3 Experimental procedures

2.3.1 Plasmid constructs and reagents

Human Gβ2, Radil and Rap1GAP cDNAs were cloned from a human brain cDNA library in the pGlue or pIRES-puro-Flag backbone vector (Angers et al., 2006b). All PCR amplified regions were verified by DNA sequencing. pCGN HA-Rap1aQ63E was obtained from J. Cooper (University of Washington in Seattle, WA), and pMT2 Rap1aG12V and pCDNA3.1 Flag-HA-AF6 were provided by J.L. Bos (University Medical Center Utrecht, The Netherlands). Detailed
Formyl-Met-Leu-Phe peptide (fMLP) was purchased from Biomol. Pertussis toxin was purchased from Tocris Bioscience. Antibodies were purchased from the following vendors: α-HA.11 (Covance), α-FLAG (Sigma), α-Gβ (T20) (Santa Cruz Biotechnology), α-Rap1a (C-17) (Santa Cruz Biotechnology) and α-Erk1/2 (Cell Signalling), α-CD29 mAb (9EG7) (BD Transduction Laboratories), α-GFP (Covance and University of Alberta), α-tubulin (Sigma) and α-Na-K-ATpase (Affinity Bioreagents).

2.3.2 Tissue culture and transfection

HEK293T and HT1080 cells were grown in Dulbecco’s modified Eagle medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS; Sigma) and penicillin/streptomycin (Sigma) in a 37°C humidified incubator with 5% CO₂. Stable cell lines were generated by calcium phosphate transfection followed by puromycin selection (2µg/ml). cDNA transfections in HT1080 cells were done using Lipofectamine 2000 (Invitrogen) at 1:2 ratio (cDNA : Lipofectamine 2000) or polyethylenimine (PEI) at 1:3 ratio at 70% cells confluency with a typical 80-90% transfection efficiency. siRNAs were delivered at a finale concentration of 50nM in HT1080 cells by reverse transfection using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer protocol. The siGenome Non-Targeting siRNA (control siRNA) and human Radil ON-TARGET plus SMARTpool siRNA (hRadil siRNA pool) were purchased from Thermo Scientific and the single siRNA targeting human Radil (hRadil siRNA #1) was purchased from Ambion Inc. The sequences for each siRNA in the ON-TARGET plus SMARTpool (Thermo Scientific) for human Radil are: GCACAGGAACCCACUACAA, GAGCGUACUCUUCCCUUGA, CGUCAUGGCGGGAAGAAGA, GAAGGUUUAUCCCGGAGGU. The sequence for human Radil siRNA # 1 (Ambion) is:
GGUUUAUCCCGAGGUUUG. The sequence for siGENOME Non-Targeting siRNA # 4 (Thermo Scientific) is: AUGAACGUGAAUUGCUCAAUU.

2.3.3 Production of Radil antibody

A rabbit polyclonal antibody was raised against full length recombinant human Radil protein. Human Radil coding sequence was cloned into pGEX vector (Promega) downstream of GST. Proteins were expressed in BL-21 E-Coli strain upon stimulation with IPTG (500µM; Sigma) in a shaker at room temperature (240 RPM) overnight. The bacterial pellet was lysed by sonication in buffer containing 20mM Tris-HCL (pH 8.0), 200mM NaCl and 5mM DTT, 1mM PMSF and proteins purified on Glutathione Sepharose 4B (GE Healthcare). The purity of the purified Radil protein was confirmed by SDS-PAGE and mass spectrometry analysis, and provided to Covance Custom Immunology Services for antibody production. The final antisera was purified first by negative selection by running the crude antisera through GST proteins immobilized on a Glutathione Sepharose 4B column followed by affinity purification on a GST-Radil-Glutathione Sepharose 4B column. The antibody was characterized for antigen specificity by ELISA (Covance Custom Immunology Services) and by western blotting lysates from Radil siRNA treated cells (See Appendix 1 for ELISA data).

2.3.4 Tandem affinity purification and mass spectrometry

HEK293T cells (2×10^8) expressing TAP-tagged Gβ2, Gγ2 or Radil constructs were used for the tandem-affinity purification procedure as described previously (Angers et al., 2006b). Briefly, cell were lysed and solubilised in TAP lysis buffer (0.1% Igepal CA 630, 10% glycerol, 50mM Hepes-NaOH; pH 8.0, 150mM NaCl, 2mM EDTA, 2mM DTT, 10mM NaF, 0.25mM NaOVO₃, 50mM β-glycerophosphate, and protease inhibitor cocktail (Calbiochem)) and extracts were incubated at 4 °C with 100µl packed streptavidin (GE Healthcare) or α-FLAG M2 (Sigma)
resin. The resin was washed and protein complexes were then eluted from the streptavidin resin in calmodulin binding buffer (50mM Hepes-NaOH, pH 8.0, 150mM NaCl, 10mM β-
marcaptoethanol, 1mM MgOAc, 1mM Imidazole, 0.1% NP-40) supplemented with 2mM biotin (Sigma). The second round of affinity purification was performed using 100 μl of calmodulin resin (GE Healthcare) followed by elution in calmodulin elution buffer (25mM EGTA, 50mM ammonium bicarbonate). α-FLAG immunoprecipitation for mass-spectrometry analysis purified proteins were eluted from the beads using 500mM ammonium hydroxide at pH 11.0. The proteins in the complex were reduced in 25mM dithiothreitol and alkylated using 100mM iodoacetamide (Sigma), and brought to 1mM CaCl₂. The proteins were then directly digested with sequenced-grade trypsin (Promega). The resulting peptide mixture was then analyzed by LC–MS/MS using LTQ-XL Linear Ion Trap Mass Spectrometer (Thermo Scientific). The acquired tandem mass spectra were searched against a FASTA file containing the human NCBI sequences using a normalized implementation of SEQUEST running on the Sorcerer platform (Sage-N Research). The resulting peptide identifications returned by SEQUEST were filtered and assembled into protein identifications using peptide and protein prophets (ISB, Seattle).

2.3.5 Affinity purification, immunoprecipitation and western blot

Cells were lysed (0.5% Igepal CA 630, 20mM Tris-HCl, pH 7.5, 150mM NaCl, 2mM EDTA and protease inhibitor) and incubated at 4°C for 20 mins to solubilise the proteins. Affinity purification and immunoprecipitations were performed using streptavidin resin (GE Healthcare) or FLAG-M2 beads (Sigma) for 1 hour at 4 °C. After extensive washes with lysis buffer the beads were eluted with 2X Laemmli sample buffer and heated at 95°C for 5 mins in the presence of β-mercaptoethanol (Sigma). Whole cell lysates or purified protein samples were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Nitrocel-
lulose Transfer Membranes (Pall Corporation). Western blotting were performed with antibodies as indicated in the figure legends, followed by chemiluminescent detection with SuperSignal West Pico (Thermo Scientific) and exposed on film.

2.3.6 Immunocytochemistry and confocal microscopy

HEK293T and HT1080 stably expressing Venus-Radil were transfected with HA-Rap1a, HA-Rap1aG12V, HA-Rap1aQ63E or Flag-Gβ2 and Gγ2 as indicated. For intracellular protein staining, cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in phosphate buffered saline (PBS) supplemented with 10% normal donkey serum. Samples were then immunostained with α-HA (HA.11, Covance) or α-FLAG (Sigma) specific mouse monoclonal antibodies in PBS supplemented with 1% normal donkey serum. Cells were subsequently labeled with secondary goat-anti-mouse antibody conjugated to Alexa Fluor 594 (Invitrogen) to visualize HA-tagged Rap1a or FLAG-tagged Gβ2. The coverslips were mounted onto slides using Vectashield mounting media (Vector Laboratories). Cells were visualized and images acquired with a Carl Zeiss LSM510 confocal microscope using a Plan-Apochromat 63X/1.4 oil immersion objective. Lasers at 488nm and 543nm wavelength were fired independently using the multi-track function of LSM510. Uncompressed images were processed using Zeiss LSM Image browser version 4.2. To magnify portions of the plasma membrane to depict co-localization, images were cropped as designated (in white boxes) using Adobe Photoshop CS3. For fMLP stimulation experiments, HT1080 cells stably expressing Venus-Radil were serum starved for 24 hours, and stimulated with fMLP (1µM) for the indicated times at 37°C. Following treatments, cells were immediately chilled on ice and washed once with ice cold PBS followed by fixation in 4% paraformaldehyde for 30 mins at room temperature.
2.3.7 Subcellular fractionation

HT1080 cells transiently transfected with the indicated cDNAs in 100mm tissue culture dishes were washed with phosphate saline buffer (PBS); pH7.4 and lysed by Dounce homogenization (20 strokes) in hypotonic buffer (5mM Tris-HCl, 2mM EDTA). A small fraction was aliquoted from each sample and labelled as inputs. The lysates were centrifuged at 1000xg for 5 mins at 4ºC to remove unbroken cells and nuclei. The post-nuclear supernatant was transferred to a new tube followed by centrifugation at 100,000xg (34,100 RPM in Ti90 rotor) for 30 mins. The supernatant was removed, and the pellet was resuspended in the above buffer and subjected to a second round of centrifugation at 100,000xg for 30 mins. The supernatant was discarded and the pellet containing the membrane fraction was resuspended in the above lysis buffer. Equal amounts of proteins for each the input and membrane fractions were then resolved by SDS-PAGE for western blot analysis. An antibody against Na-K-ATPase was used as internal loading control.

2.3.8 Cell spreading assay

HT1080 cells were plated on glass coverslips and transfected with GFP alone, Venus-Rap1GAP or GFP together with different expression plasmids coding for HA-Rap1aQ63E, HA-Radil or HA-Gb and untagged Gγ. 48 hours after transfections cells were fixed in 4% paraformaldehyde and immunostained with α-HA monoclonal antibody to label the overexpressed proteins, followed by secondary detection with goat-anti-mouse conjugated Alexa 594. Cells were mounted on slides and visualized under 63X oil immersion lens using a Carl Zeiss LSM 510 confocal microscope. Spreading of cells co-expressing GFP and the overexpressed protein of interest was quantified by measuring the area of each cells using Image J software. To acquire more quantitative and real-time insights into cell adhesion and spreading we employed change in im-
pedance as a measure of degree of cell attachment and spreading using the xCelligence system (Roche Applied Science). HT1080 cells were transfected with the different plasmids. 48 hours after transfections, cells were trypsinized and resuspended in DMEM containing 10% FBS. 25,000 cells from each condition were seeded onto a 96-well microtitre xCELLigence assay plate (E-Plate) (ACEA Biosciences Inc.), and placed on the Real-time xCELLigence Cell Analyzer (Roche) platform at 37°C to measure the “Cell Index” every 5 mins. The cell index unit is defined as \((R_n-R_b)/15\); where \(R_n\) is the cell-electrode impedance of the well when it contains cells and \(R_b\) is the background impedance of the well with the media alone.

### 2.3.9 Cell adhesion assay

96-well flat-bottom plates (BD Biosciences) were coated with 5\(\mu\)g/ml of Fibronectin from bovine plasma (Sigma) for 16 hours at 4°C. Wells were washed and blocked with a solution of 2% filtered BSA in PBS for 1 hour at room temperature. 48H after transfection with cDNA or siRNAs, HT1080 cells were dissociated from culture plates with trypsin, washed twice with PBS and resuspended in serum free DMEM supplemented with 0.1% BSA and 20mM HEPES, pH 7.4. Before the adhesion assay, the cells were incubated at room temperature for one hour on a nutating shaker. Where indicated, cells were incubated at 37°C for 1 hour with the indicated drugs. For pertussis toxin (PTX) treatment, cells were incubated overnight in 10% FBS/DMEM media containing 100ng/ml PTX. Cells were plated in triplicates at a density of 2.5 x 10^4 per well and allowed to adhere for 8 mins at 37°C followed by washing with warm 0.2% BSA in PBS. For each conditions input controls were let to adhere for 30 mins followed by aspiration of the media and fixation. Cells were fixed with gluteraldehyde (Sigma) for 20 mins and stained with hematoxyline Gill’s No.1 reagent (Sigma) followed by washing with acid alcohol (0.5% hydrochloric acid in 70% ethanol) to remove residual stain and 0.04% ammonium hydrox-
ide for color maturation. Cell adhesion was quantified by taking a minimum of 3 random pictures per well. Cells in each picture were then counted using an automated macro algorithm created in ImageJ ver. 1.41 software.

2.3.10 Integrin activation assay

HT1080 cells were re-suspended as described for adhesion assay, and let to recover at 37°C for 45 mins. Transfected or drug treated cells were incubated for 1 hour in the presence of α-CD29 (9EG7) antibody (BD Biosciences). Cells were washed in PBS three times and incubated in the presence of Dylight™-488-conjugated Donkey α-Rat IgG (Jackson ImmunoResearch Laboratories) for 45 mins, followed by three washes in PBS. Cells were then fixed in 4% paraformaldehyde for 15 mins and resuspended in PBS. Flow cytometry analysis was performed on a Beckman Coulter Cytomics FC500 MPL analyzer using the CXP 2.2 software. Post-acquisition data analysis was done with FlowJo Version 7.2.5 software (Tree Star Inc).

2.3.11 Rap activity assay

HT1080 cells were serum starved for 16h, followed by stimulation with 100nM fMLP for different time points as indicated. Following three washes with PBS, the cells were lysed in TAP lysis buffer containing protease and phosphatase inhibitors (10mM NaF, 0.25mM NaOVO3, 50mM β-glycerophosphate). 800μg of proteins was then incubated with GST-RalGDS-RBD coupled to Glutathione Sepharose 4B beads for 1 hour at 4°C. Beads were then washed three times in TAP lysis buffer and bound proteins eluted in 15μl of 2X Laemmli buffer containing β-mercaptoethanol (Sigma) and samples analyzed by western blot analysis using α-Rap1a antibodies. A fraction of whole cell lysate was also analyzed by western blot to assess total Rap1a.
2.3.12 Statistical Analysis

Data, reported as ±S.E.M. were analyzed by analysis of variance (ANOVA) followed by Tukey’s post-hoc multiple comparison tests (Graphpad Prism 5 software). Statistical significance was measured at $p < 0.05$ for all analysis.
2.4 Results

2.4.1 Proteomic identification of Radil and Rap1a as novel effectors of Gβγ subunits of heterotrimeric G-proteins

To identify novel effectors of Gβγ subunits of heterotrimeric G-proteins, Gβ2 and Gγ2 cDNAs were cloned downstream of Streptavidin- and Calmodulin-binding peptides (Strep and CBP respectively) in the pGLUE vector (Angers et al., 2006a) and stable human embryonic kidney (HEK293T) cells expressing low levels of Gβ or Gγ fusion proteins were derived. Extracts from these cells were subjected to tandem-affinity chromatography to isolate Gβ2 and Gγ2 protein complexes. These protein complexes were digested in solution with trypsin and the resulting peptide mixture analyzed by liquid chromatography-tandem-mass spectrometry (LC-MS/MS). The detailed list of proteins identified in the complexes is listed in the Table 2-1 and schematically represented in Figure 2-1A. Many peptides corresponding to several Gα subunits were predictably found in both Gβ and Gγ purified complexes. Furthermore, most Gγ subunits and Gβ subunits were found to associate with Gβ2 and Gγ2 respectively. Based on the total number of peptides identified and derived sequence coverage, the data also support previous reports that DnaJ (Dupre et al., 2007; Magga et al., 2000), the TCP complex (Humrich et al., 2005) and phosducin-like proteins (Willardson and Howlett, 2007) associate with either fully formed or nascent Gβγ subunits.

In terms of total peptides identified, the recently identified Rap effector Radil (Smolen et al., 2007) was one of the most abundant proteins found in both Gβ2 and Gγ2 protein complexes. Of note, a few peptides attributed to Rap1a were also present in the Gβ2 pull-down experiment (Figure 2-1A and Table 2-1). The reciprocal analysis of Radil protein complexes by mass spectrometry agreed with the previous showing that Radil interacts with the Rap family of small
GTPases (Smolen et al., 2007) and confirmed the association of Radil with Gβγ heterodimers since several peptides corresponding to the different Rap isoforms as well as Gβ and Gγ subunits were identified (Figure 2-1A and Table 2-1). The Radil affinity-purification/mass spectrometry experiments were performed in HEK293T and HT1080 fibrosarcoma cells with similar results (Table 2-1). Co-affinity purification and western blot experiments were then performed to support and validate the mass spectrometry data. Lysates from control HEK293T cells or from cells stably expressing Strep-HA-Gγ2 or Strep-HA-Radil were subjected to affinity purification using sepharose-streptavidin followed by western blotting using antibodies specific to Radil, HA, Gβ or Rap1. Confirming the mass spectrometry results, endogenous Gβ and Rap1 were detected in purified Radil complexes (Figure 2-1B, top panel) and endogenous Radil and Gβ proteins were detected in Gγ2 samples (Figure 2-1B, bottom panel).

Radil is a protein of 1075 amino acids composed of a RA (Ras-associating) domain known to bind small GTP binding proteins of the Ras family, a central DIL (Diluted) domain of unknown function and a PDZ domain at the C-terminus (Figure 2-1C). To test for specificity of association between Radil and Gβγ, the interaction between AF6 and Gβγ was addressed (Figure 2-1C). AF6 is a protein related to Radil sharing its domain architecture and has been similarly reported to interact with Rap1a and play important roles in cell adhesion (Kooistra et al., 2007; Su et al., 2003; Zhang et al., 2005). Whereas endogenous Gβ could be co-immunoprecipitated with Radil, it was absent from AF6 or control immunoprecipitates (Figure 2-1C, compare lane 6 with lanes 4 and 5). We conclude that the Rap effector Radil is a novel Gβγ-associated protein.
Figure 2-1. Identification of Radil as a novel interactor of Gβγ-subunits of heterotrimeric G proteins.
(A) Shown is the protein-protein interaction network of Gβ2, Gγ2 and Radil. Single headed arrows represent interactions found in Gβ2 (red), Gγ2 (blue) and Radil (purple) pull-down experiments, color coded according to the color of the bait. Dark double sided arrows represent proteins reciprocally identified using the other as the bait. Gβ2 (n=3), Gγ2 (n=2) and Radil pull-downs (n=2) were performed in HEK293T and HT1080 cells. Analysis of the tandem affinity purified Gβ2 or Gγ2 protein complexes using mass spectrometry reveals several known Gβγ interactors. The small GTP binding protein Rap1a and the newly characterized protein Radil were also identified in the Gβ2 complexes. The reciprocal analysis of Radil protein complexes confirmed Radil as a Gβγ-associated protein and also revealed that it binds small G proteins of the Ras family. (B) Streptavidin affinity purification of Strep-HA-Radil (top panel) and Strep-HA-Gγ2 (bottom panel) and immunodetection of co-purified endogenous Gβ, Rap1a or Radil as indicated validates the mass spectrometry results. (C) Left panel, HEK293T cells were transiently transfected with expression vectors coding for FLAG-GFP, FLAG-Radil or the closely related FLAG-AF6 and proteins were immunoprecipitated using α-FLAG M2 conjugated agarose beads followed by western blot with α-FLAG (top-panel) or α-Gβ (bottom panel) antibodies (n=3). Gβ co-immunoprecipitates with FLAG-Radil but not with FLAG-GFP or FLAG-AF6. Right panel, Schematic representation of Radil and AF6 proteins showing the similarity between the two proteins containing RA, DIL and PDZ domains.
2.4.2 Radil recruits G\(\beta\gamma\) and activated Rap1a in the same protein complex

Since only a few peptides for Rap1a were identified in the G\(\beta\) and G\(\gamma\) pull-downs and several hundred in Radil complexes, the interaction between G\(\beta\) and Rap1 is likely indirect and mediated by Radil. To examine this possibility, G\(\beta2\) was purified from cells exogenously expressing Rap1 alone or from cells overexpressing both Rap1 and Radil. Whereas trace amounts of Rap1 were co-purified with G\(\beta2\) in cell expressing these proteins, the overexpression of Radil strongly promoted the association of Rap1 with G\(\beta2\) (Figure 2-2A, compare lanes 2 and 3). Radil was previously shown to selectively interact with the activated GTP-bound form of Rap1 and exhibit negligible affinity for its inactive GDP-bound form (Smolen et al., 2007). To determine if the activation state of Rap1 is important for the binding of G\(\beta\) to Radil we first established that overexpression of Rap1GAP, a GTPase activating protein (GAP) specific for Rap1 (Jeon et al., 2007a; Rubinfeld et al., 1991), could block the association of Radil with Rap1a (Figure 2-2B, compare lanes 2 and 3). Similarly, whereas G\(\beta\) is efficiently co-purified with Radil in control cells, this association is markedly attenuated in Rap1GAP overexpressing cells (Figure 2-2B). We also performed the reciprocal experiment and showed that expression of the constitutively activated Rap1aQ63E promoted the association of G\(\beta\gamma\) with Radil (Figure 2-2C). We conclude that following Rap1 activation, Radil interacts with Rap1 and can recruit G\(\beta\gamma\) into the complex.
Figure 2-2. Interaction between Gβγ, Rap1a and Radil.

(A) Radil physically connects Gγ2 and Rap1a. HEK293T cells were transfected with different plasmid combinations coding for Strep-HA-Gβ2, Gγ2, HA-Rap1a and Venus-Radil. Strep-HA-Gβ2 containing complexes were affinity purified with streptavidin-sepharose beads and the association with Rap1a and Radil was monitored by western blot with α-HA and α-GFP antibodies respectively. The efficiency of Gβ2 purification and expression was also followed by western blot using HA antibodies. A fraction of the lysates for each samples was probed with α-HA or α-GFP antibodies to assess protein expression. The association of Rap1a with Gβ2 was enhanced when Radil was overexpressed (compare lanes 2 and 3) (n=3). *Non-specific band.

(B) Formation of Gβγ-Rap1a-Radil complex requires active Rap1a. HEK293T cells were transfected with expression plasmids for Strep-HA-Radil and HA-Rap1a only (lane 2) or together with a vector coding for FLAG-Rap1GAP (lane 3). Strep-HA-Radil was purified using streptavidin-sepharose beads and its association with Rap1 and Gβ monitored by western blot using α-HA and α-Gβ antibodies respectively. The expression of Rap1GAP was followed using α-FLAG antibodies. While HA-Rap1a and Gβ bound to Strep-HA-Radil (lane 2), these interactions were compromised in the presence of overexpressed FLAG-Rap1GAP (lane 3) (n=4).

(C) Expression of a constitutively active Rap1a mutant (Rap1aQ63E) promotes the interaction of Gβ with Radil. Strep-HA-Radil expressing stable cells were transfected or not with HA-Rap1aQ63E. 48hours after transfection, Radil was affinity purified with streptavidin-sepharose beads for 1.5 hours and the eluates were analyzed by western blot using the indicated antibodies. Untransfected HEK293T cells were used as negative control for streptavidin purification. AP: Affinity purification. *M*: Molecular weights, K, ×1000.
2.4.3 Gβ and Rap1 promote the recruitment of Radil at sites of cell-matrix adhesion

The activation of several GPCRs has previously been shown to activate Rap1a in multiple contexts (Bos et al., 1997; Carey et al., 2000; Citro et al., 2007; Durand et al., 2006; Feller et al., 1998; McLeod et al., 2002; Watanabe et al., 2008). Our results describing Gβγ forming a protein complex with Rap1a and its effector Radil is consistent with Gβγ participating in Rap1a-dependent processes. Upon activation, Rap1 translocates from a cytoplasmic pool to the plasma membrane (Bivona et al., 2004; Li et al., 2006). Rap effectors are subsequently recruited to this activated membrane pool of Rap1 to in turn exert their biological functions (Arthur et al., 2004; Boettner et al., 2000). To test if the subcellular localization of Radil changes upon Rap1 activation and Gβγ overexpression we performed subcellular localization microscopy experiments in HT1080 fibrosarcoma cells stably expressing a Venus-Radil fusion protein. We chose this model system since Radil expression is known to be induced following an oncogenic translocation in a pediatric sarcoma (Smolen et al., 2007) and its functions may be particularly relevant in this cellular context. In the resting state, Radil appears to be mainly localized in a cytoplasmic pool with minimal plasma membrane localization (Figure 2-3A panel c). In contrast, overexpression of wild-type Rap1a (Figure 2-3A panels e-g) or two different constitutively active Rap1a mutants (Figure 2-3A panels h-m) led to the translocation of Radil to the plasma membrane with extensive co-localization with the activated pool of Rap1a. Note that although expressing wild-type Rap1a was sufficient to translocate Radil, this effect was significantly stronger with the constitutively active Rap1a mutants (data not shown). These results support previous data that Radil forms a complex with the GTP-bound form of Rap1a (Figure 2-2B) (Smolen et al., 2007). Interestingly, at the plasma membrane, Radil appears to be enriched in both lamellipodia and filopodia (Figure 2-3A, insets) a subcellular localization compatible with a role in cell-matrix interac-
tions and cell migration. We next asked if overexpression of \( \text{G} \beta \gamma \), impacts on the localization of Radil. \( \text{G} \beta \gamma \) is predominantly localized to the plasma membrane in these cells (Figure 2-3A, panel o) and overexpression of G\( \beta \)2 and G\( \gamma \)2 phenocopied Rap1a expression in that it resulted in translocation of Radil to the plasma membrane (Figure 2-3A panels n-p). As control, we showed that overexpression of activated Rap1aQ63E did not lead to the membrane enrichment of wild-type GFP which localizes to the cytosol and nucleus of these cells (Figure 2-9). Also, we demonstrated that overexpression of an active form of Rac1, which is a Rho family GTPase with described roles in cell migration and spreading (Fukata et al., 2003; Niggli et al., 2009), did not promote the plasma membrane recruitment of Radil (Figure 2-9). To quantitatively support our immunofluorescence data we performed biochemical fractionation experiments. While a modest amount of Venus-Radil was found in the membrane pool under resting conditions, expression of Rap1aQ63E and G\( \beta \gamma \) markedly promoted the association of Radil with the membrane fractions (Figure 2-3B, compare lanes 2&3 with lane 1). We thus conclude that following G\( \beta \gamma \) and Rap1a activation, the Rap effector Radil is translocated to the plasma membrane at sites of cell-matrix contacts likely through the formation of the G\( \beta \gamma \)-Rap1-Radil protein complex.
Figure 2-3. Rap1a-GTP and Gβγ promote the translocation of Radil at cell-matrix contacts in HT1080 cells. (A) Shown is localization of GFP (a and b), Venus-Radil (c and d), or Venus-Radil co-expressed with: wild-type HA-Rap1 (e and g), HA-Rap1aQ63E (h and j), HA-Rap1G12V (k and m) or FLAG-Gβ2/Gγ1 (n and p). HA- and FLAG-tagged Rap1a or Gβ2 + Gγ (untagged) were transiently transfected in HT1080 cells stably expressing Venus-Radil as indicated. Cells were fixed followed by immunodetection with α-HA (e and m) or α-FLAG (n and p) mAb, followed by secondary detection with goat-anti-mouse conjugated to Alexa 594 antibody. Cells were visualized using a Zeiss LSM 510 confocal microscope under 63X oil immersion objective. White arrows depict plasma-membrane co-localization. White boxes indicate magnified sections cropped in Adobe Photoshop CS3. Images shown are representative of 10-15 cells analyzed in three independent experiments. Bars, 50µm. (B) Membrane fractions show enrichment of Venus-Radil when Rap1aQ63E and Gβγ are expressed. HT1080 cells stably expressing Venus-Radil were transiently transfected to express the indicated proteins. Cells were lyzed in hypotonic buffer and subjected to subcellular fractionation as described under “Experimental Procedures”. Membrane fractions and the inputs were analyzed by western blot to determine the amount of Venus-Radil present in the membrane pool when HA-Rap1aQ63E and Gβγ are co-expressed. The blot for the membrane fractions was stripped and re-probed using α-Na-K-ATPase antibody to provide for an internal loading control.
2.4.4 The Gβγ-Rap1-Radil complex regulates cell spreading and cell-matrix adhesiveness

Research in the last ten years has established Rap1a as an important factor in the regulation of cell-matrix and cell-cell adhesion (Bos, 2005; Bos et al., 2003; Bos et al., 2001). Rap1a activation has been implicated in inside-out signalling leading to the activation of integrins and increases in cell-matrix adhesion (Bos, 2005; Bos et al., 2003; Bos et al., 2001; Kooistra et al., 2007; Reedquist et al., 2000). Radil was recently described as a Rap effector with a role in the control of cell-matrix adhesion (Smolen et al., 2007). Consistent with these notions, we noted that overexpression of WT or constitutively active Rap1a or Radil led to increased cell spreading (Figure 2-4A and B), a phenotype commonly associated with increased cell-matrix adhesiveness (Arthur et al., 2004). Interestingly, the over-expression of Gβγ also led to an increased spreading of these cells (Figure 2-4A and B). To quantitatively monitor the effects of Rap1a, Radil or Gβγ overexpression on the spreading of HT1080 cells in real time, we employed the xCELLigence impedance system (Atienza et al., 2005). This system provides a cell index which increases as a function of cell attachment, spreading and cell growth. As Figure 2-4C shows, the overexpression of Rap1WT, Gβγ, Radil and Rap1aQ63E led to progressively higher cell indices compared to control-transfected cells over the first two hours. We conclude that the induced formation of the Gβγ-Rap1-Radil complex increases the spreading of HT1080 cells.

These results led us to test the hypothesis that the Gβγ-Rap1a-Radil protein complex may represent a cellular machinery regulating cell adhesion downstream of GPCR activity. To test this, we optimized a cell-matrix adhesion assay to measure the adhesion of HT1080 cells to the extracellular matrix protein fibronectin. In this assay, overexpression of Rap1aQ63E predictably led (since it promotes inside-out integrin signalling) to a strong increase in the adhesive proper-
ties of these cells (Figure 2-5A, quantified in 2-5B). Inhibiting Rap1a activation by expressing Rap1GAP on the other hand did not lead to any increase in cell adhesion.

After having determined the dynamic range of this assay, we tested whether the overexpression of the other members of the Gβγ-Rap1a-Radil- complex was sufficient to promote adhesion of these cells. Both Radil and Gβγ overexpression led to a strong potentiation of cell-matrix adhesion (Figure 2-5A and B) to levels similar to that observed with Rap1aQ63E expression (Figure 2-5A and B). Consistent with the interaction data between Gβγ, Radil and Rap1a (Figure 2-2B), the increase in adhesion promoted by Radil or Gβγ expression depended on Rap1a activity. Indeed, coexpression of Rap1GAP strongly inhibited both Gβγ- or Radil-promoted cell-matrix adhesion (Figure 2-5A and B). We conclude that the Gβγ-Rap1a-Radil protein complex is sufficient to promote cell-matrix adhesiveness.

As stated previously, Radil is composed of RA, DIL and PDZ domains. We next studied the individual requirement of the RA and PDZ domains of Radil for their ability to promote cell-matrix adhesion. Whereas overexpression of full length Radil led to a robust increase in adhesion (Figure 2-5C and D), equivalent expression (Figure 2-5D inset) of ∆RA or ∆PDZ mutant Radil proteins had no effect. Since the RA domain is known to interact with GTP-bound Rap1a, these results further support the requirement for the Rap1a-Radil interaction for this process and indicate that the PDZ domain is also required possibly through the interaction of an as yet unidentified PDZ ligand-containing effector protein.
Figure 2-4. The Gβγ-Rap1-Radil complex increases HT1080 cell spreading. (A) HT1080 cells were plated on coverslips and transiently transfected with cDNA for GFP alone or together with HA-Rap1aQ63E, HA-Radil, HA-Gβ2+Gγ. Cells transfected with Venus-Rap1GAP alone were also used as control. 48h post-transfection cells were fixed and visualized using a confocal microscope under 63X oil immersion objective. Bars, 50µm. (B) The spreading of cells co-expressing GFP and the indicated proteins was quantified by taking the total surface area for each cells using the Image J software. A total of 50-70 cells were analyzed. Error bars represent ± S.E.M. Statistical significance was analyzed using one-way ANOVA followed by Tukey’s multiple comparison test; p<0.05. (C) HT1080 cells were transfected with the indicated cDNAs. 48h post-transfections cells were trypsinized and resuspended in DMEM/10% FBS media. Cells were counted and 25,000 cells were plated on a 96-well xCELLigence microtitre plate (E-Plate) and the change in cell index was measured using the xCELLigence impedance system. Error bars represent ± S.D. Data shown is representative of two independent experiments. Wt, wild type.
Figure 2-5. Radil and G\(\beta\gamma\) promote adhesion of HT1080 cells on fibronectin matrix in a Rap1a dependent manner. (A) Shown are representative pictures of HT1080 cell adhesion assays in response to overexpression of different proteins as indicated. FLAG-GFP cDNA was transfected as control. Top panels show remaining adherent cells after washes. The bottom panels demonstrate the total cells input for each conditions. (B) Quantified representation of the adhesion assay as fold stimulation compared to control. (C) Representative pictures comparing the ability of ΔRA and ΔPDZ Radil mutants to promote HT1080 cells adhesion. (D) Quantified representation of C. The inset shows expression of the different Radil proteins in one representative experiment. Each experiment was done in triplicates and several pictures from random fields were taken from each well of 96-well plate. Cells were counted (using an automated cell counting macro created in ImageJ software) from each field and expressed as fold of control from each experiment. The results from three independent experiments were pooled for the quantification. Each dot on the graph represents cell counts from a single field. Error bars, ±S.E.M. Statistics, One-way ANOVA followed by Tukey’s multiple comparison test; p<0.05. Asterisks indicate statistical significance compared to control.
2.4.5 GPCR activation leads to Rap1 activation, Radil translocation to the plasma membrane and increased cell-matrix adhesion

Having established that the formation of the Gβγ-Rap1a-Radil protein complex was sufficient to promote cell-matrix adhesion, we next wanted to assess its requirement in the context of a GPCR known to modulate cell adhesion. A previous report described that HT1080 cells endogenously express functional Formyl Peptide Receptors (FPRs) and that their activation with the formylated peptide fMLP strongly potentiate cell matrix adhesion (VanCompemolle et al., 2003). We first repeated this experiment and showed that fMLP treatment of these cells also caused an approximately 5 fold increase in adhesion using our experimental conditions (Figure 2-6A). We next asked whether fMLP-promoted cell adhesion requires active Rap1a. To do this, we overexpressed Rap1GAP and asked whether fMLP could still potentiate adhesion. As shown in Figure 2-6A, expression of Rap1GAP strongly antagonized the ability of fMLP to induce cell adhesion. We conclude that FPRs utilize a Rap1a-dependent pathway to regulate cell adhesion to the fibronectin matrix. Supporting this, fMLP treatment of these cells led to a rapid and robust activation of Rap1a (Figure 2-6B). This activation is sustained, peaks at 2 minutes and slowly wanes over time. Learning from the localization experiments (Figure 2-3) that activated Rap1 and Gβγ translocates Radil to the plasma membrane, we tested the prediction that fMLP treatment of the cells would also lead to a translocation of Radil to the plasma membrane. As shown in Figure 2-6C Venus-Radil was enriched at the plasma membrane within 2-3 minutes following FPRs activation, a kinetic that mirrored Rap1a activation. We conclude that FRP activation by fMLP leads to Rap1a activation and to the recruitment of Radil to the plasma membrane.
2.4.6 The Gβγ-Rap1a-Radil signalling axis regulates adhesion downstream of GPCR activation

Our next goal was to determine whether Radil was required downstream of FPRs activation for the control of cell adhesion using a RNAi loss of function approach. We first validated the efficiency of a pool of 4 individual siRNA sequences targeting Radil to inhibit the expression of Venus-Radil (Figure 2-7A, left blot compare lanes 2 and 1) and more importantly to target the levels of endogenous Radil proteins in HT1080 cells (Figure 2-7A, right blot). Using these reagents we then assessed the ability of fMLP to promote cell-matrix adhesion when Radil levels are reduced. Whereas control and Radil siRNA treated cells exhibited background level of adhesion on their own, Radil siRNAs strongly inhibited the ability of fMLP to promote adhesion when compared to control siRNA treated cells (Figure 2-7B). Importantly, we also screened several individual Radil siRNAs and found one (Radil siRNA#1) that effectively targeted Radil expression (Figure 2-7A, compare lanes 3 and 1). Similar results were obtained using this single siRNA in the functional adhesion assay (Figure 2-7B). To evaluate the implication of Gaι-containing heterotrimeric G proteins for the fMLP-promoted increase in adhesion, we pre-treated the cells with 100ng/ml of pertussis toxin (PTX) for 16 hours, a concentration known to inactivate Gaι. While fMLP induced the expected 5 fold increase in control-treated cells, PTX completely inhibited the fMLP-mediated cell adhesion (Figure 2-7C). We conclude from these results that a Gaι-containing heterotrimeric G protein and Radil are required downstream of FPRs for the control of cell-matrix adhesion.
Figure 2-6. fMLP promotes Rap1-dependent HT1080 cell adhesion, Rap1 activation and Radil translocation to the plasma membrane. (A) HT1080 cell adhesion on fibronectin matrix with or without fMLP (100nM) stimulation in the presence or absence of Rap1GAP. Rap1aQ63E (used as a positive control) for increase in cell adhesion (n=3). (B) Shown is the time course of Rap1 activation by fMLP (100nM) in HT1080 cells. The top panel shows GTP-bound Rap1a, purified using GST-RalGDS-RBD pull-downs. The bottom panel shows total Rap1 in a fraction of whole cell lysates from each sample. Western blotting (WB) was done using α-Rap1a antibodies. (C) HT1080 cells stably expressing Venus-Radil were serum starved for 24 hours and stimulated or not with fMLP (1µM) for (a) 0 min, (b) 2 mins (c) 3 mins at 37°C. Arrows highlight regions of Venus-Radil enrichment at the plasma membrane. Images were captured by a blinded observer using 100X oil-immersion lens. Images shown are representative of two independent experiments. Bars, 20µm.
Figure 2-7. Radil is required for the fMLP-promoted HT1080 cell adhesion on fibronectin matrix. (A) Efficiency of Radil knockdown. HT1080 cells stably expressing Venus-Radil were treated with control, a pool of 4 Radil siRNAs (hRadil siRNA pool), or a single Radil siRNA (hRadil siRNA # 1) (Left panel) and Radil expression was monitored by western blot using GFP antibodies. Immunoblotting with total Erk1/2 antibodies was performed as loading controls. Endogenous hRadil knockdown efficiency in HT1080 cells using the hRadil siRNA pool (Right panel). Cells transfected with control or hRadil siRNA pool were lysed, and equivalent amount of lysates were immunoprecipitated using α-Radil rabbit polyclonal antibodies followed by detection in western blot using the same antibody. (B) fMLP-promoted cell adhesion in HT1080 cells treated with control or hRadil siRNAs (n=4). Each dot on the graph represents counts from a single field. Asterisks at means correspond to statistical significance compared to control siRNA treatment. (C) Treatment of HT1080 cells with PTX (100ng/ml) inhibits fMLP-mediated cell adhesion. Each experiment was done in triplicates and several pictures from random fields were taken from each well of a 96-well plate. Cells were counted from each field and expressed as fold of control from each experiment. Data from all experiments were pooled. Error bars, ±S.E.M. Statistical significance was assessed using one-way ANOVA followed by post-hoc analysis using Tukey’s multiple comparison test; p<0.05.
2.4.7 The Gβγ-Rap1a-Radil complex regulates inside-out signalling leading to integrin activation

We were then interested to address the mechanism by which GPCR activation and the Gβγ-Rap1a-Radil complex modulate cell adhesion. Cell-matrix adhesiveness is a dynamic process regulated through the interaction of integrin receptors with extracellular matrix components. Both integrin availability and affinity for their ligands can be regulated. The latter has been showed to be controlled in part by signalling through the small GTPase Rap1a in a process known as inside-out signalling (Banno and Ginsberg, 2008; Kinbara et al., 2003; Mor et al., 2007). HT1080 cells are known to express several integrins including the α5β1 subtypes which form the receptor for fibronectin. Since GPCRs have previously been shown to regulate integrin affinity (Alon and Ley, 2008; Etzioni and Alon, 2004; Laudanna et al., 2002), we investigated whether the Gβγ-Radil-Rap1a protein complex regulates cell-matrix adhesion through inside-out signalling and integrin activation. To perform this experiment we took advantage of a conformationally sensitive monoclonal antibody (9EG7) that selectively recognizes β1-integrin when activated (Bazzoni et al., 1995; Wei et al., 2005) and initially asked whether Rap1aQ63E, Radil, or Gβγ overexpression or fMLP treatment led to integrin activation. When expressed in HT1080 cells, these proteins increased cell spreading (Figure 2-4) and adhesion (Figure 2-5). After expression of these proteins or treatment of the cells with fMLP, a substantial increase in β1-integrin activation was measured (Figure 2-8A and B) to levels approaching 50-60% of what is obtained after treatment of the cells with the divalent cation Mn2+ used as a positive control to maximally activate integrins. Importantly and consistent with the requirement of activated Rap1a for the Gβγ-Rap1a-Radil complex assembly and function, the overexpression of Rap1GAP inhibited integrin activation in each case (Figure 2-8A and B).
To address the requirement for Radil in fMLP-induced integrin activation, we treated HT1080 cells with control or two independent Radil siRNAs and compared integrin activation patterns upon fMLP stimulation. Although fMLP promoted integrin activation by approximately three fold in control siRNA treated cells, depletion of Radil severely impaired the ability of fMLP to activate integrins (Figure 2-8C and D) but did not affect the Mn$^{2+}$ response (data not shown). These findings support the view that the G$\beta$$\gamma$-Radil-Rap1a complex controls cell adhesiveness downstream of GPCR activation by regulating inside-out signalling leading to integrin activation.
Figure 2.8. β1 Integrin activation. (A) Representative flow cytometry analysis showing β1 integrin activation upon the indicated treatments. Gray area represents control staining with 9EG7 mAb antibodies that specifically recognize activated β1-integrins. Black area corresponds to the treated population. Rap1aQ63E, Mn2+ and Rap1GAP are used as controls. (B) Average of three independent experiments performed as in (A) showing β1 integrin activation upon the different treatments. Error bars, ±S.E.M. (C) Flow cytometry analysis showing β1 integrin activation in the presence or absence of fMLP in HT1080 cells treated with control or hRadil siRNAs. (D) Average of three independent experiments performed as in (C). Error bars, ±S.E.M. Statistics, One-way ANOVA followed by post-hoc analysis using Tukey’s multiple comparison test; p<0.05.
2.5 Discussion

Several GPCRs have been implicated in the control of cell motility and adhesion. However, the intracellular signalling pathways underlying these functions remain poorly defined. We present here the functional characterization of a novel protein complex functioning downstream of GPCR signalling to regulate cell-matrix adhesion. We have identified Radil, a Rap1 effector, as a multi-domain protein linking GPCRs, G\(\beta\gamma\) and Rap1-GTP to integrin activation. First, G\(\beta\gamma\) or Radil overexpression is sufficient to activate mesenchymal cell spreading, adhesion to fibronectin and to promote the active conformation of integrins. Second, G\(\beta\gamma\) and active-Rap1a promote the localization of Radil to sites of cell-matrix adhesion within the plasma membrane. Third, the stimulation of cell adhesion by fMLP receptors was abrogated in Radil knockdown cells and when Rap1a function was inhibited with the overexpression of Rap1GAP. Our results thus unveil a new signalling axis downstream of Rap1 activation by GPCRs and implicate a function for the G\(\beta\gamma\)-Rap1a-Radil protein complex in the inside-out signalling cascade leading to integrin activation.

Other Rap1 effectors have previously been identified and shown to be important in regulating cell adhesion. RAPL, which is enriched in lymphoid tissues, has been shown to be important for the polarized relocalization of LFA1, the major leukocyte integrin (Katagiri et al., 2003), to the leading edge. Another effector important for T cell functions is RIAM which regulates adhesion by the modulation of actin dynamics through its interaction with Profilin and Ena/Vasp proteins (Lafuente et al., 2004). Similar to Radil, overexpression of RAPL or RIAM also leads to enhanced adhesion and integrin activation (Katagiri et al., 2003; Watanabe et al., 2008). Additional studies will be needed to understand how Rap1 selectively activates different effectors in different contexts. One possibility is that the expression of the different Rap1 effec-
tors is restricted to specific cell types. Supporting this, RAPL and RIAM are expressed highly in lymphoid cells but are either absent or expressed at low levels in other cell types (Katagiri et al., 2003; Lafuente et al., 2004). Although Radil is expressed in all epithelial and fibroblast cell lines that we examined (data not shown) and is broadly expressed in zebrafish embryos (Smolen et al., 2007), we do not know its level of expression in lymphocytes and whether it is functionally redundant with RAPL or RIAM in this setting. Alternatively, the recruitment of Rap1 and one of its effectors within a protein complex could account for the specificity of action. GPCR-activated G\(\beta\gamma\) subunits in this design could serve to insulate the activation of Radil from the other Rap1 effectors following Rap1 activation. Supporting this possibility, we found that G\(\beta\gamma\) interacts with Radil but not with its close homologue AF-6 (Figure 2-1C).

Since Rap1 regulates cell-cell and cell-matrix adhesion in different contexts, its role in tumourigenesis and cancer progression has been closely scrutinized (Hao et al., 2008; Hattori and Minato, 2003; Itoh et al., 2007; Till et al., 2008). Recent findings suggest a role for aberrant Rap1 activity in prostate cancer progression as activation of Rap1 increased prostate cancer cells migration and invasion and its inhibition had the opposite effect (Bailey et al., 2009). Interestingly, in the same study, integrins were shown to be involved in the mechanism of Rap1-mediated migration and invasion of prostate cancer cells. Further supporting a role for Rap1 activation for cancer dissemination, Rap1GAP expression has been found to be downregulated in several cancers (Nellore et al., 2009; Tsygankova et al., 2007; Zhang et al., 2006). Roles for Rap1 effectors in the context of cancers have not been explored. As mentioned earlier, Radil was originally identified as a factor induced by a chromosomal translocation in a sarcoma (Smolen et al., 2007). Interestingly, a recent analysis of prostate cancer biopsies demonstrated that Radil expression is upregulated during prostate cancer progression (Savli et al., 2008). Together this suggests that
Rap1 signalling through Radil may be important for cancer initiation/progression. Given Radil’s assembly in the Gβγ-Rap1-Radil complex, the role of Radil may thus be particularly important in the context of GPCR signalling during metastasis. Indeed, chemokine receptors for example are known to direct tumour cells metastasis and tropism to different secondary sites. Further studies examining the role of this protein complex during the progression of different cancers are thus needed.

This study unveils a previously unappreciated role for Gβγ in regulating cell adhesion through its recruitment in the Gβγ-Rap1-Radil complex. Given that the formation of this protein complex requires the activation of Rap1, one function for this complex, which we characterized in the present study, occurs downstream of Rap1 activation by GPCRs. One important question that remains unclear and that was beyond the focus of our study is how GPCRs lead to Rap1 activation. Further studies are thus needed to precisely map the sequence of events leading to Rap1 activation by GPCRs and to evaluate whether context-dependent signalling pathways may contribute to Rap activation and engagement of the Gβγ-Rap1-Radil complex to regulate cell adhesion.

In conclusion, this study identifies the Gβγ-Rap1-Radil complex as an important component of GPCR signalling regulating cell-matrix adhesion. Although we found that Radil leads to integrins activation, how exactly Radil regulates this process and whether it has other functions is not known. Perhaps the identification of which PDZ ligand containing binding partners interact with Radil will help to further understand how this protein complex works. Since Rap1 is also known to function in cell-cell adhesion and cell invasion, which are also known to be regulated by GPCR signalling, more work is also needed to examine the role of the Gβγ-Rap1-Radil complex in these processes.
Figure 2-9. (Supplementary Figure) Radil translocates to the plasma membrane with activated Rap1a only. HT1080 cells were co-transfected with GFP and HA-Rap1aQ63E (A-C). HT1080 cells stably expressing Venus-Radil were transfected with HA-Rap1aQ63E (D-F), HA-Rac1G12V (G-I) or pcDNA3.1 (J-K). Cells were fixed, immunostained and visualized under 63X oil immersion lens using a confocal microscope. Bars 50µm.
### Table 2-1.

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**Supplementary table.** Peptide identifications by mass-spectrometry analysis from representative purification experiments of (A) Strep-CBP-HA-Gy2 in HEK293T cells, (B) Strep-CBP-HA-Gy2 in HEK293 cells, (C) Strep-CBP-HA-Radil in HEK293T cells and (D) Flag-Radil in HT1080 cells. Proteins are shown in descending orders of percentage sequence coverage of the proteins.
Chapter 3:
KIF14 negatively regulates Rap1a-Radil signalling during breast cancer progression

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Majority of the experiments and data analysis was performed by Syed M. Ahmed except for phage display assay which was performed in collaboration with Dr. Maruti Uppalapati and Dr. Sachdev Sidhu. In vivo experiments were done in collaboration with Dr. Brigitte Theriault and Dr. Brenda Gallie. Catherine Chui helped with flow cytometry based data collection.
3.1 Abstract

The small GTPase Rap1 regulates inside-out integrin activation and thereby influences cell adhesion, migration and polarity. Several Rap1 effectors have been described to mediate the cellular effects of Rap1 in a context-dependent manner. Radil is emerging as an important Rap effector implicated in cell spreading and migration but the molecular mechanisms underlying its functions are unclear. We report that the kinesin KIF14 associates with the PDZ domain of Radil and negatively regulates Rap1-mediated inside-out integrin activation by tethering Radil on microtubules. The depletion of KIF14 leads to increased cell spreading, altered focal adhesion dynamics and inhibition of cell migration and invasion. We also show that Radil is important for breast cancer cell proliferation and for metastasis in mice. Our findings provide evidence that the concurrent upregulation of Rap1 activity and increased KIF14 levels in several cancers is needed to reach optimal levels of Rap1-Radil signalling, integrin activation and cell-matrix adhesiveness required for tumour progression.

3.2 Introduction

The small G-protein Rap1 is an important mediator of integrin inside-out signalling which plays a pivotal role in adhesion, spreading and migration of cells (Arthur et al., 2004; Bos, 2005; Bos et al., 2003; Kinashi and Katagiri, 2004, 2005). Rap1 acts as a molecular switch that cycles between active GTP-bound and inactive GDP-bound states. Rap1 activity is regulated by guanine nucleotide exchange factors (GEFs) such as Epac1 (de Rooij et al., 1998) and GTPase activating proteins (GAPs) such as Rap1GAP (Rubinfeld et al., 1991). Upon activation, Rap1 has the ability to increase the affinity of integrins for their extra-cellular matrix (ECM) ligands and to promote their clustering (Han et al., 2006; Kim et al., 2011; Lafuente et al., 2004; Sebzda et al., 2002). In recent years the identification and characterization of downstream Rap effector
proteins such as RIAM (Lafuente et al., 2004), RapL (Katagiri et al., 2003), Krit1 (Glading et al., 2007) AF-6 (Boettner et al., 2000) and Radil (Smolen et al., 2007) have shed light on the molecular mechanisms underlying the cellular effects mediated by Rap1. We previously identified the Rap1 effector Radil as a protein associating with Gβγ subunits of heterotrimeric G-proteins (Ahmed et al., 2010). Radil was found to be required for the Rap1a-mediated inside-out activation of integrins, adhesion and spreading of human fibrosarcoma cells (Ahmed et al., 2010). Radil is also known to have important functions in Epac1-mediated spreading of lung carcinoma cells (Ross et al., 2011) and to be indispensable for the migration of neural crest cells during zebrafish development (Smolen et al., 2007).

The control of cell-matrix adhesion plays a fundamental role in controlling cancer cell migration during metastasis (Arjonen et al., 2011; Desgrosellier and Cheresh, 2010; McLean et al., 2005). The implication of Rap1 signalling in the modulation of integrin activity has thus provided a framework to study its implication in tumour progression. Both hyper-activation as well as decreased Rap1 activity is known to affect the migration of breast, melanoma and prostate cancer cells (Bailey et al., 2009; Kim et al., 2012; Zheng et al., 2009). This highlights that precise control of cellular adhesion by Rap1 and its effectors is required for efficient cell movements. This requirement for the fine-tuning of Rap1-mediated inside-out signalling for optimal control of cell-matrix adhesion implies the existence of positive and negative mechanisms of regulation. Although, how Rap1 leads to integrin inside-out activation is becoming better defined, the identification of mechanisms buffering or negatively impinging this process is not well understood. Such regulators may be especially relevant in the context of aggressive cancer cells to optimally adjust Rap1 activity where it is known to be elevated (Bailey et al., 2009; Freeman et al., 2010; Huang et al., 2012; Lorenowicz et al., 2008; Lyle et al., 2008; Zheng et al., 2009).
Kinesins are molecular motors associated with intracellular transport (Hirokawa et al., 2009; Verhey and Hammond, 2009). Kinesin superfamily proteins (KIFs) are important molecular motors that transport various cargoes along microtubules tracks. Several kinesins have been implicated in cancer progression due to their role in mitotic cell division (Huszar et al., 2009). Recently, kinesins were uncovered as playing important regulatory roles in adhesion and migration of cells (Uchiyama et al., 2010; Zhang et al., 2010). Blocking kinesin-1 activity using inactivating antibodies was also shown to lead to increase in the size and number of substrate adhesions (Kaverina et al., 2002a; Kaverina et al., 1997). Although the precise mechanisms are unclear, kinesins were suggested to control the delivery of factors at adhesion sites to retard their growth or promote their disassembly. KIF14 was initially characterized as a protein involved in cytokinesis by interacting with Protein Regulating Cytokinesis-1 (PRC1) and Citron kinase (Gruneberg et al., 2006). KIF14 was also demonstrated to be highly up-regulated in several cancers including retinoblastomas, breast cancers, lung cancers, and ovarian cancers and its high expression levels has been clinically correlated with increased breast cancer invasiveness and mortality (Corson and Gallie, 2006; Corson et al., 2005; Corson et al., 2007; Theriault et al., 2012).

We previously established that the C-terminal PDZ domain of Radil was critical for its function but the identity of the protein(s) binding to the Radil PDZ domain and how it contributed to Rap1-Radil signalling was however not addressed. PDZ domains are present in many scaffolding proteins and are involved in the organization of multi-protein complexes important for numerous biological processes including cell adhesion, spreading, migration and polarization (Baumgartner et al., 2008; Fanning and Anderson, 1999; Hall et al., 1998; Harris and Lim, 2001; Humbert et al., 2003; Kim and Sheng, 2004; Ludford-Menting et al., 2005; Meerschaert et al., 2007; Radziwill et al., 2007; Sheng and Sala, 2001). In this study, we use an integrative proteomic approach based on phage display and mass spectrometry to identify KIF14 as a novel PDZ
binding protein that interacts with Radil. Our results describe KIF14 as a negative regulator of Rap1-Radil-mediated signalling and uncover an essential role for Radil in breast cancer progression.

3.3 Material and Methods

3.3.1 Plasmid constructs and reagents

The pGlue-Radil and pGlue-Radil-ΔPDZ expression plasmids (the GLUE cassette contains a streptavidin tag, an HA epitope and a calmodulin binding peptide) were described previously (Ahmed et al., 2010). The Radil and Radil-ΔPDZ coding sequences were also cloned into pLentiGlue-HA-mCherry lentiviral vector. Mouse Radil (mRadil) and mRadil-ΔPDZ coding sequences were amplified by PCR from MGC mouse I.M.A.G.E clone # 5696312 and inserted into the PSL9-FLAG lentiviral vector. Paxillin was also cloned downstream of Venus in the PSL9 lentiviral vector. The eGFP-KIF14 expression plasmid was a kind gift from Dr. Francis Barr (Max Planck Institute of Biochemistry, Martinsreid, Germany) (Gruneberg et al., 2006). The eGFP-KIF14-IQAA mutant was generated by Quickchange PCR mutagenesis to convert the last two amino acids of eGFP-KIF14 from WV to AA. pGEX-Radil-PDZ encodes for the last 206 amino acid residues of Radil that includes the PDZ domain fused to GST. Flag-KIF14-ctail-IQWV and Flag-KIF14-ctail–IQAA that encodes for the last 166 amino acid of human KIF14 were cloned into the pIRES-puro vector downstream of a Flag tag. All cDNA constructs were verified by sequencing and detailed maps are available on the lab website: http://phm.utoronto.ca/angers/.

Antibodies were purchased from the following vendors: mouse anti-HA.11 clone 16B12 (Covance), mouse anti-FLAG, rabbit anti-FLAG, mouse anti-vinculin clone hVin-1, mouse anti-β-tubulin clone TUB 2.1 and mouse anti-acetylated-tubulin clone 6-11B-1 (Sigma-Aldrich), rab-
bit anti-GFP (University of Alberta), mouse anti-cortactin clone 4F11 (Millipore), rabbit anti-β1-integrin C-terminal antibody (Millipore), rabbit anti-KIF14 (Bethyl Laboratories), rat anti-CD29 clone 9EG7 (active β1-integrin antibody) (BD Transduction Laboratories) and rabbit anti-CDC25C (Santa Cruz Biotechnology). The Radil polyclonal antibody was developed in house and described previously (Ahmed et al., 2010). Rabbit anti-KIF7 antibodies were a kind gift from Dr. C.C. Hui (Sickkids, Toronto, Canada). Secondary antibodies conjugated to AlexaFluor 488 and 594 were purchased from Life Technologies. Phalloidin conjugated to CF647 dye was purchased from Biotium Inc. Secondary antibodies conjugated to horseradish peroxidase were purchased from Jackson ImmunoResearch Laboratories, Inc. Fibronectin from bovine plasma was obtained from Sigma-Aldrich. Matrigel growth factor reduced basement membrane matrix was purchased from BD Biosciences.

KIF14 MISSION shRNA clones in the lentiviral plasmid pLKO.1-puro were acquired from Sigma. TRCN0000113816 and TRCN0000113817 labeled as KIF14 shRNAs #816 and #817 respectively throughout the paper, were the most efficient according to our validation using western blot analysis (Figure 3-10F). Radil MISSION shRNA clone TRCN0000155169 which led to effective knockdown (Radil shRNA#4) was also purchased from Sigma. Another shRNA targeting human Radil (Radil shRNA#206) was designed in pLKO.1-puro with the following sequence: (ccggggcccaacagcgcaactcaactcagctcagttcttcttttgggtggtttttg). A scrambled non-targeting control shRNA was also created in the pLKO.1-puro vector with the sequence: cccggtctcataaggttacgctcctgtcctctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcctgtcct
calcium phosphate precipitation as described elsewhere (Ahmed et al., 2011; Jordan et al., 1996). MDA-MB-231 cells were transiently transfected with Lipofectamine LTX supplemented with PLUS reagent according to manufacturer guidelines (Life Technologies). All lentiviral particles were produced in HEK293T cells by co-transfection of VSV-G (3µg), psPAX2 (6µg) and lentiviral plasmids (8µg) in 30-40% confluent monolayer cell culture grown in 10cm plates. Media was changed 12 to 16h post-transfection and virus subsequently collected after 24h and 48h. Target cells were transduced in the presence of 10µg/ml polybrene (Sigma-Aldrich). 24h after infection the viral media was replaced with fresh DMEM/10% FBS media. Where appropriate cells were selected with puromycin for 48 hours after viral infection and typically used for our experiments within 4-5 days after transduction.

### 3.3.3 Phage display analysis

Recombinant Radil PDZ domain was purified from E. Coli as a GST-fusion protein and used as the bait in phage display selections (Tonikian et al., 2008). Briefly, phage-displayed C-terminal peptide libraries containing >10^10 unique, random heptapeptides were used to isolate ligands for the Radil PDZ domain. To obtain a concise representation of the preferred binding motif sequence of the Radil PDZ domain, amino acid sequence enrichment analysis was performed on 25 unique binding peptide sequences using a web based sequence logo generator WebLogo (http://weblogo.berkeley.edu/) (Crooks et al., 2004; Schneider and Stephens, 1990). The resulting sequence logo was used to find proteins in the human proteome ending with the given sequence pattern using PHI-BLAST algorithm to search the UniProt knowledge-base/Swiss-Prot database employing Prosite's ScanProsite tool.
3.3.4 RT-PCR and quantitative real-time PCR

Frozen sections were obtained from surgical samples of 99 breast tumours and 10 reduction mammoplasties from the Manitoba Breast Tissue Bank (MBTB) (Liu et al., 2010; Watson et al., 1996). All tumours were from female patients who had not received any therapy at the time of resection. The University Health Network Research Ethics Board and the MBTB Access Review Committee approved this study, and all subjects provided informed consent to the MBTB.

Total RNA was extracted from breast tumours, and normal breast tissue samples using TRIzol (Life Technologies) according to manufacturer’s instructions. The concentration and quality of total RNA were determined using a Nanodrop-1000 spectrophotometer (Thermo Scientific). For cDNA synthesis, 1μg of total RNA was reverse transcribed using random primers (Invitrogen) and Superscript II Reverse Transcriptase (Life Technologies).

Real-time PCR was performed in a 7900HT Fast Real-Time PCR system using 1.5μl of the synthesized cDNA product plus the Universal PCR Master Mix in a final volume of 20μl (Applied Biosystems). Taqman gene expression assays (Applied Biosystems) were employed to measure the mRNA expression of Radil (Hs01020348_m1), KIF14 (Hs_00978216_m1) and GAPDH (Hs99999905_m1) in triplicate. Mean relative gene expression was determined using the ΔΔCt method built into the SDS 2.2 software (Applied Biosystems). GAPDH was used as the endogenous control gene.

3.3.5 Immunofluorescence microscopy and Image acquisition

Cells grown on coverslips were fixed with 4% paraformaldehyde in 5% sucrose-PBS pH 7.4, for 15 minutes. Cells were permeabilized with 0.5% Triton X-100 followed by blocking in 2% BSA for 30 min. β1-integrin staining was performed in non-permeabilized cells. For micro-
tubule disruption experiments cells were plated on fibronectin-coated coverslips (0.5μg/ml) overnight and treated with nocodazole (10μM) or DMSO for 20 minutes. Cells were subsequently washed with PBS gently and processed for immunostaining as described above. Slides were mounted on coverslips using Vectashield mounting media (Vector Laboratories). For total internal reflection fluorescence microscopy (TIRF), cells were imaged directly on 18mm circular coverglass (Fisher Scientific).

Laser scanning confocal images were acquired using a Plan-Apochromat 63X/1.4 oil immersion objective on a Carl Zeiss LSM 510 Meta confocal microscope. eGFP/Alexa-488 and mCherry/Alexa-594 fluorophores were excited individually with 488nm and 543nm lasers respectively with appropriate filter sets. Uncompressed images were processed with Zeiss LSM Image Browser version 4.2 and Image J software version 1.44p. Where indicated, images were taken as z-stacks and rendered as 3D projection for detailed visualization of the microtubule networks. To measure the distribution of fluorescence in cells, a line was drawn from end to end on a single confocal slice and measured using the Plot Profile function in ImageJ.

Images of focal adhesions were obtained by TIRF microscopy using Olympus cell-TIRF/Lambert FLIM microscope. Images were acquired with a 60X/1.49 NA APON objective and an inverted microscope (Olympus IX81) equipped with Hamamatsu C9100-13 back-thinned EM-CCD camera operated by Volocity version 4 software. For fixed cell cell TIRF imaging cells were kept in PBS. Evanescent field depth for TIRF microscopy was kept at ~200nm. Focal adhesion size and abundance were quantified using the particle analysis function of ImageJ from thresholded images using a fixed region of interest (27.5x27.5μm²) from two different areas at the cell periphery. For live cell imaging, temperature was maintained at 37°C and CO₂ at 5% using a live cell chamber (Chamlide). Images were captured every three minutes for 54 minutes.
Percentage dynamics was calculated by overlaying images from time 0 min and 54 min and counting the percentage of focal adhesions that disappeared within 54 minutes as described previously (Matsumoto et al., 2010). Image contrasts to generate figures were adjusted using ImageJ or Volocity version 6 softwares.

3.3.6 Affinity purification, immunoprecipitation and western blot analysis

Cells were lysed and protein complexes affinity purified on streptavidin sepharose column, or immunoprecipitated with the indicated antibodies as described previously (Ahmed et al., 2011). Briefly, cells were solubilized in buffer containing 0.5% Igepal CA630, 20mM Tris-HCl; pH 7.5, 150mM NaCl, 2mM EDTA, 10mM NaF, 0.25mM NaOVO₃, 100mM β-glycerophosphate and protease inhibitors cocktail (Sigma) for 1 hour at 4°C. Streptavidin affinity purification was performed on streptavidin sepharose resin (GE Healthcare) for 16h, followed by extensive washing of the beads in lysis buffer. For immunoprecipitations the lysates were incubated with the antibodies and Protein A agarose beads (Sigma) for 16h. α-FLAG pull-downs were performed using α-FLAG-M2 agarose beads (Sigma). Co-purified proteins were eluted from the beads at 95°C for 5 min using 2x Laemmlli buffer containing β-mercaptoethanol (Sigma), resolved by SDS-PAGE and transferred onto nitrocellulose or PVDF membranes (Pall) for western blot analysis.

3.3.7 In Vitro binding assay

Flag-KIF14-ctail-IQWV and Flag-KIF14-ctail–IQAA were transiently transfected and expressed in HEK293T cells. Cells were lysed in buffer containing 0.5% Igepal CA630, 20mM Tris-HCl; pH 7.5, 150mM NaCl, 2mM EDTA and protease inhibitor cocktails and immunopurified using Flag-M2 beads (Sigma) for 2 hours. Purified proteins bound to Flag beads were rinsed twice in the above buffer also containing 0.1% SDS and 0.5% sodium deoxycholate to
remove any non-covalent interactors bound to the bait proteins. For *in vitro* binding, equimolar amounts of GST-Radil-PDZ and Flag-tagged KIF14 tails immobilized on Flag-M2 beads were mixed in the same buffer and incubated with gently rocking at room temperature for 1 hour. Beads were subsequently washed with lysis buffer three times and binding analyzed by western blotting.

3.3.8 Microtubule sedimentation assay

Approximately 6x10⁶ MDA-MB-231 cells were lysed in 500μl of BRB80 buffer (80mM PIPES, 1mM MgCl₂ 1mM EGTA, pH6.8 with KOH and protease inhibitors) followed by sonication for 15 sec. Lysates were then centrifuged at 100,000 x g for 30 minutes at 4°C using Beckman Optima ultracentrifuge with MLA-130 rotor. All following steps were carried out at room temperature. Tubulin in the supernatant was polymerized by adding 2mM GTP (Sigma), 2mM MgCl₂ and stepwise addition of paclitaxel (Sigma) to final concentration of 20μM. Kinesins were then allowed to bind by adding 2mM AMP-PNP and letting the reaction incubate at 37°C for 30 minutes. Tubulin-kinesin complex was pelleted by centrifugation at 165,000 x g for 1 hour and the gelatinous precipitate was resuspended in SDS sample buffer and analyzed by SDS-PAGE and western blotting.

3.3.9 LC-MS/MS analysis of protein complexes

HEK293T cells or MDA-MB-231 cells expressing FLAG-mRadil or FLAG-mRadilΔPDZ were lysed in TAP lysis buffer (0.1% Igepal CA630, 10% glycerol, 50mM HEPES-NaOH, pH 8.0, 150mM NaCl, 2mM EDTA, 2mM dithiothreitol, 10mM NaF, 0.25 mM NaOVO₃, 100mM β-glycerophosphate and protease inhibitor cocktail), and immunoprecipitated with α-FLAG-M2 beads overnight at 4°C. Purified proteins were eluted from the beads using 500mM ammonium hydroxide at pH 11.0. The proteins in the complex were reduced in 25mM
dithiothreitol and alkylated using 100mM iodoacetimide (Sigma), and brought to 1mM CaCl\(_2\). The proteins were then directly digested with sequenced-grade trypsin (Promega). Tryptic peptides were analyzed by LC–MS/MS using an LTQ-XL Linear Ion Trap Mass Spectrometer (Thermo Scientific) (Ahmed et al., 2010). The tandem mass spectra generated were searched against the human NCBI protein sequences using SEQUEST (Eng et al., 1994) running on the Sorcerer platform (Sage-N Research). The peptides identified by SEQUEST were validated using PeptideProphet\textsuperscript{TM} and assigned a protein identification using ProteinProphet\textsuperscript{TM} (Keller et al., 2002). The identified proteins were further filtered against other unrelated FLAG pull-down experiments to subtract background proteins using ProHits (Liu et al., 2010).

3.3.10 Cell spreading

Plates or coverslips were coated with fibronectin (0.5μg/ml) at 4°C overnight, and rinsed with PBS twice the following day and dried before performing the experiments. Cells were incubated at 37°C and allowed to adhere and spread for different times. Several phase contrast images of cells were taken from random fields for each condition with a 10X objective using a Nikon Eclipse TS100 inverted microscope. The cell areas were measured by outlining the perimeters of each cell using ImageJ software version 1.44p. Curve was fitted and the rate constant estimated by non-linear regression analysis using the one-phase association equation:

\[ Y = Y_0 + (Plateau - Y_0)(1 - e^{-kx}) \]

where \( Y \) is the cell area and \( x \) is time, plateau is the size of the cell when fully spread. Quantified results are reported as ± S.E.M.
3.3.11 Integrin activation assay and flow cytometry

MDA-MB-231 cells were detached from plates using PBS containing 4mM EDTA followed by centrifugation at 500xg. Cells were rinsed 2X in PBS, resuspended in DMEM containing 0.2% BSA and 20mM HEPES pH 7.2 and were allowed to recover for 1h with gentle agitation on a rocker. Cells from each experimental condition were then incubated at 4°C for 40 min with α-CD29 (9EG7) antibody in the presence or absence of 10mM MnCl2. Cells were then washed in PBS three times followed by fixation in 4% paraformaldehyde containing 5% sucrose in PBS for 10 min and followed by three more washes in PBS before labeling with Alexa-Fluor 647 conjugated goat α-rat IgG (Life Technologies) for 30 min. Flow cytometry analysis was performed with BD FACSCanto II flow cytometer running on BD FACSDiva software 6.1.3. Post-acquisition data analysis was performed using FlowJo version 7.6.5.

3.3.12 Cell migration and invasion assays

For migration assays cells (5x10^4) were seeded on the top chamber of Falcon cell culture transwell inserts with 8µm pores (BD Falcon). For Matrigel invasion assays, 50µg of growth factor reduced Matrigel (50µl) (BD Biosciences) was polymerized on the top chamber of the transwell inserts for 2 hours at 37°C and used immediately as described for migration assays. Cells were allowed to settle on the transwell inserts with serum free media in the bottom chambers for 1h. After one hour, the media in the bottom chamber was supplemented with 20% FBS and cells were incubated at 37°C for 10-12h. At the end of this time point cells at the bottom of the transwell inserts were fixed with 2% glutaraldehyde (Sigma) for 15 min followed by staining with 0.1% crystal violet in 200mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (Sigma) for 1h. The cells that did not invade or migrate were carefully wiped off the top of the membrane with a cotton-swab. Cell migration and invasion were quantified by averaging cell counts.
from pictures taken from 4-5 random areas on the membranes for each condition using an inverted microscope with 10X objective. Quantified results are presented as ± S.E.M

3.3.13 Rap1 activity assay

MDA-MB-231 cells expressing scrambled, Radil or KIF14 specific shRNAs were lysed in RBD lysis buffer (0.5% Igepal CA630, 150 mM NaCl, 25mM Tris-HCl; pH 7.5, 10mM MgCl2, 50mM NaF, 2mM sodium vanadate and protease inhibitor cocktails) and solubilized. 600μg of protein from each condition were then incubated with GST-RalGDS-RBD coupled to glutathione-Sepharose 4B beads for 1h at 4ºC and GTP bound Rap1 levels assessed by western blotting.

3.3.14 Mouse xenograft experiments

For *in vivo* metastasis experiments, 6-10 weeks NOD-SCID mice were injected via the tail-vein with MDA-MB-231-RLuc cells treated with the indicated shRNAs at a density of 1x10^6 per mice. Cell metastasis and homing to the lung was monitored by injecting mice intraperitoneally with XenoLight RediJect Coelenterazine h (Caliper) and measured by *in vivo* bioluminescence imaging using an IVIS Spectrum Bioluminescence system (Xenogen). Animals were sacrificed 45 days post-inoculation, lung tissues collected and fixed in 3.7% paraformaldehyde followed by 70% ethanol. Lungs were stained with Bouin's stain (Sigma-Aldrich) overnight. Visible tumour nodules were counted to assess the extent of tumour metastasis. To assess effects on tumour growth 1 x 10^6 MDA-MB-231 cells treated with Scrambled shRNA or Radil shRNA #4 were implanted subcutaneously into 6 weeks old NOD-SCID mice. After letting tumours grow for 31 days the mice were sacrificed, the tumours were dissected and photographed using a fluorescence stereomicroscope. Each tumour was weighed and their volume measured by liquid displacement. All animals were studied using protocols approved by the Animal Care Committee
of the Ontario Cancer Institute, and are in accordance with Canadian Council on Animal Care guidelines.

3.3.15  **In Vitro** cell proliferation assay

*In vitro* cell proliferation was measured using MTT assay described previously (Alley et al., 1988). Briefly, on day 0 cells were counted and plated on 24 well plates. Subsequently one plate was taken out each day over the time course, and media replaced with 600μl of fresh media containing 200μg/ml of Methylthiazolyldiphenyl-tetrazolium bromide (Sigma-Alrich). Absorbance was measured at 570nm with background subtraction at 630nm using Beckman DU 730 UV/Vis spectrophotometer.

3.3.16  Statistical analysis

Data is generally reported as ± S.E.M. and analyzed by analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison tests (Graphpad Prism 5 software), unless otherwise stated. Data in Figure 3-11A is reported as ± SD. All statistical analysis was considered significant at p<0.05. Student's t-test was performed to analyze the data represented in Figure 3-8 C and D.

3.4  Results

3.4.1  Radil associates with Kinesin Family Protein 14 (KIF14)

To gain further insights into Radil function and regulation, we set out to identify proteins interacting with its C-terminal PDZ domain. We first used a phage display approach to define the preferred carboxyl-terminal peptides recognized by the Radil PDZ domain. We panned a phage-displayed peptide library with recombinant Radil PDZ domain fused to GST (Figure 3-1A) and repeated multiple rounds of binding selection. Binding phages were isolated and the
sequences of the displayed peptides were determined (Figure 3-1A). The analysis of the 25 best binding sequences revealed that the Radil PDZ domain recognizes hydrophobic C-terminal ligands with a strong consensus to the sequence [FI]-[FWT]-WV for the last four amino acids as represented by the motif enrichment diagram in Figure 3-1A. Most PDZ domains prefer ligands containing either S/T (class I) or hydrophobic amino acids (class II) at the -2 position (third residue from the end) (Tonikian et al., 2008). Radil appears to contain an atypical PDZ domain, because it can bind to both ligands classes. Because the Radil PDZ domain can accommodate both hydrophobic and hydrophilic residues at the -2 position, we performed a BLAST search against the Swiss-prot human proteome database to assemble a list of natural proteins with C termini matching the consensus sequence [FI]-x-WV. We compared this list of putative Radil PDZ domain ligands to a list of proteins that we identified in FLAG-Radil complexes using immunoprecipitation and mass spectrometry (Figure 3-1A, Table 3-1a), and we found that KIF14 was present in both lists. Consistent with a physical association between these proteins, the immunoprecipitation of Radil from HEK293T cells led to the co-precipitation of KIF14 (Figure 3-1B). The phage display results suggest that KIF14 binds to the PDZ domain of Radil. To confirm this result in a cellular context, lysates from control HEK293T or from cells stably expressing Strep-HA-Radil or Strep-HA-RadilΔPDZ were subjected to affinity purification using sepharose-streptavidin followed by western blotting using antibodies specific to KIF14. Whereas endogenous KIF14 co-purifies with full-length Radil, it is absent in control or RadilΔPDZ affinity-purified samples (Figure 3-1C, compare lane 2 with lanes 1 and 3). To validate that KIF14 interacts with Radil via its atypical PDZ binding motif, we co-expressed Strep-HA-Radil with wild-type KIF14 or a mutant KIF14-IQAA in which the C-terminal tryptophan and valine were substituted with alanine residues, and we assessed their interaction by affinity purification. Whereas wild-type KIF14 associates with Radil, KIF14-IQAA does not
Figure 3-1. KIF14 interacts with the Radil PDZ domain. (A) Recombinant GST-RadilPDZ domain was used as bait in phage display selections using a library of random heptapeptides. Twenty-five unique binding peptides were recovered from the screen and analyzed using a sequence logo generator to identify the preferred binding sequence. The resulting C-terminal binding motif [FI]-x-WV was searched against UniProtKB/Swiss-Prot human database; and the C-termini of five proteins were found to match this sequence. (B) KIF14 co-immunoprecipitates with Radil. Lysates from HEK293T cells were immunoprecipitated (IP) using α-Radil or control IgG antibodies, followed by western blotting (WB) with α-Radil or α-KIF14 antibodies. (C) Radil interacts with KIF14 via its PDZ domain. Lysates from HEK293T cells stably expressing Strep-HA-Radil or Strep-HA-Radil∆PDZ were affinity purified (AP) using streptavidin sepharose followed by WB using α-HA or α-KIF14 antibodies. (D) The KIF14 PDZ ligand is required to bind to Radil. HEK293T cells stably expressing Strep-HA-Radil were transiently transfected with eGFP-KIF14 wild-type (wt) or eGFP-KIF14-IQAA (WV->AA mutant) constructs. Lysates were subjected to AP with streptavidin beads followed by western blotting with α-KIF14 and α-HA antibodies. (E) Radil binds to KIF14 directly. Recombinant GST-RadilPDZ was incubated with purified Flag-tagged KIF14-ctail (wild-type or IQAA mutant) that were immobilized on FLAG-M2 beads. After 1 hour of binding, beads were washed and protein binding determined by western blotting using α-Radil and α-FLAG (rabbit) antibodies.
(Figure 3-1D. compare lanes 2 and 3). To also test for the specificity of the Radil-KIF14 interaction we performed affinity purification of Radil and blotted for the unrelated kinesin family protein KIF7 and showed that these proteins do not interact (Figure 3-10A). We also showed that KIF14 does not interact with AF-6, which is closely related to Radil in terms of domain architecture and also possess a C-terminal PDZ domain whose sequence is 33% identical and 53% homologous to the PDZ domain of Radil (Figure 3-10B-C). To determine if the interaction between KIF14 and Radil is direct, we performed \textit{in vitro} binding experiments using recombinant proteins. While recombinant C-terminal domain of wild type KIF14 binds to the PDZ domain of Radil, the IQAA mutant did not (Figure 3-1E, compare lane 8 and 10). From these experiments we conclude that Radil and KIF14 directly associate through a PDZ domain-ligand interaction.

3.4.2 Radil localizes with KIF14 on the microtubule network

Kinesins are ATP-consuming motor proteins that travel along the microtubule network to deliver proteins or organelles as cargo to different cellular locations (Hirokawa et al., 2009; Verhey and Hammond, 2009). We hypothesized that Radil could associate with microtubules through its binding to KIF14. To address this we used confocal microscopy to study the subcellular localizations of Radil and KIF14 in interphase cells. The strong co-localization of KIF14 with $\beta$-tubulin and acetyl-tubulin validates the presence of KIF14 on microtubules (Figure 3-10D). When co-expressed in HEK293T cells, mCherry-Radil and eGFP-KIF14 co-localize on microtubules (Figure 3-2A, panels a-c). The mutant KIF14-IQAA also localizes on microtubules (Figure 3-2A, panel e) but fails to recruit Radil (Figure 3-2A. panels d-f). Similarly, Radil$\Delta$PDZ does not co-localize with KIF14 on microtubules (Figure 3-2A, panels g-i). To support these findings, we performed microtubule sedimentation experiments from MDA-MB-231 cells to
determine the subcellular localization relationship of endogenous Radil and KIF14. In cells expressing scrambled shRNAs, KIF14 and Radil co-sediment with microtubules (Figure 3-2B). Sedimentation of KIF14 with microtubules is not affected when Radil’s levels are reduced using shRNA (Figure 3-2B and Figure 3-10E) but knockdown of KIF14 inhibits the sedimentation of Radil in microtubule fractions (Figure 3-2B and Figure 3-10F). We conclude that KIF14 recruits Radil to the microtubule network through a physical association.
Figure 3-2. KIF14 recruits Radil on microtubules. (A) Shown is localization of mCherry-Radil with eGFP-KIF14 (a-c), mCherry-Radil with eGFP-KIF14-IQAA (d-f), and mCherry-RadilΔPDZ with eGFP-KIF14 (g-i). HEK293T cells were co-transfected with low amounts of Radil expression plasmids together with KIF14 cDNA constructs. (B) Localization of endogenous Radil on microtubules is KIF14 dependent. MDA-MB-231 cells expressing scrambled- Radil or KIF14 shRNA were lysed in BRB80 buffer and cytosolic tubulin was polymerized by addition of GTP (2mM) and paclitaxel (20μM). Rigor binding of motor proteins was allowed by addition of AMP-PNP (2mM) and the microtubule-motor protein mixture pelleted by centrifugation. The fractionated proteins were detected by western blotting. P = Pellet fraction; S = Supernatant fraction.
3.4.3 KIF14 negatively regulates Radil function in cell spreading

KIF14 was first identified as an oncogene in retinoblastoma and is overexpressed in several cancers. Interestingly, KIF14 is highly overexpressed in breast tumours and its expression correlates with a worst outcome of the disease (Corson and Gallie, 2006; Corson et al., 2005). We extended these data by measuring KIF14 levels using qPCR in 103 individual primary breast tumours and show that KIF14 expression is increased on average 12.32 fold (Figure 3-11A). In contrast, Radil mRNA expression remains unchanged among different tumour grades (data not shown). We could not evaluate KIF14 and Radil levels during breast cancer progression since we did not have access to the metastatic tissues but elevated KIF14 expression in primary breast tumours is known to correlate with the severity and invasiveness of cancer (Corson and Gallie, 2006). High levels of KIF14 expression in many primary breast tumours and its correlation with breast cancer severity prompted us to measure its abundance in three different breast cancer cell lines. KIF14 expression was 1.6, 5 and 14 fold higher in the transformed MCF7, MDA-MB-468 and MDA-MB-231 breast cancer cell lines, respectively, compared to normal breast tissues (Figure 3-11B). Interestingly, KIF14 expression in these cell lines appears to correlate with their migratory and invasive properties since MCF7 and MDA-MB-468 cells are poorly invasive compared to MDA-MB-231 (Meng et al., 2000; Naik et al., 2008; Wang et al., 2007). We established that the Radil-KIF14 complex exists in MDA-MB-231 cells using co-immunoprecipitation (Figure 3-3A). We also analyzed Radil and Radil∆PDZ protein complexes isolated from MDA-MB-231 cells using mass spectrometry. As expected several peptides corresponding to Rap1 and Gβγ subunits were identified in both wild-type Radil and Radil∆PDZ but KIF14 peptides were only identified in Radil complexes. (Figure 3-3B and Table 3-1B). These results confirm that KIF14 interacts with the PDZ domain of Radil and suggest that the Radil∆PDZ mutant is not grossly misfolded since it still interacts with most
interactors but selectively lost the ability to bind KIF14. These observations led us to examine a potential functional role for Radil in breast cancer and test the possibility that KIF14 overexpression modulates Radil functions during tumourigenesis.

Given the established role of Rap1a-Radil signalling in cell adhesion and spreading in other contexts (Ahmed et al., 2010; Ross et al., 2011; Smolen et al., 2007), we next tested its requirement for MDA-MB-231 cell spreading. We validated two shRNAs (#4 and #206) that knockdown Radil mRNA levels by more than 75% and that efficiently decrease Radil protein levels (Figure 3-10E). The depletion of Radil led to a marked impairment in the ability of cells to spread on fibronectin-coated coverslips (Figure 3-3C & Fig. Figure 3-11C), a phenotype similar to cells expressing Rap1GAP and therefore have low Rap1a activity (Figure 3-3C). Notably, 120 min after plating, most of the scrambled-shRNA expressing cells are fully spread whereas Radil-depleted cells and Rap1GAP-expressing cells still exhibit a rounded morphology (Figure 3-3C, quantification of the rate of spreading in right panel). The Radil shRNA is on-target since expressing a knockdown resistant mouse Radil cDNA rescued the spreading defects induced by both shRNAs (Figure 3-11D). However, expression of mouse Radil∆PDZ could not rescue the spreading defects (Figure 3-11D). We conclude that Radil is required for ECM-and Rap1a-mediated spreading in breast cancer cells.

Since KIF14 interacts physically with Radil we were interested to test whether it was important for cell spreading. Following knockdown of KIF14 using shRNAs (Figure 3-3D and Figure 3-10F), MDA-MB-231 cells behave opposite to cell in which Radil was depleted, exhibiting increased spreading efficiency and accelerated kinetics (Figure 3-3D, compare cell spreading at 45 min). Indeed, KIF14-depleted cells are on average three times the area of control shRNA-expressing cells (Figure 3-3D, compare cells at 180 min and quantification on right
panel). To explore the functional relationship between KIF14, Rap1a and Radil, we asked if blocking Rap1a activity or decreasing Radil expression could modify the enhanced cell spreading phenotype that manifest upon KIF14 depletion. Overexpression of Rap1GAP or depletion of Radil with shRNA both reversed the enhanced spreading phenotype of KIF14 depleted cells (Figure 3-3E, quantification in right panel). These findings indicate that KIF14 negatively regulates cell spreading by controlling Rap1a-Radil activity.
Figure 3-3. KIF14 negatively regulates Radil during MDA-MB-231 cell spreading. (A) The KIF14-Radil complex is present in MDA-MB-231 cells. Immunoprecipitation was performed using α-Radil or control IgG antibodies followed by western blotting (WB) with α-Radil or α-KIF14 antibodies. (B) Shown is a protein-protein interaction heat map for FLAG-mRadil and FLAG-mRadilΔPDZ. Lysates from cells stably expressing FLAG-mRadil or FLAG-mRadilΔPDZ were subjected to pull-downs with α-FLAG M2 beads and the purified protein complexes were subsequently analyzed by LC-MS/MS. Colors indicate the number of experiments in which the identified proteins were detected as depicted in the legend (right). (C) Rap1-Radil signaling is required for MDA-MB-231 cell spreading. Cells expressing control shRNA, Radil shRNA#4, or overexpressing Rap1GAP cDNA were plated on fibronectin for the indicated times and cell spreading was quantified by measuring the cell area (right panel). (D) KIF14 negatively regulates cell spreading. Cells expressing KIF14 shRNA#816 were plated on fibronectin coated plates and cell spreading was monitored over the time points indicated by quantifying cell surface areas. Images were captured from 5 random fields for each time point. The curves were fitted by one phase association model (see materials and methods). (E) KIF14 negatively regulates Rap1a-Radil signaling. The increased cell spreading observed in cells expressing KIF14 shRNA is rescued by co-expressing Radil shRNA or Rap1GAP. Cells were plated on fibronectin coated surface (0.5μg/ml) and allowed to adhere and spread for 180 min. At the end of the experiment cells were imaged (left) and their spreading quantified (right). Also see Figure 3-11. Bars = 50μm. Error bar ± S.E.M.
3.4.4 KIF14 negatively regulates Rap1a and Radil-dependent inside-out signalling

Through the engagement of different effectors such as Radil, Rap1a regulates cell spreading by modulating inside-out signalling influencing integrin activation. Using the 9EG7 antibody that specifically recognizes activated β1-integrin, we show that Radil knockdown impairs integrin activation during spreading in MDA-MB-231 cells (Figure 3-4A, quantification in 4B). We note that this is likely an underestimation since cells with efficient Radil knockdown have impaired adhesion and were washed-off during the experiment. Consistent with the enhanced spreading phenotype, depletion of KIF14 leads to hyperactivation of β1-integrin when MDA-MB-231 cells are plated on fibronectin (Figure 3-4A and B). This results from increased Rap1a-dependent inside-out signalling since integrin activation returns to normal when Radil expression is reduced within KIF14 shRNA-expressing cells (Figure 3-4A). We confirmed that the effects are due to change in integrins activation rather than expression since β1-integrin levels did not vary (Figure 3-4B).

Integrin engagement by ECM proteins leads to their clustering and to the recruitment and activation of several proteins implicated in the ontogeny and maturation of focal contacts. Hence, we predicted that Radil and KIF14-depleted cells would have altered focal contact properties given their defects in spreading and integrin activation. We thus visualized focal adhesions by imaging vinculin in cells expressing control, Radil or KIF14 shRNAs (Figure 3-4C, Figure 3-12A-B), quantified their abundance and size in individual cells (Figure 3-4D) and calculated the average focal adhesion sizes in all the cells examined (Figure 3-4E). Knockdown of Radil results in cells with fewer and smaller focal contacts whereas KIF14 depletion dramatically increases focal contact numbers and results in enlarged focal adhesions that are on average approximately twice the size of control cells (Figure 3-4C and E). Following the stabilization of
adhesion sites, F-actin is known to increase in density leading to formation of compact bundles of stress fiber, which in turn leads to focal adhesion maturation (Amano et al., 1997; Chrzanowska-Wodnicka and Burridge, 1996; Gardel et al., 2010; Geiger et al., 2009; Oakes et al., 2012; Parsons et al., 2010). Predictably, hyper-activated integrin signalling and the presence of large mature focal adhesions observed in KIF14-depleted cells correlate with increased stress fiber formation (Figure 3-4F, Figure 3-12A-B). In all three staining conditions we also noted that Radil, as well as KIF14 depleted cells, have unpolarized morphology when compared to control shRNA-infected cells. Presence of large focal adhesions is indicative of mature adhesion and suggests defective adhesion disassembly. To test this hypothesis we performed time lapse imaging experiments to look at the dynamics of the focal adhesion marker Paxillin fused to Venus (Figure 3-5A). Approximately 80% of adhesion foci in control cells displayed rapid and dynamic turnover of Venus-Paxillin (Figure 3-5A and B). On the other hand, KIF14-shRNA expressing cells exhibit stable and large focal adhesions that seldom disassembled over a period of approximately one hour (Figure 3-5A and B). We conclude that KIF14 is a negative regulator of inside-out integrin signalling regulated by Rap1 and its effector Radil.
Figure 3-4. Radil and KIF14 differentially regulate integrin activation and focal adhesion dynamics. (A) Shown are representative TIRF images of active β1 integrin (9EG7) staining in MDA-MB-231 cells expressing scrambled shRNA, Radil shRNA #4, KIF14 shRNA #816 or Radil shRNA #4 + KIF14 shRNA #816. Cells were plated on fibronectin coated coverslips (0.5μg/ml) for 120 min, fixed and stained with 9EG7 antibody. (B) FACS analysis showing increase in activated β1 integrin (9EG7) upon depletion of KIF14. Total integrin expression levels were determined by western blot using a β1 integrin antibody that recognizes the cytoplasmic tail. β-tubulin was used as loading control. (C) Focal adhesion sites were imaged using TIRF microscopy and vinculin staining in cells treated as above. Threshold images were used to quantify FA areas and numbers. (D) Graphical representation of FA area versus number in each cells quantified. (E) Average FA area for all cells quantified in D. (F) Confocal images of F-actin in cells treated as indicated and following spreading for 120 min on fibronectin coated coverslips. A line was drawn across cells and the intensity profile plotted (below) to show the relative distribution of actin stress fibers in cells. Also see Figure 3-12. Bars = 20μm. Error bars ± S.E.M.
Figure 3-5. KIF14 knockdown alters focal adhesion dynamics. (A) Venus-Paxillin dynamics was assessed by time lapse TIRF microscopy in MDA-MB-231 cells expressing scrambled or two different KIF14 shRNAs. Cells were plated on fibronectin (0.2μg/ml) coated glass slides. Each cell was observed over 54 min and a picture was taken every 3 min. Merge shows overlay of images taken at time 0 and 54. Red = 0 min; Green = 54 min. (B) Quantification of Venus-Paxillin dynamics shown in (A). Bars = 5μM. Error bars ± S.E.M.
3.4.5 KIF14 spatially restricts Radil from interacting with activated Rap1a

Expression of the constitutively active Rap1a-Q63E mutant leads to plasma membrane recruitment of Radil (Ahmed et al., 2010). Having established that KIF14 negatively regulates inside-out signalling and recruits Radil on microtubules, we reasoned that Rap1a activity could modulate the recruitment of Radil on microtubules. Supporting this prediction, expressing the Rap1a-Q63E mutant leads to the dislodging of Radil from the microtubules and to its recruitment at the plasma membrane (Figure 3-6A). The release of Radil from microtubules in KIF14-shRNA expressing cells could thus explain the increased inside-out integrin signalling observed in these cells. We therefore monitored the localization of Radil in MDA-MB-231 cells while varying the levels of KIF14. Since they express high levels of KIF14, Radil is predominantly localized on microtubules in MDA-MB-231 (Figure 3-6B, panel c). Upon knockdown of KIF14, Radil is redistributed at the cell cortex, presumably at sites of Rap1a activation (Bivona et al., 2004; Li et al., 2009), whereas the localization of the control protein Dishevelled2 is not affected (Figure 3-6B, compare panel b and d). Likewise, disrupting microtubule networks with nocodazole results in Radil redistribution at the cell periphery (Figure 3-12C). We also tested if Rap1 activity is affected in the knockdown conditions. Rap1-GTP pull-down assays did not reveal any change in Rap1 activity in KIF14-depleted cells compared to control cells but depletion of Radil leads to a slight decrease in Rap1 activity (Figure 3-12D). These observations suggest that KIF14 controls inside-out integrin activation by tethering the Rap1a effector Radil on microtubules and thereby spatially controlling Rap1 signalling.
Figure 3-6. **Rap1a activation releases Radil and KIF14 from microtubules.** (A) Confocal images of HEK293T cells expressing mCherry-Radil and eGFP-KIF14 with or without HA-Rap1aQ63E or FLAG-Rap1GAP. Expression of constitutively active Rap1a leads to dislocation of Radil and KIF14 from microtubules and recruitment of Radil to the plasma membrane. Bars = 10µm. (B) Confocal images of MDA-MB-231 cells expressing FLAG-Dishevelled2 (FLAG-Dsh2) or FLAG-Radil transduced with scrambled shRNA or KIF14 shRNA #816. A line was drawn across each cell and the profile of protein distribution depicted below each image. Bars = 20µm
3.4.6 The tuning of Rap1a-Radil mediated inside-out signalling is required for efficient breast cancer cell migration and invasion

Both decreased or hyperactivated Rap1a or integrin signalling inhibit cell migration and invasion (Bailey et al., 2009; Freeman et al., 2010; Lyle et al., 2008; Tsygankova et al., 2007). With this in mind, together with the known role of Radil in neural crest cell migration (Smolen et al., 2007) we tested the requirement of Radil for the migration of MDA-MB-231 cells. Knockdown of Radil with two different shRNAs (#4 and #206) severely impairs the migratory (Figure 3-7A) and invasive (Figure 3-7B) properties of these cells. Interestingly, depletion of KIF14 using two independent shRNAs (#816 and #817), which we show leads to hyper-activated Rap1-Radil signalling, also leads to decreased MDA-MB-231 cell migration and matrigel invasion (Figure 3-7D and E). Several lines of evidence support the specificity of the phenotypes that we observed. First we obtained similar results with two independent Radil and KIF14 shRNAs. Second, we were able to rescue the migration defects by expressing the RNAi-resistant mouse Radil cDNA or the eGFP-KIF14 coding sequence since the KIF14 shRNAs target the 3’UTR region (Figure 3-7C and F). In contrasts, expression of mouse Radil∆PDZ and eGFP-KIF14-IQAA mutants fails to rescue the cell migration defects associated with Radil and KIF14 depletion (Figure 3-7C and F). From these findings we conclude that the optimal balance of Rap1a-Radil signalling controlled by KIF14 is required to sustain the efficient migration and invasion of breast cancer cells.
Figure 3-7. Radil and KIF14 are required for MDA-MB-231 cell migration and invasion. (A) MDA-MB-231 cells were transduced with the indicated shRNAs. 72 hours after transduction 5x10^4 cells were seeded on the upper chamber of transwells and 20% fetal bovine serum (FBS) in DMEM was applied to the lower chamber. Cells were allowed to migrate for 10-12 hours and migrated cells counted from pictures of 4-5 random fields. (B) Cell invasion was assessed as above except the transwells coated with 1µg/ml of matrigel were used. Cells were allowed to invade through the matrigel for 20 hours. (C) MDA-MB-231 cells stably expressing the shRNA-resistant murine full-length mRadil or mRadilΔPDZ were transduced with scrambled, or two different Radil shRNAs. The migratory potential of these cells was assessed as described above. Expression levels of FLAG-mRadil and FLAG-mRadilΔPDZ are shown on the right panel. Cortactin used as loading control. (D) Transwell cell migration and (E) invasion assays with cells expressing KIF14 shRNAs. Cells were processed as above. (F) MDA-MB-231 cells were transduced with scrambled shRNA or KIF14 shRNA #816 in the presence of FLAG-GFP, eGFP-KIF14 or eGFP-KIF14-IQAA. The different cells were then subjected to transwell assays. Expression levels of eGFP-KIF14 and eGFP-KIF14-IQAA are shown on the right panel. KIF14 shRNA #816 targets the 3' UTR and was thus utilized for rescue experiments. Bars; mean, ± S.E.M.
3.4.7 Knockdown of Radil blocks breast cancer progression.

Since knockdown of Radil efficiently blocks cell migration and invasion of breast cancer cells \textit{in vitro}, we further studied the requirement of Radil during breast cancer cell metastasis using a mouse model. MDA-MB-231 cells constitutively expressing Renilla luciferase (MDA-MB-231-Rluc) were derived to assess metastatic activity to the lungs using bioluminescence imaging. Control- or Radil-shRNA expressing cells were injected in the tail-veins of immunodeficient mice. Prior to injections, we validated the functional knockdown of Radil by measuring the \textit{in vitro} migratory properties of the cells using transwell assays (Figure 3-8A). Following injections, we monitored lung metastasis by bioluminescence imaging on days 10, 20 and 35 and began to detect bioluminescence signals on day 35 only in control shRNA-treated animals (Figure 3-8B). By day 45 some of the control shRNA-treated animals exhibited signs of respiratory distress and lethargy at which point the experiment was terminated. Gross anatomical review confirmed massive tumour growths in the lungs of several control shRNA treated mice, accompanied by extensive vascularization and increase in organ sizes (Figure 3-8C and D). In contrast, all the animals injected with Radil shRNA-expressing cells appeared healthy on day 45 and only a few metastatic nodules were detected on the lungs of 6 of the 11 animals (Figure 3-8C and D).

It is well established that integrins (Cruet-Hennequart et al., 2003; Kuwada and Li, 2000), Rap1 (Dao et al., 2009) as well as KIF14 (Gruneberg et al., 2006; Theriault et al., 2012) are also important during cell proliferation. We were therefore interested to determine if Radil controls the proliferation of breast cancer cells. Cells expressing Radil shRNA show 42.5% inhibition of cell proliferation when cultured in vitro for 4 days (Figure 3-8E). We also assessed their growth \textit{in vivo} by injecting cells expressing control or Radil shRNAs subcutaneously in nude mice. 31
days after injection, subcutaneous tumours from all mice were retrieved and their weights and volumes were measured. Radil shRNA-expressing tumours were on average 44% lighter than control shRNA-expressing tumours (Figure 3-8F and G, Figure 3-13A and B). We conclude that Radil is required during breast cancer progression by controlling cell proliferation and cell dissemination to the lungs.
Figure 3-8. Radil is required for breast cancer cell metastasis. (A) MDA-MB-231 cells stably expressing Renilla luciferase were transduced with scrambled shRNA or Radil shRNA #4 and their migratory potential was tested using transwell assays prior to injection in mice. (B) MDA-MB-231 cells were injected via tail-vein in NOD-SCID mice. Metastasis and homing of cells to the lungs was monitored over time using in vivo bioluminescence imaging. Shown are images from Day 35. (C) Mice were sacrificed 45 days after inoculations. Lungs from mice injected with saline or with scrambled shRNA or Radil shRNA expressing cells were collected, fixed and stained with Bouin’s solution. Shown are representative images from each condition (top). Quantification of the visible tumor nodules is provided in the table (below). (Scrambled shRNA n=12, Radil shRNA #4 n=11), statistics; Student’s t-test. (D) Total number of nodules counted on the lungs of each animal. Error bar +/- S.E.M. (E) In vitro proliferation of MDA-MB-231 cells treated with scrambled or Radil shRNA #4. (F) Quantification of tumor end volume (in cm$^3$) on day 31. (Scrambled shRNA n=5, Radil shRNA #4 n=5). Red line denotes median value. Bars; interquartile range. (G) Quantification of tumor end weight (in grams) on day 31.
3.5 Discussion

Our results identify the oncogene KIF14 as a negative regulator of Rap1a-Radil signalling. We provide evidence that KIF14 tethers Radil on microtubules and thereby controls inside-out integrin activation by tuning the availability of this Rap effector for binding Rap1-GTP at the plasma membrane. Since KIF14 is frequently up-regulated in several human cancers, this led us to study the role of Radil in cancer progression. Our data show that depletion of Radil and KIF14 using shRNAs affects the migratory and invasive properties of breast cancer cells in vitro. Using xenograft models, we further demonstrated the requirement of Radil for tumour cell proliferation and for the efficient dissemination of breast cancer cells to lungs.

The regulation of Rap1-Radil signalling by KIF14 highlights the necessity for the fine regulation of integrin activation and cell-matrix adhesion during cancer cell migration. Indeed, it is known that reduced and hyperactivated Rap1 signalling similarly lead to impaired cell motility (Bailey et al., 2009; Freeman et al., 2010; Huang et al., 2012; Lorenowicz et al., 2008; Lyle et al., 2008; Zheng et al., 2009). Accordingly, we show that Radil- and KIF14-depleted cells that respectively exhibit impaired and hyperactivated integrin signalling do not migrate efficiently. In addition to increased spreading and reduced migration, KIF14-depleted cells have lost the polarized morphology that characterizes highly migratory cancer cells. We propose that the spatial control of Radil availability, controlled by microtubules and KIF14, enables rapid changes in localized Rap1-Radil signalling and integrin activation that are known to be critical for the regulation of cell-matrix adhesion and the establishment of cell polarity (Arthur et al., 2004; Freeman et al., 2010; Gerard et al., 2007; Itoh et al., 2007; Jeon et al., 2007b). The modulation of Rap1 signalling through the availability of Radil thus represents another regulatory step for the inside-out activation of integrins during breast cancer cells migration.
Although mechanisms differ, other Rap1 effectors localize and are regulated by microtubules. For example, the Rap1 effectors RapL (Fujita et al., 2005) and Krit1 (Beraud-Dufour et al., 2007) directly interact with microtubules from where they are dislodged and recruited to the plasma membrane when Rap1 is activated. Although a kinesin protein may not be needed in these cases, this suggests that the control of Rap1 effectors by microtubules may be a general mechanism to govern spatiotemporal Rap1 signalling in various contexts.

At present the mechanism by which Radil leads to inside-out activation of integrins is not completely clear. Although other Rap1 effectors such as RIAM have been shown to mediate the formation of an integrin-activation complex containing Rap1 and Talin (Lee et al., 2009) that can directly associate with integrins, our mass spectrometry interrogation of the Radil complex did not reveal the presence of Talin or other integrin-associated proteins. However, we previously identified Gβγ subunits of heterotrimeric G proteins as Radil interactors and demonstrated that Gβγ overexpression leads to Rap1a-dependent inside-out activation of integrins and cell spreading (Ahmed et al., 2010). Interestingly, Gγ subunits were previously shown to localize to the tip of stress fibers and focal adhesions (Hansen et al., 1994). Following G Protein Coupled Receptor activation, the release of Gβγ could thus direct the assembly of Gβγ-Rap1a-Radil signalling complex leading to spatiotemporal activation of integrins. Especially relevant to our findings showing that the depletion of Radil inhibits the metastasis of MDA-MB-231 cells to lungs, activation of the CXCR4 receptor (Muller et al., 2001) and Gβγ subunits (Kirui et al., 2010; Tang et al., 2011) were previously shown to be required for this process. Additionally, given that loss of function for the ERM protein Ezrin was recently demonstrated to be required for the Rap1-induced spreading to similar extent than Radil (Ross et al., 2011), studying the functional relationship between these proteins may reveal further clues about their mechanisms.
Figure 3-9. Proposed model describing the roles of Radil and KIF14 during cancer cell migration. (A) In highly motile cancer cells, KIF14 is upregulated and sequesters Radil on microtubules. This enables optimal Radil-Rap1 signaling, inside-out integrin signaling and cell-matrix adhesive properties required for efficient cell migration. (B) Depletion of Radil inhibits integrin activation, reduces cell-matrix adhesion and causes loss of traction and cell motility. (C) Depletion of KIF14 leads to the release of Radil from microtubules thereby increasing the pool of Radil available to associate with activated Rap1 at the plasma membrane. This leads to hyperactivated integrins, increases focal adhesion formation and decreases motility.
As the expression of Rap1GAP and knockdown of Radil similarly lead to decreased activation of integrins, impaired focal adhesion formation and defects in spreading of breast cancer cells, our results strongly implicate a requirement for Rap1-Radil signalling in the context of cancer cell migration.

We previously established that the PDZ domain of Radil was important for its activity in cell adhesion (Ahmed et al., 2010). The integration of the phage-display screen to identify the preferred PDZ ligand for the Radil PDZ domain and the mass spectrometry analysis of Radil-containing protein complex enabled the identification of KIF14 as a protein interacting with the Radil PDZ domain. The C-terminal of KIF14 contains the atypical hydrophobic [F/I]-x-W-V motif revealed by phage display and was found to be critical for its binding to Radil and for recruitment of the latter on microtubules. Further studies will be needed to understand how the binding of Radil to KIF14 is modulated. One possibility is that the recruitment of Rap1-GTP to the N-terminal RA domain of Radil could modulate the availability of the C-terminal PDZ ligand for binding to KIF14.

KIF14 is frequently overexpressed in several human cancers. Its overexpression in breast cancer was previously shown to be a predictor of poor survival as it correlates with tumour grade and invasiveness (Corson and Gallie, 2006; Corson et al., 2005). KIF14 in a complex with citron kinase and PRC1 were identified as a mitotic kinesin with a role during cytokinesis since its knockdown led to binucleated Hela cells (Carleton et al., 2006; Gruneberg et al., 2006). Interestingly, Rap1 activity and integrin signalling need to be optimally regulated for normal cell division (Dao et al., 2009). Indeed, at the onset of mitosis, most adherent cells undergo cell retraction characterized by the disassembly of focal adhesions and actin stress fibers, processes that are controlled by Rap1 and integrins. Given the importance of Radil as a Rap1 effector and
its regulation by KIF14 we show that Radil, perhaps not surprisingly, controls cell proliferation and tumour growth. Our results thus position the KIF14-Rap1-Radil signalling axis as an important mediator of tumour progression as it affects both cell proliferation and migration (Bailey et al., 2009; Freeman et al., 2010; Huang et al., 2012).

The ability of cells to dynamically change their adhesion to the ECM during cell migration is a property optimized by cancer cells to support metastasis and cancer progression (Desgrosellier and Cheresh, 2010; Felding-Habermann, 2003; Felding-Habermann et al., 2001). In the light of our findings, KIF14 overexpression detected in several human cancers may reflect this optimization process required to tune the level of Rap1-Radil signalling and resulting integrin activation needed for efficient cell motility and tumour progression. We validated that KIF14 is highly upregulated in a cohort of primary breast tumours and showed that KIF14 overexpression in breast cancer cell lines correlates with their invasive properties. In contrast, Radil expression is not significantly changed in breast tumours or in cell lines but Rap1 activity is known to be highly upregulated in cancer cells (Banerjee et al., 2011; Sjoblom et al., 2006; Zheng et al., 2009). Interestingly, similar to KIF14, Sharpin was recently identified as an inhibitor of β1-integrins by preventing the interaction with Talin and Kindlin thereby inhibiting the dynamic switching between active and inactive conformations (Rantala et al., 2011). Sharpin levels are also upregulated in different cancers and this was found to be important for cell migration.

The creation and regulation of traction forces in response to interactions of the extracellular matrix with different regions of the cell are intrinsic to cell migration and proliferation (Dao et al., 2009; Kaverina et al., 2002b). Regulated cycles of assembly and disassembly of focal adhesions and focal complexes at adhering and retracting sides of the cells produce the tractions
required for cells to move. It is becoming increasingly clear that the precise control of focal adhesion dynamics is important for efficient cell migration (Gardel et al., 2008; Gardel et al., 2010; Nagano et al., 2012). As such, the presence of fewer focal complexes at the periphery and large focal adhesions are signs of reduced adhesion dynamics and cell movement. The depletion of Radil in MDA-MB-231 impairs integrin activation and leads to reduced focal adhesions whereas KIF14 depletion leads to dramatically enlarged focal adhesions and increased stress fibers (Figure 3-4). Consistent with this notion, both Radil and KIF14-depleted cells exhibit reduced cell motility (Figure 3-7).

In summary this study identifies the oncogene KIF14 as a PDZ-ligand-containing protein interacting with Radil. KIF14 negatively regulates Rap1a signalling and inside-out integrin activation by tethering Radil on microtubules. Our results describe the role of Radil in cancer progression, and suggest that it is an important nexus integrating GPCR, Gβγ and Rap1 signalling in this context. Given that KIF14 is frequently overexpressed in several cancers, further studies examining the requirement of Radil for the progression of other cancers will be important. Furthermore, guided by other studies showing the targeting of PDZ-mediated interactions with small molecules (Houslay, 2009; Patra et al., 2012; Thorsen et al., 2010) and synthetic peptides (Tonikian et al., 2008; Zhang et al., 2009), inhibiting KIF14-Radil interaction could provide a novel strategy for the treatment of cancer.
Figure 3-10. (Supplementary figure) Radil specifically associates with KIF14. (A) Lysates from control HEK293T cells or stably expressing Strep-HA-Radil were affinity purified with streptavidin sepharose and probed for endogenous KIF14 or KIF7. While KIF14 co-purifies with Strep-HA-Radil, KIF7 did not. (B) Sequence alignment of the AF-6 and Radil PDZ domains. AF-6 and Radil share 33% identical and 53.4% homology sequences. (C) Lysates for HEK293T wild-type cells or transfected with FLAG-Radil or FLAG-AF6 were immunoprecipitated using α-FLAG M2 antibodies and probed for KIF14 using western blotting. (D) KIF14 localizes on microtubules. eGFP-KIF14 was transiently expressed in HEK293T cells. Cells were fixed followed by immuno-detection of endogenous β-tubulin (a-c) or acetylated-tubulin (d-f). Merged images are shown to depict colocalization in panels c and f. Images of cells were taken as z-stacks at 1μm intervals using a confocal microscope and a 63×/1.4 oil immersion objective. The images are 3D renderings. Bars = 10μm. (E) Knockdown efficiencies of two different Radil shRNAs were determined by Taqman qPCR (left panel) or Western blotting using α-Radil antibodies (right panel). (F) Knockdown efficiencies for two independent KIF14 shRNAs were determined using western blotting. KIF14 shRNA #816 targets the 3’UTR whereas shRNA#817 targets a sequence within the coding sequence. α-Cortactin antibody was used as loading control.
**Figure 3-11. (Supplementary figure) KIF14 is highly expressed in breast cancer cells.** KIF14 mRNA levels were quantified by Taqman qPCR from (A) 99 primary breast cancer tumors and compared with 10 matched normal breast tissue samples or (B) in three different breast cancer cell lines. (C) Effects of Radil and KIF14 knockdown on cell spreading. MDA-MB-231 cells expressing two different Radil-specific shRNAs (Radil shRNA #4 or Radil shRNA #206) were dissociated, plated on fibronectin (0.5µg/ml) coated plates and allowed to spread for 180 min. Representative images of cells are shown and the cell area quantified (right panel). (D) Expression of a shRNA-resistant mouse Radil full-length cDNA (FLAG-mRadil) rescues the spreading defects caused by expression of the two Radil shRNAs, whereas FLAG-mRadilΔPDZ does not. Quantifications of cell areas are on the right panel. (E) Two different shRNAs targeting KIF14 (KIF14 shRNA #816 and KIF14 shRNA #817) were expressed in MDA-MB-231 and tested for their effect on cell spreading as described above. Quantification of cell areas (right panel).
Figure 3-12. (Supplementary Figure) Effect of Radil and KIF14 depletion on focal adhesion and stress fibers. MDA-MB-231 cells treated with scrambled shRNA or Radil shRNA #206 (A), or KIF14 shRNA #817 (B) were plated on fibronectin (0.5µg/ml) coated coverslips, allowed to adhere for 120 minutes and stained for either F-actin or vinculin. F-actin images were acquired using confocal microscopy whereas vinculin images were taken using TIRF microscopy. Panel e in A) shows an epifluorescence image of cells stained with α-vinculin to depict its expression but the absence of recruitment within focal adhesion at the cell matrix interface (panel d). Bar = 20μm. (C) Disruption of microtubules affect Radil localization. MDA-MB-231 cells expressing FLAG-Radil were treated with DMSO or Nocodazole (10µM) for 20 minutes. Cells were then fixed and immunostained with α-FLAG antibodies. Bottom panel is shown in pseudocolors to illustrate protein re-localization upon nocodazole treatment. (D) RalGDS pull-downs to assess Rap1 activity in scrambled, Radil shRNA #4 and KIF14 shRNA #816 expressing cells. Total Rap1 in whole cell lysates and captured Rap1-GTP levels were determined by western blotting using α-Rap1 antibodies.
Figure 3-13. (Supplementary Figure) Knockdown of Radil reduced MDA-MB-231 tumor progression in mice. (A) Assessment of Radil knockdown prior to subcutaneous injections in mice. (B) Representative subcutaneous tumors generated from scrambled or Radil shRNA #4 treated MDA-MB-231 cells. Images show GFP expression driven by the shRNA vector. Bar = 5mm.
A. FLAG-Radil pull-down in HEK293T cells

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<th>Gene ID</th>
<th>Protein name</th>
<th>Unique peptides</th>
<th>Total peptides</th>
<th>Probability</th>
<th>Coverage</th>
<th>No. of Pull-downs</th>
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<td>969</td>
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<td>40.00%</td>
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<td>3845</td>
<td>c-K-ras2 protein isoform b</td>
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B. FLAG-mRadil and FLAG-mRadilΔPDZ pull-downs in MDA-MB-231 cells

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<tr>
<th>Gene ID</th>
<th>Protein ID</th>
<th>Protein description</th>
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<td>9411</td>
<td>ARHGAP29</td>
<td>Rho GTPase activating protein 29</td>
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<td>1.00 (57-14-10.60)</td>
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<td>RADIL</td>
<td>Ras association and OIL domains</td>
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<td>KIF14</td>
<td>kinesin family member 14</td>
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<td>1.00 (21-18-13.50)</td>
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<td>5906</td>
<td>RAP1A</td>
<td>RAP1A, member of RAS oncogene family</td>
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<td>1.00 (14-6-47.80)</td>
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<td>5908</td>
<td>RAP1B</td>
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<td>1.00 (11-5-62.00)</td>
</tr>
<tr>
<td>2782</td>
<td>GNB1</td>
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<td>RRAS</td>
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<td>51232</td>
<td>CRIM1</td>
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<td>0.99 (2-3-7.70)</td>
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Table 3-1. (Supplementary table) Radil-associated proteins identified by LC-MS/MS. (A) Compilation of proteins identified in FLAG-Radil complexes isolated from HEK293T cells. (B) Shown is a comparison of proteins that associate with FLAG-Radil and FLAG-RadilΔPDZ complexes isolated from MDA-MB-231 cells. The table lists the name of the proteins identified in a representative pull-down experiment with the number of unique and total peptides identified as well as their percent coverage of the protein sequences. Also listed is the number of independent experiments in which the different proteins were identified by mass spectrometry.
Chapter 4
General Discussion
Over the past several years, the βγ-subunits of the heterotrimeric G proteins have been clearly implicated in several aspects of cell motility. But how exactly βγ elicit these functions is not very well understood. Recently, there has been increased interest to decipher these mechanisms leading to discovery of novel effectors of Gβγ and ways they transmit βγ-mediated signalling during cell motility (see section 1.2.2.4).

I started my thesis work looking for novel interactors of Gβγ by taking a mass spectrometry-based proteomic approach. In doing so, we identified Radil and showed that it is a scaffold protein that forms a complex interfacing the association between Gβγ and Rap1. We also found that the interaction of Radil with Gβγ depends on the activation state of Rap1a, as its inactivation by Rap1GAP can completely obliterate the binding. We tried performing reciprocal experiments by blocking the interaction of Gβγ with Radil using βγ-scavengers such as β-adrenergic receptor kinase c-tail (Jing et al., 1999) or Ga1 (Federman et al., 1992). Neither of these inhibitors of Gβγ affected the interaction of Radil with Gβγ (data not shown), suggesting either a very high affinity interaction between these partners or that the binding epitope for Radil on Gβγ is distinct from that of the other effectors mentioned above. Indeed such examples showing Gβγ’s ability to interact with multiple molecules via alternative binding site exist in the literature (Davis et al., 2005; Yuan et al., 2007). Targeting to disrupt the binding of Radil and Gβγ will therefore require an understanding of the binding interface of Radil on Gβγ, which we did not address in our studies. Affinity purification and protein localization experiments indicated that Radil is recruited to the plasma membrane upon activation of Rap1a or expression of Gβγ alone. The fact that expression of activated Rap1 or Gβγ is sufficient to recruit Radil to the plasma membrane, and Gβγ binding to Radil is Rap1-dependent, suggests the presence of either basal Rap1 activity or that Gβγ overexpression leads to Rap1 activation. Considering Gβγ-dependent PI3Kγ activation
can trigger Rap1 activation (Woulfe et al., 2002), it could be a plausible explanation for these observations.

To my knowledge, the study described in chapter 2 is the first direct evidence showing a direct link between Rap1 and Gβγ within a single protein complex. Since most described functions of Rap1 are related to cell adhesion and spreading, the discovery of this new molecular complex raises the possibility that GPCR-activated Gβγ may directly modulate cell motility or adhesion via the Rap1-Radil signalling axis.

In our functional studies, overexpression of both Gβγ and Radil were sufficient to increase cell adhesion and spreading on fibronectin; comparable to constitutively active Rap1 mutant, Rap1Q63E (Arthur et al., 2004; Lafuente et al., 2004). The increased cell adhesion promoted by Gβγ or Radil overexpression was reversed by Rap1GAP suggesting that Rap1 activity is required for this effect. Similarly, endogenous stimulation of Formly Peptide Receptors (FPR) with fMLP promoted cell adhesion in a Rap1 and Radil-dependent manner, as assessed by overexpression of Rap1GAP or knockdown of Radil with siRNAs respectively. Previously, VanCompernolle et al., made the observation that non-leukocyte cells, such as the HT1080 fibrosarcoma cells, are responsive to fMLP stimulation and leads to enhanced adhesion and migration of these cells in a dose dependent manner. The authors showed fMLP stimulation was followed by increased Ca²⁺ release and caused transient actin filamentation (VanCompernolle et al., 2003). Whether Rap1, which is known to be activated during fMLP-mediated neutrophil adhesion (M'Rabet et al., 1998), also plays a role in HT1080 cell adhesion was not addressed. Using the same system, we now show that fMLP-mediated integrin activation and adhesion signals are Rap1 and Radil dependent. Addition of fMLP to HT1080 cells leads to induction of Rap1 activity, which peak within 2 minutes after stimulation and slowly diminishes over 30 minutes, kinet-
ics similar to that observed previously for Rap1 in the context of several GPCRs in different cell types (Ghandour et al., 2007; Holinstat et al., 2009; Jeon et al., 2007a; M'Rabet et al., 1998). Of note, stimulation of cells with fMLP also led to a dramatic recruitment of Radil to the plasma membrane and induced cell spreading contemporaneous with peak Rap1 activity (2-3 minutes). Based on these observations we concluded that Rap1 and Radil are crucial for cell adhesion and spreading downstream of Gαi-coupled GPCRs such as FPRs. A similar observation has been also reported for the pertussis toxin sensitive LPA receptor, whose stimulation increases cell spreading in Gβγ-dependent manner (Ueda et al., 2001). In our study we also show the FPRs in HT1080 cells are pertussis toxin-sensitive receptors, but could not provide direct evidence implicating Gβγ in cell adhesion and spreading due to technical difficulties we faced with blocking Gβγ-Radil interaction. However, the fact that we can induce Radil recruitment to the plasma membrane by overexpressing βγ-subunits, and could block βγ-mediated adhesion with Rap1GAP is suggestive that they are involved in Radil-promoted cell adhesion and spreading. Furthermore, we also showed Rap1GAP blocks Gβγ as well as fMLP mediated β1-integrin activation to similar levels further adding support for the above conclusions. Whether this signalling axis that we characterized, in the context of fMLP receptor activation can be extended to other GPCRs, is currently unknown and requires further experimental testing. In its support; however, a recent study showed that Epac1-mediated cell spreading of lung adenocarcinoma cells requires Radil (Ross et al., 2011). Epac1 is activated by cAMP, which in turn can be activated by Gαs (Hansen and Casanova, 1994) as well as by Gβγ (Diel et al., 2006). The study by Ross et al., did not address these upstream activators, but rather directly activated Epac1 using a highly specific cAMP analogue.

As discussed in section 2.5, whether Radil works in concert with other Rap1 effectors in a given cell type, or if their effects are somewhat cell-type and lineage specific remains to be de-
terminated. Although we did not experimentally address this possibility, recently it was shown that in epithelial cells only the knockdown of Radil but not of other RA domain containing proteins, displayed defects in cell spreading in response to Epac1 activation (Ross et al., 2011). This observation suggests that Radil is the primary Rap1 effector in this context. While other Rap1 effectors, like RIAM and RAPL, have been studied mostly in the context of hematopoietic cell lineages, using RT-PCR I found they also express in the cell lines used in this study (data not shown). Consistent with this finding a recent study showed metastatic human melanoma cells also express RIAM where it plays important roles during proliferation and metastasis of the tumours (Hernandez-Varas et al., 2011). The precise functional interplay between the different Rap1 effectors expressed in different cells will thus require further investigation.

The regulation of cell adhesion through GPCRs is obviously important in different contexts, ranging from developmental morphogenesis to leukocyte biology, wound healing and cancer cell motility. The molecular mechanisms in place downstream of GPCR activation to regulate cell adhesion and spreading are poorly understood. The study presented in chapter 2 shows that $\gamma\beta\gamma$ plays a previously unrecognized role for this process and unveils the function of a new $\gamma\beta\gamma$/Radil/Rap1 protein complex in cell adhesion signalling. The working model proposed from these studies is that following GPCR activation, $\gamma\beta\gamma$ subunits are mobilized to interact with their effectors and Rap1 is activated by GPCR-dependent or independent mechanisms. Activated Rap1 and $\gamma\beta\gamma$ captures Radil and recruits it to the plasma membrane, possibly to sites of GPCR activation where the protein complex can promote the activation of integrins causing adhesion and spreading signals (Figure 4-1). In support of this hypothesis, $\gamma\beta\gamma$ has been previously reported to localize at sites of focal adhesion (Hansen et al., 1994), induce focal adhesion and stress fibers formation (Ueda et al., 2000), and the calcium-sensing GPCR has been recently
Figure 4-1. Working model shows how G\(\beta\gamma\)-Radil-Rap1 complexes activates integrins and promotes cell adhesion. Stimulation of cells with fMLP cause G\(\beta\gamma\) and Rap1-GTP to enter a protein complex mediated by Radil. The complex then promotes \(\beta\)1 integrin inside-out activation leading to increased cell spreading and adhesion. The formation of G\(\beta\gamma\)-Radil-Rap1 complex is Rap1-dependent as its inhibition by Rap1GAP blocks the interaction of Rap1 and G\(\beta\gamma\) with Radil, and reverses adhesion phenotypes observed by overexpression of the individual components of the complex. The mechanism by which Radil promotes integrin activation remains elusive.
found to form a physical complex with integrins (Tharmalingam et al., 2011). In the light of these observations, one possibility is that $G\beta\gamma$ guides and captures the Rap1-Radil complex to sites of GPCR activation, which then can locally stimulate cell-matrix adhesion by activating integrins.

While it is known that Rap1 is a potent activator of inside-out signalling leading to integrin activation and thereby to cell adhesion and spreading, how Rap1 cooperates with its effector Radil to exert these functions is not yet clear. RAPL directly binds to integrins and RIAM recruits the adaptor protein talin during integrin activation; however, our mass spectrometry experiments did not provide evidence that Radil also bind to these proteins. In spite of that, it should be noted that the cell lysis and affinity pull-down conditions employed in our experiments may not be amenable to retrieve or preserve such interactions. From co-localization studies we noticed that activated Radil frequently localizes with Rap1 at membrane ruffles or in lamellipodia, regions where adhesion and spreading is initiated. In fact, active but unligated integrins have been found to freely diffuse in the membrane (Galbraith et al., 2007). As such, it is possible that Rap1-Radil signalling is involved in initiating adhesion and spreading by activating integrins at the periphery of cells, but somehow must be quickly disassembled and recycled at the membrane, which is why they do not appear in dense adhesion plaques. This is consistent with our finding that Radil promotes initial spreading in two different cells line (chapters 2 and 3). Additionally, I found that disruption of microtubules lead to Radil accumulation in dense plaques at the plasma membrane (section 3.4.5), which colocalize with the ends of actin stress fibers and vinculin (data not shown). Indeed, microtubule network dissolution is known to elicit enhanced focal adhesion assembly (Bershadsky et al., 1996) and RhoA mediated stress fiber formation (Chang et al., 2008), attributable to the lack of microtubules and hence inability to support focal adhesion disassembly (Kaverina et al., 1998). In chapter 3, we provided evidence that microtu-
bules are important tether vessels for Radil; therefore, it is conceivable that in their absence Radil at adhesion sites (discussed more below). While it is possible that Radil may activate integrins in a conventional fashion as reported for other Rap1 effectors, it could also be involved in strengthening integrin inside-out activation by alternative mechanisms such as promoting catch bond behavior by modulating the cytoskeleton (see section 1.6.3.2). This other possibility has not been investigated in our studies.

We have mapped the interaction of Rap1 with Radil to the RA domain as expected (Appendix 2). Likewise, we found Gβγ also interacts with the N-terminus of Radil, and truncation of the RA domain obviates this interaction (Appendix 2). Structure-function experiments revealed that deletion of the RA domain blocks the ability of Radil to promote cell adhesion on fibronectin implicating that the binding with Rap1 and/or Gβγ is required for Radil-mediated cell adhesion. Similarly, we saw that the PDZ domain was also important for Radil’s function in adhesion. As described in section 3.4 we have identified KIF14 as the PDZ ligand-containing protein interacting with Radil. Interestingly, KIF14 contains an atypical PDZ ligand. As shown in Table 1-2 most PDZ domains prefer ligands, which contain S/T (class I), D/E (class II) or hydrophobic amino acid at -2 position (Hung and Sheng, 2002; Tonikian et al., 2008). However, the c-terminal amino acid sequence of KIF14 contains a polar neutral side chain (Q) at the -2 position. Looking at Radil PDZ domain structure and its peptide binding groove, it’s selectively for WV at the extreme C-terminal end as a PDZ ligand can possibly be explained by the existence of an arginine group sitting right above the binding pocket created by the GLGM motif (Figure 4-2). This arginine group likely participates in π-cation interactions with the tryptophan at the -1 position of the PDZ binding motif to stabilize its binding. The PDZ domain of HtrA2 as shown in Figure 1-11 was previously identified to have similar ligand sequence preference. However, in this case the position of the aromatic group containing amino acid residue was not strictly
Figure 4-2. Radil PDZ binding site. (A) Shown is the ligand binding groove in the PDZ domain of Radil (PDB 1UM1). Sticks (right) show the conserved PDZ motif GLGM which creates the binding pocket on PDZs. Also shown in sticks (left) is an arginine residue which sits right above the ligand binding groove of Radil. (B) Shown is a plausible binding mode with the peptide IQWV manually docked on to Radil PDZ binding pocket. The front-view depicts the peptide fitting almost perfectly into the groove. The side-view illustrates π-cation interaction between the W residue of the ligand and residue R in position 82 of Radil PDZ.
required to be in -1 position. Moreover, the HtrA2 PDZ domain does not contain the same arginine group found in the Radil PDZ domain; as such the ligand appears to bind via van der Waal’s interactions in this case. For a serine protease like HtrA2, this makes sense because it has to maintain a certain degree of promiscuity for ligand recognition to be able to efficiently do its job as a protease. On the other hand, Radil requires a specific partner, and the presence of an arginine group sitting right above the binding groove is most likely a way to ensure the specificity of interaction. Remarkably, the PDZ domain of AF-6, which is a close relative of Radil in terms of its domain architecture, also does not have the arginine residue above its ligand binding groove and does not interact with KIF14.

KIF14 as mentioned earlier is gained either by genomic amplification or by increased expression levels in several cancers including breast cancers (Corson et al., 2005). One of the cell lines used in my study, MDA-MB-231, characterized as one of the most aggressive and migratory breast cancer cell lines exhibits very high KIF14 expression level compared to normal breast cells. Given that the interaction of KIF14 and Radil is maintained in these cells we first tested to see effects on initial cell spreading upon loss of function of Radil by RNAi or inhibition of Rap1 activity by expression of Rap1GAP. Our data indicated that Rap1 activity and Radil are required for MDA-MB-231 cell adhesion and spreading. In contrast, when we knocked down KIF14 we observed faster and enhanced cell spreading, suggesting that KIF14 is a negative regulator of Radil. In support of this hypothesis we showed that depletion of Radil or Rap1 activity rescued the enhanced spreading phenotype in KIF14 null cells.

Other Rap1 effectors, such as RAPL and KRIT1 have been previously demonstrated to also localize on microtubules, but dislodge and relocate to plasma membrane upon Rap1 activation (Fujita et al., 2005; Liu et al., 2011). We reasoned that while Radil is sequestered on micro-
tubules by KIF14, it probably also relocates to the plasma membrane upon Rap1 activation. Indeed, co-expression of constitutively active mutant Rap1Q63E with KIF14 and Radil translocated them from microtubules to the cell cortex. Furthermore, depletion of KIF14 or disruption of microtubules with nocodazole also led to accumulation of Radil at the cell periphery. These observations suggest that Radil is regulated similarly to the other Rap effectors by microtubules but in this case the tethering occurs via KIF14 rather than direct binding to microtubules. Mechanisms by which binding of Rap1-GTP to Radil cause KIF14 and Radil to fall off the microtubules remain to be understood. Conformational changes induced by Rap1 and inhibited by KIF14 are possible mechanisms that could be pursued. Since KIF14 is a motor protein we attempted to visualize if Radil may also be shuttled to sites of Rap1 activation in a microtubule dependent fashion. Using time-lapse confocal microscope we could not reproducibly observe movement of Radil and KIF14 on microtubules. While we bear in mind that this could be due to technical limitations, whether the motor activity of KIF14 is required for its functional effect remains to be determined.

The function of KIF14 as a negative regulator of Radil prompted us to inquire if depletion of KIF14 in MDA-MB-231 cells would lead to increased β1-integrin activation as observed with Radil overexpression in HT1080 cells. Indeed, KIF14 knockdown cells showed enhanced staining of β1-integrin with the activation-specific 9EG7 integrin antibody, while total integrin levels remained unchanged. While activation of integrin explains the fast adhesion kinetics it does not provide much information on the nature of cell adhesion. As such, we decided to directly visualize focal adhesions by staining for vinculin. KIF14-depleted cells were found to have large concentric adhesion foci and to adopt an unpolarized morphology. Interestingly, these phenotypes are similar to those when microtubules in cells are disrupted using microtubules, or kinesin-1 is inhibited using antibodies (Krylyshkina et al., 2002; Rape et al., 2011).
large focal adhesions together with increased stress fibers are indicative of mature and stable adhesions, likely due to impaired turnover. This was confirmed in our live cell imaging experiments that revealed reduced venus-paxillin dynamics in KIF14 depleted compared to control cells. Taken together these data led us to propose that in KIF14-depleted cells, Radil is released from microtubules and free to engage with activated Rap1 at the cell periphery to initiate constitutive adhesion signals causing strong and stable adhesions. Tensions generated by constant integrin mediated adhesions can subsequently signal the formation of large end-to-end actin cables (stress fibers) and associated focal adhesions signifying entrenched anchorage of the cell to the extracellular matrix (discussed in detail in section 1.6.3). Whether KIF14 can also shuttles other factors important for focal adhesion turnover remains to be addressed.

MDA-MB-231 cells are highly aggressive with invasive and migratory properties. Since KIF14 expression is dramatically elevated in these cells when compared to less aggressive breast cancer cell lines or non-transformed breast mammary epithelial cells, we reasoned that Radil and KIF14 may fulfil important functional roles in this context. Knockdown of Radil resulted in decreased migration and invasion of MDA-MB-231 cells. This we reasoned is due to reduced cell adhesion and lack of traction required for cells to move. Consistent with these in vitro observations Radil depletion dramatically reduced lung metastasis in vivo in a xenograft mouse model. This suggests that Radil is possibly required for extravasation and homing of breast cancer cells to the lung. In addition to defects in metastasis, knockdown of Radil also exhibited defects in cell proliferation in culture as well as reduced tumour volume in mice. Our findings are consistent with previous reports showing that loss-of-function of β1 integrin (Huck et al., 2010) and RIAM (Hernandez-Varas et al., 2011) also cause reduced tumour growth in mice, in addition to defects in tumour dissemination. In fact, perturbations of integrins (Reverte et al., 2006) and Rap1 (Dao et al., 2009) functions are also known to affect cell division as forces generated by
integrin mediated adhesion is crucial for spindle assembly and cytokinesis. Therefore, it is conceivable that Radil loss-of-function, which causes reduced integrin-dependent cell adhesion and spreading also, impede cell’s ability to proliferate. Given, that we also observed defects in cell migration and invasion *in vitro*, we conclude that Radil depletion may contribute to lack of tumour progression by impinging partly on both metastatic properties and proliferation of tumours. The tropism of breast cancer cells to lung has been shown to depend on the SDF1-CXCR4 signalling axis (Muller et al., 2001). One possibility is that Radil-depleted cells cannot adequately translate these chemotactic cues due to defects in cell adhesion. Although we haven’t directly tested the implication of Radil downstream of CXCR4 we showed that it is important for fMLP-promoted cell adhesion.

Knockdown of KIF14 also resulted in reduced MDA-MB-231 cell migration. This outcome is conceivable since we know that the depletion of KIF14 leads to defects in focal adhesion dynamics and unpolarized cell morphology. Defects in focal adhesion disassembly are well known to prevent cell movements (Garzon-Muvdi et al., 2012; Wu et al., 2008) (also see section 1.6.3.3). Likewise, perturbations of proper functions of other kinesins as described in section 1.8.2 have been also reported to cause migration defects. Whether the unpolarized morphology we observe is a result of altered focal adhesion dynamics or due to some other mechanisms is not clear. However, overexpression of constitutively active Rap1G12V was recently reported to result in a similar outcome (Freeman et al., 2010). Therefore it is plausible that desultory accumulation of a Rap1 effector such as Radil at the cell periphery, results in loss of cell polarity.

Fine tuning of integrin and Rap1-mediated adhesion is integral to cell migration. In this thesis I present a novel mechanism by which an effector of Rap1, as well as an activator of integrin is sequestered to provide optimal balance of Rap1-Radil signalling and therefore integrin-
mediated cell-matrix adhesion. Interestingly, Rap1 activity tends to be higher in several cancers and one mechanism governing this at the genomic level is by virtue of Rap1GAP downregulation via its promote hypermethylation (Banerjee et al., 2011; Zheng et al., 2009). Whether, shutting down Rap1GAP is a compensatory mechanism for cells to curb aberrant migration of cells is now known. Yet many cancer cells at some point of their cycle acquire the ability to invade through the basement membrane and interstitial matrices and migrate to distant sites in spite of elevated Rap1 activity (Bailey et al., 2009). It is possible that in some of these aggressive cancer cells, KIF14 is upregulated to compensate for high Rap1 activity to adjust the availability of Radil to levels that benefit cell migration, invasion and cancer progression. Indeed, KIF14 has been shown to be particularly upregulated in the more aggressive cancer cells, and positively correlated with their invasiveness (Corson and Gallie, 2006). The interrelationship of Rap1GAP and KIF14 regulation at the genomic level could be an interesting avenue for future investigation to further our understanding of how cancer cells gain the ability to migrate by modulating cell-matrix adhesion.

We have clearly established that the PDZ domain of Radil serves to interact with KIF14 on microtubules and leads to the inhibition of its activity. However, we also showed that expression of a ΔPDZ mutant of Radil was unable to promote cell adhesion or rescue the spreading deficits of Radil-depleted cells. Our interpretation of these results is that the Radil PDZ domain is required for the onset of cell spreading and cell adhesion possibly through interaction with other proteins. Our mass spectrometry analysis of Radil pull-downs revealed an interesting candidate, ArhGAP29. As no peptide corresponding to ArhGAP29 was found in our mass spectrometry analysis of the Radil-ΔPDZ mutant, this interaction is likely mediated through the PDZ domain. ArhGAP29 is a RhoA specific GTPase activating protein (Xu et al., 2011). Given the antagonis-
tic relationship between RhoA and Rac1 during initial cell spreading, and Rap1’s ability to induce Rac activity (Arthur et al., 2004), it is possible that Radil interacts with ArhGAP29 to locally inhibit RhoA and promote Rap1-dependent Rac1 activation and cell spreading. Further experiments are needed to test this hypothesis.

Understanding how Radil activates α5β1 integrin and whether it also has other integrin targets is important and will be of interest in the future. It is possible that Radil activated by Rap1 and Gβγ directly associates with integrins and induces their activation. Alternatively, it could elicit other signalling activities such as modulation of the actin cytoskeleton that may eventually indirectly influence integrin activation. As for the interaction between Radil and KIF14, it remains to be addressed if KIF14 is also involved in the trafficking Radil on microtubules. Identifying additional cargoes of KIF14 will also be crucial to better understand its specific role in focal adhesion dynamics as observed in my studies. Mutations in Radil affecting the RA domain have been recently reported in liver fluke-associated cholangiocarcinomas; however, the functional importance of these mutations is currently unknown (Ong et al., 2012). Future experiments are required to delineate the functional consequences of these mutations both mechanistically and in the context of cancer biology. Radil knockout or transgenic mice expressing Radil mutants will be helpful in understanding Radil’s function in a more physiological setting. Radil knockout or transgenic mice expressing Radil mutants in the presence of tissue specific expression of activated oncogenes may provide insights Radil’s requirement during cancer progression.

Finally, efforts need to be leveraged to see if the findings reported here can be used to treat cancer. Metastasis is the primary cause of cancer mortality. While, markers for cancers such as upregulation of HER2, estrogen receptor and/or progesterone receptor in breast cancer
have been characterized and patients are being treated accordingly with some success, there are other breast cancers that do not express these markers, termed triple negative breast cancers (TNBC) (Carey et al., 2010). Hence there currently are few treatment regimens available for these patients except traditional chemotherapies, radiation, and when possible surgical resection. Poor prognosis of patients with TNBC and the proclivity of these cancers to relapse with metastasis warrant need for additional therapies. Dysregulation of integrin, Rap1, and GPCR signalling have all been implicated in cancer and in cell motility during metastasis. In light of this thesis, the association and function of KIF14 with Radil is an attractive candidate whose interaction can be targeted for disruption using small molecule inhibitors. KIF14 is emerging as an important early biomarker of several cancers including breast cancers whose expression appears to be independent of other known marker of mammary tumour mentioned above (Corson and Gallie, 2006; Corson et al., 2005). As such, in the absence or scarcity of adequate therapeutic opportunities to treat these types of tumours, disruption of Radil-KIF14 interaction may prove to be a fruitful opportunity for therapeutic intervention.
References


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Appendices
Appendix 1. ELISA analysis of antibody production bleed.

Species: NZW Rabbit  
Immunogen: RADIL  
Plate Coating Ag: RADIL  
Concentration: 1μg/ml

<table>
<thead>
<tr>
<th>Samples</th>
<th>Control (negative)</th>
<th>Control (positive)</th>
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<td>Production</td>
<td>Bleed Type: Pre</td>
</tr>
<tr>
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<td>22/08/2008</td>
<td>Bleed Date: 07/01/2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anti-ovalbumin (rabbit) diluted at 1:1000</td>
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</table>

<table>
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<tr>
<th>Animal ID</th>
<th>50% Titre</th>
<th>Mean Value (O.D.)</th>
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<td>2,200</td>
</tr>
<tr>
<td>TO 032</td>
<td>48,000</td>
<td>400</td>
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</table>

Blank: 0.036  
Blank Std Dev: 0.005  
Noise Cutoff: 0.050

Performed by Covance Immunology Services
Appendix 2: Radil deletion analysis.

**A**

<table>
<thead>
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<th>Construct</th>
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<th>PDZ</th>
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<tr>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Strep-HA-RADIL ΔRA</td>
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<td>+</td>
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<tr>
<td>Strep-HA-RADIL ΔPDZ</td>
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</tr>
<tr>
<td>Strep-HA-RADIL PDZ</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**B**

The N-terminus of Radil is essential for Rap1a and Gβγ binding. (A) The above constructs were transiently transfected in HEK293T. The different Radil construct’s binding to HA-tagged Rap1a or endogenous Gβγ were examined by streptavidin affinity purification followed by western blotting using the indicated antibodies. (B) Expression of Rap1Q63E does not translocate RadilΔRA to the plasma membrane, whereas wild-type Radil and RadilΔPDZ can. Bar = 20 μm.
Copyright Acknowledgements

1. Figure 1-5 was adapted from: Guo, W., and Giancotti, F.G. (2004). Integrin signalling during tumour progression. Nature reviews Molecular Cell Biology 5, 816-826, with permission from Macmillan Publishers Ltd. Copyright 2004


