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Abstract

Syndecans are transmembrane proteoglycans that act as receptors for extracellular matrix molecules and co-receptors for growth factors, cytokines and morphogens. Syndecan-4 is a ubiquitously expressed member of the syndecan family and encompasses a unique PKCα-binding motif within its variable region. Engagement of syndecan-4 by fibronectin regulates Rho family guanosine triphosphatase (Rho GTPase) activity to promote focal adhesion formation and reorganisation of the actin cytoskeleton that is dependent on syndecan-4-mediated PKCα activity. Recent work in our laboratory has demonstrated a role for syndecan-4 in the dynamic recycling of other fibronectin receptors, α5β1 and αVβ3 integrins. Here, it is demonstrated that phosphorylation of syndecan-4 mediated by another PKC isoform, PKCδ, and a non-receptor tyrosine kinase, Src, regulates syndecan-4-dependent GTPase activity, heterodimer-specific integrin recycling and focal adhesion formation, providing a mechanism for spatiotemporal control of cell migration.

Src is associated with processes regulating adhesion disassembly and cell migration, and aberrant activation of Src contributes to neoplastic progression. Using in vitro kinase assays, syndecan-4 was identified as a target for Src-mediated phosphorylation. Mutagenesis studies coupled with mass spectrometric analysis of phosphorylation indicated the existence of two novel Src phosphorylation sites within the syndecan-4 cytoplasmic domain, tyrosine180 (Y180) and tyrosine197 (Y197). Importantly, modulation of the phospho-competence of the PKCδ-phosphorylation site (Serine179 (S179)) within syndecan-4 cytoplasmic domain increased Src-mediated syndecan-4 phosphorylation by promoting preferential phosphorylation of Y180 over Y197. Thus, PKCδ primes syndecan-4 for Src-mediated phosphorylation, functioning as a molecular switch to regulate phosphorylation of alternative tyrosine residues.

In cells, Src-mediated phosphorylation of Syn4Y180 suppressed activity of the small GTPase Arf6. Similarly, phosphomimetic mutation of the PKCδ-phosphorylation site within syndecan-4 inhibited Arf6 activation in response to fibronectin engagement. These results suggested that PKCδ-dependent phosphorylation of syndecan-4 regulated Arf6 activation by controlling Src-dependent Y180 phosphorylation. Furthermore, suppression of Arf6 activity or perturbation of the S179 residue promoted membrane delivery of internalised αVβ3 integrin but not α5β1. Thus, syndecan-4-dependent Arf6 activity...
regulates differential recycling of α5β1 and αVβ3 integrins and is controlled by phosphorylation by PKCδ and Src.

As, the other identified Src kinase target, Y197, is located within the PDZ-binding motif of syndecan-4, it was hypothesised that phosphorylation of this residue may regulate the interaction of syndecan-4 with PDZ-domain-containing proteins. Protein binding to GST-syndecan-4 constructs was assessed in pull-down assays. Interestingly, binding of syntenin, a PDZ-domain containing syndecan-4 binding partner, was inhibited in the presence of a phosphomimetic Y197 residue. By contrast, syntenin binding was enhanced in PKCδ-phosphorylation site mutants of syndecan-4 that decrease Src-dependent Y197 phosphorylation. Therefore, the phosphorylation state of Y197 is a critical determinant of syndecan-4-PDZ-binding motif interactions. Intriguingly, cells expressing a syndecan-4 receptor defective for PDZ interactions exhibited constitutive activation of Arf6 and a severe defect in syndecan-4 and integrin receptor internalisation. These data suggest that syndecan-4 endocytic pathways may be intimately associated with integrin internalisation and that interactions at the PDZ-binding motif of syndecan-4 may be critical for regulating syndecan-4 internalisation.

Finally, it was demonstrated that ECM engagement of syndecan-4 induces a transient wave of PKCα activity and that this is dependent on the integrity of the PKCδ-phosphorylation site. Furthermore, perturbation of S179 or siRNA-mediated knockdown of PKCδ resulted in suppressed Rac1 activity and enhanced focal adhesion formation. Thus, PKCδ-mediated phosphorylation of syndecan-4 appears to regulate several key processes involved in cell migration including cell-surface expression of integrin heterodimers, GTPase activity and focal adhesion formation. Consequently, cells expressing a syndecan-4 receptor with mutations in the PKCδ-phosphorylation site exhibited defects in migration speed and persistence on cell-derived matrices.

Together, these data suggest that in migrating cells PKCδ activity is essential to coordinate differential phosphorylation of syndecan-4 by Src on two separate residues, to spatially and temporally restrict GTPase activity, heterodimer-specific integrin recycling and focal adhesion formation.
Declaration

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<td>50K</td>
<td>Recombinant fragment of fibronectin comprising type III repeats 6-10</td>
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<tr>
<td>AEBSF</td>
<td>4-(2-aminoethyl)benznesulphonyl fluoride</td>
</tr>
<tr>
<td>ABTS</td>
<td>2, 2'- azino-bis (3-ethylbenzthiazone 6-sulphonic acid)</td>
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<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dock180</td>
<td>Dedicator of cytokinesis 180</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunoabsorbant assay</td>
</tr>
<tr>
<td>ELMO</td>
<td>Engulfment and cell motility</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FA</td>
<td>Focal adhesion</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FERM</td>
<td>Four point one, ezrin, radixin, moesin domain</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FLIM</td>
<td>Fluorescence lifetime imaging microscopy</td>
</tr>
<tr>
<td>Fn</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence (Förster) resonance energy transfer</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDI</td>
<td>Guanosine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine disphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GGA3</td>
<td>Golgi-localized, gamma-ear containing Arf6 binding protein-3</td>
</tr>
<tr>
<td>GIPC1</td>
<td>carboxy-terminal GAIP interacting protein</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GSK-3</td>
<td>Glycogen synthase kinase-3</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphatase</td>
</tr>
<tr>
<td>H/0</td>
<td>Recombinant fragment of fibronectin comprising type III repeats 12-15</td>
</tr>
<tr>
<td>HA</td>
<td>Nonapeptide sequence YPYDVPDYA derived from the human influenza virus hemagglutinin protein</td>
</tr>
<tr>
<td>HEPES</td>
<td>(4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid</td>
</tr>
<tr>
<td>HFF</td>
<td>Human foreskin fibroblasts</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulphate proteoglycan</td>
</tr>
<tr>
<td>IB</td>
<td>Immunoblot</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL2Ra</td>
<td>Interleukin-2 receptor alpha subunit</td>
</tr>
<tr>
<td>ILK</td>
<td>Integrin-linked kinase</td>
</tr>
<tr>
<td>Im</td>
<td>Immortolised</td>
</tr>
<tr>
<td>INF-γ</td>
<td>Interferon- gamma</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitate</td>
</tr>
<tr>
<td>LB medium</td>
<td>Luria Bertani medium</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>LPA</td>
<td>lysophosphatidic acid</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>mDia</td>
<td>mammalian homologue of the drosophila gene diaphanous 1 (mDia1)</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MesNA</td>
<td>2-Mercaptoethane sulfonate sodium</td>
</tr>
<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>Nrp1</td>
<td>Neuropilin-1</td>
</tr>
<tr>
<td>PAK</td>
<td>P21-activated kinase</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>Pull-down</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PDZ</td>
<td>Post synaptic density protein (PSD95), Drosophila disc large tumour suppressor (DlgA), and Zonula occludens-1 protein (Zo-1)</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PI3-K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PIX</td>
<td>PAK-interactive exchange factor</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PNRC</td>
<td>Perinuclear recycling compartment</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PSI</td>
<td>Plexin-semaphorin-integrin</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine-binding</td>
</tr>
<tr>
<td>PTP</td>
<td>Protein tyrosine phosphatase</td>
</tr>
<tr>
<td>Rab11FIP/FIP</td>
<td>Rab11 family interacting protein</td>
</tr>
<tr>
<td>Rac1</td>
<td>Ras-related C3 botulinum toxin substrate 1</td>
</tr>
<tr>
<td>RCP</td>
<td>Rab-coupling protein</td>
</tr>
<tr>
<td>Rho</td>
<td>Ras homolog</td>
</tr>
<tr>
<td>RIAM</td>
<td>Rap1-GTP-interacting adaptor molecule</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated coiled-coil kinase</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family kinase</td>
</tr>
<tr>
<td>SH</td>
<td>Src-homology domain</td>
</tr>
<tr>
<td>SHP-2</td>
<td>Src homology 2 domain-containing protein tyrosine phosphatase 2</td>
</tr>
<tr>
<td>siRNA</td>
<td>Silencing RNA</td>
</tr>
<tr>
<td>Src/c-Src/v-Src</td>
<td>Sarcoma/ cellular sarcoma/ viral sarcoma</td>
</tr>
<tr>
<td>Sulfo-MBS</td>
<td>Sulfo-3-maleimidobenzoyl-N-hydroxysulphosuccinimide ester</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>Tiam1</td>
<td>T-cell lymphoma invasion and metastasis gene 1</td>
</tr>
<tr>
<td>TIRF</td>
<td>Total internal reflection microscopy</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>VASP</td>
<td>Vasodilator-stimulated phosphoprotein</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskot-Aldrich syndrome protein</td>
</tr>
<tr>
<td>WAVE</td>
<td>WASP family verprolin homologous proteins</td>
</tr>
</tbody>
</table>
1. Introduction

Cell migration is essential for embryogenesis, tissue repair and homeostasis and control of the immune response (Duncan and Su, 2004; Martin and Parkhurst, 2004; Randolph et al., 2005). The loss or perturbation of mechanisms that would normally control migration is associated with a number of diseases. For example, in rheumatoid arthritis, a chronic inflammatory disease, the continuous infiltration of immune cells into the joint leads to the destruction of bone and cartilage tissue (Aidinis et al., 2005). Furthermore, cancer progression is aided by dysregulation of cell-matrix contacts that under normal circumstances restrict cell movement within tissue of origin (Demuth and Berens, 2004). Elucidating the mechanisms controlling cell migration is therefore essential to understanding many physiological and pathological processes.

Cell migration can be initiated by chemical and physical factors in the microenvironment, chemotaxis and haptotaxis, respectively, and both types of migration are involved in development, repair and disease processes. Chemotactic migration is initiated and directed by an external signal, such as a chemical gradient. For example, vascular endothelial cells migrate down a vascular endothelial growth factor (VEGF) gradient to form new capillaries during the process of angiogenesis (Risau, 1997) and leukocytes transmigrate across the endothelium barrier in response to cytokine secretion (Fernandez-Borja et al., 2009). Haptotactic migration is implicated in wound healing and represents the cell’s intrinsic ability to migrate on an extracellular network in the absence of a defined chemical stimulus. The mechanisms involved in regulation of haptotaxis are not as clearly defined as chemotaxis; however, it is clear that cellular interaction with the extracellular matrix (ECM) is a vital requirement for cell migration.

Cell migration requires the coordination of cell-ECM interactions, membrane protrusion and cellular contraction. Cells adhere to ECM via adhesion complexes – dynamic macromolecular assemblies – that provide a physical link between ECM and the cell cytoskeleton and exhibit high levels of molecular heterogeneity. In this way, information on environmental changes, such as application of tension or changes in ECM composition, can be passed rapidly to the inside of the cell (Bershadsky et al., 2003; Chen et al., 2004a; Geiger et al., 2001; Geiger et al., 2009; Ridley et al., 2003; Sastry and Burridge, 2000). In order for cells to migrate, adhesion contact dynamics are coordinated in a spatially and temporally restricted manner. Membrane protrusions (filopodia and lamellipodia), formed
as a result of actin remodelling and polymerisation, are stabilised by cell adhesion to the substratum, and serve as a means for the cell to extend forward (Friedl, 2004; Vicente-Manzanares et al., 2009a). Actin stress fibres, the most prominent actin structures observed in well-spread cells, together with myosin II chains and α-actinin, form the contractile actomyosin machinery of the cell (Ridley et al., 2003; Zimerman et al., 2004). The actomyosin machinery uses cell-matrix adhesion sites to transfer contractile forces, generating traction for cell movement and facilitating adhesion disassembly at the cell rear (Citi and Kendrick-Jones, 1987; Li et al., 2005; Nobes and Hall, 1999). Importantly, engagement of adhesion receptors elicits signals to control adhesion contact dynamics and the protrusive and contractile cytoskeletal machinery. Thus, adhesion to the ECM is pivotal for cellular migration and is mediated by transmembrane receptors that extend out from the cell surface. The principal cell-surface receptors that mediate cell-ECM adhesion are members of two gene families – the integrins and the syndecans (Berrier and Yamada, 2007; Couchman and Woods, 1999; Morgan et al., 2007).

For efficient cell migration to occur, the cell must balance formation of adhesions at membrane protrusions, against turnover of adhesion complexes and cell retraction at the rear. The key mechanisms that regulate adhesion complex dynamics and how this impinges on cell migration will be described in the following chapters. In particular, the role of ECM receptors, integrins and syndecans in the spatial and temporal regulation of adhesion turnover will be discussed. It is believed that signalling cascades activated by integrin and syndecan ECM-engagement display a synergistic relationship whereby their signals converge to control many aspects of cell migration.

### 1.1 Extracellular matrix and cell migration

The three-dimensional (3D) arrangement of macromolecules and proteoglycans that comprise the ECM defines tissue architecture and the localised cellular environment. The diverse roles of ECM represent the specialised function of a particular organ and arises from tissue-specific expression of ECM components. ECM provides physical support for cells, sequesters growth factors and establishes chemokine gradients. Thus, the extracellular environment provides a platform for cell migration during development and tissue repair, and regulates the cellular response to external stimuli by restricting and localising growth factor signals (Hynes, 2009).
One of the many challenges in studying cell migration has been to assemble appropriate migration platforms that mimic the different ECM environments observed in vivo, i.e. dense connective tissue, loose connective tissue and the tightly packed basement membrane. The composition and topography of these extracellular environments determines cell migration by constraining cell adhesion sites in response to physical cues (Even-Ram and Yamada, 2005; Pankov et al., 2005; Petrie et al., 2009). Different cell types employ different mechanisms to migrate into and within the ECM. Mesenchymal cell migration is characterized by a cyclic process whereby cells advance forward by extending membrane protrusions (lamellipodia, or filopodia) from the leading edge. Protrusions are stabilised by cell adhesion to the substratum followed by maturation of adhesions, while the release of adhesions at the back pulls the cell rear forward (Li et al., 2005; Petrie et al., 2009).

Fibroblasts plated on two-dimensional (2D) substrates exhibit multiple large lamellipodia and filipodia that allow exploration of the local environment and a random mode of cell motility in the absence of a defined chemical signal. Conversely, when plated on a fibrillar 3D cell-derived matrix (CDM), which more accurately resembles ECM in vivo (Cukierman et al., 2001), fibroblasts adopt an elongated morphology, lose fibrillar adhesions and exhibit a higher level of migrational persistence, which is dependent on matrix architecture and the fibronectin (Fn) receptors α5β1 integrin and syndecan-4 (Bass et al., 2007b; Cukierman et al., 2001; Pankov et al., 2005).

The topography of ECM can also define the mechanisms of cancer cell invasion. Use of Matrigel (reconstituted basement membrane derived from Engelbreth-Holm-Swarm (EHS) mouse) tumour has demonstrated the ability of tumour cells to adopt an amoeboid phenotype to negotiate the ECM barrier (Friedl and Wolf, 2003). However, recent work has highlighted a fundamental difference in the macromolecular network of Matrigel compared to that of an extracted physiological basement membrane. The structural integrity of the physiological basement membrane is characterised by covalently cross-linked type IV collagen, a property not exhibited by the collagen matrix of Matrigel. Thus, it has been argued that in a physiological basement membrane cancer cells do not adopt an amoeboid morphology but rather migrate through the ECM by remodelling the basement membrane in a protease dependent manner (Hotary et al., 2006; Sodek et al., 2008). Together, these studies highlight the requirement of studying cell migration in an environment that more closely resembles those found in vivo. Furthermore, ECM
composition and architecture are equally important in defining cell response to the extracellular environment.

1.1.1 Cell-ECM adhesions and migration

The majority of ECM components contain putative binding sites for both integrin and syndecan receptors, and substantial evidence suggests that the engagement of both receptors is required for adhesion formation and actin reorganisation (Bass et al., 2007a; Longley et al., 1999; Saoncella et al., 1999; Woods and Couchman, 1998; Woods and Couchman, 2000). Clustering of adhesion receptors following ECM engagement recruits adaptor and signalling proteins to adhesion sites termed focal complexes (small dot-like structures (1 µm assemblies)) at the leading edge of lamellipodia (Fig. 1.1). The maturation of these small protein assemblies into focal adhesions (FAs) serves to anchor actin stress fibres and is vital for generation of contractile forces required for cell movement. FAs were first visualized in 1971 by electron microscopy (Abercrombie et al., 1971) and appear as electron-dense areas in leading lamella of migrating cells representing intimate points of contact between the cell and ECM. These structures contain integrins, together with specialized cytoskeletal, signalling and membrane components that regulate adhesion-mediated cell responses (Berrier and Yamada, 2007; Geiger et al., 2001; Geiger et al., 2009; Wegener and Campbell, 2008).

**Figure 1.1. Immunofluorescence images of a mouse embryonic fibroblast spread on fibronectin.** Vinculin is a major component recruited to focal complexes (green arrow) and FAs (yellow arrow) in response to ECM engagement. Focal complexes (punctuate vinculin staining) are found at leading edge of lamellipodia, whereas, larger vinculin-containing FAs appear at the end of actin stress fibres (organised actin cytoskeleton).
The dynamic turnover of these adhesions (FAs and focal complexes) stabilises protrusions at the leading edge of migrating cells, allows application of tension to the ECM and retraction of the cell rear (Fig. 1.2).

Other sites of cell-ECM contacts have been identified. The centripetal translocation of α5β1 integrin, a principal Fn receptor, from mature FAs towards the body of the cell precedes formation of fibrillar adhesions – sites of Fn fibrillogenesis (Clark et al., 2005; Mao and Schwarzbauer, 2005; Pankov et al., 2000). Here, the composition of adhesions is very different and is represented by an absence of typical FA proteins such as phosphotyrosines and vinculin, and no physical link to actin stress fibres is observed (Katz et al., 2000; Zamir et al., 2000). Lastly, specialised adhesions podosomes and invadopodia are found in monocytic cell types such as macrophages and osteoclasts and in transformed cells, respectively. These adhesions contain actin-rich structures and mediate adhesion to the substratum and matrix degradation and as such are normally confined to invasive cell types (Linder and Aepfelbacher, 2003).

Data compiled from different cell types and consolidation of data obtained from all types of integrin-mediated adhesion complexes (including FAs, focal contacts and fibrillar adhesions) identified in literature have highlighted a total of 180 proteins - including adaptors, cytoskeletal components, kinases, phosphatases and lipids - as components of
cell-ECM adhesion sites (Zaidel-Bar and Geiger, 2010; Zaidel-Bar et al., 2007a). However, this is not to say that every component identified as part of the “integrin adhesome” is recruited to all cell-ECM contacts; instead, recruitment of these proteins is dynamic and dependent on cellular context, integrin heterodimer specificity and on which ECM ligand is being engaged. The complexity and dynamic nature of adhesion component recruitment ensures that cells are able to adapt and respond rapidly to changes in their external environment, a characteristic that is central to regulation of cell migration. The intracellular components of FAs serve two major functions; adaptor molecules, such as paxillin, α-actinin, vinculin and talin, provide structural links from integrins to the actin cytoskeleton and recruit other FA components; enzymatic proteins, such as tyrosine kinases (e.g. focal adhesion kinase (FAK) and Src) and serine/threonine kinases (e.g. protein kinase C, (PKC)), mediate many distal signalling processes downstream of integrin-ECM engagement including regulation of Rho guanosine triphosphatase (Rho GTPase) activity, thus controlling cytoskeletal organisation and cell morphology and behaviour (Geiger et al., 2001; Wozniak et al., 2004; Zaidel-Bar et al., 2007a). Indeed, dynamic phosphorylation of FA components mediated by kinases and phosphatases is an important mechanism modulating adhesion turnover and cell migration and will be discussed in later chapters (section 1.5). Currently, 18 tyrosine kinases and phosphatases and 15 serine/threonine kinases and phosphatases have been identified as part of the adhesome (Zaidel-Bar and Geiger, 2010). It is believed that interaction between many FA components is transient, and this is regulated in part by kinase and phosphatase switches within adhesions to allow fast and dynamic response to extracellular cues. Therefore, elucidating the mechanisms by which an environmental signal is propagated within the cell will require intimate knowledge of kinase and phosphatase function and their downstream targets.

There has been a long debate about the physiological relevance of FAs and whether they are an artefact of cell adhesion to culture surfaces. Cells plated onto a physiological, 3D fibrillar matrix exhibit FAs of different size and composition compared to cells cultured onto substrates in solution, highlighting concerns over transferring information gathered from *in vitro* studies to physiological contexts. However, immunoelectron microscopy techniques have identified comparable structures *in vivo*, such as the adhesions formed by aortic endothelial cells with their underlying basement membrane (Kano et al., 1996) and those observed at myotendinous junctions formed by skeletal muscle cells (Turner et al., 1991) and more recently in embryonic 3D matrices (Cukierman et al., 2001). It has been
suggested that FAs studied in vitro may in fact be “exaggerated versions” of in vivo adhesions (Even-Ram and Yamada, 2005). Nevertheless, physical interaction with ECM whether in vitro or in vivo is a vital requirement for control of cell survival and migration, and much of our current understanding of cell-matrix adhesion comes from studying FAs. Thus, studying the processes that regulate FA dynamics may allow us to dissect the events that regulate cell-ECM engagement in a more spatially and temporally restricted manner in vivo.

1.1.2 Fibronectin: an ECM molecule contains cell-binding domains

Fn is an abundant, ubiquitously distributed ECM glycoprotein and one of the first proteins to be assembled in ECM during wound healing. The provisional matrix deposited at sites of tissue repair is primarily composed of covalently linked fibrin and Fn molecules (Dallas et al., 2006; Midwood et al., 2004). Fn is secreted by cells as a soluble disulphide-bonded dimer. Once at the cell surface, these dimers are assembled into insoluble multimeric Fn fibrils resulting in a dense meshwork and a complex extracellular environment (Singh et al., 2010; Wierzbicka-Patynowski et al., 2004; Wierzbicka-Patynowski and Schwarzbauer, 2003). Abnormal expression, degradation or organisation of Fn has been associated with a number of pathologies including tumourogenesis, cancer metastasis and fibrosis (Clark et al., 2000; Maniotis et al., 1999; Williams et al., 2008; Zhang and Lee, 1997; Zhang et al., 1997b). Mice lacking the Fn gene die at embryonic day 8.5 due to severe defects in cell migration, proliferation and differentiation (George et al., 1993; Hynes and Wagner, 1996), demonstrating the importance of Fn in regulating cell behaviour. Importantly, deletion of α5 integrin, a Fn receptor, results in embryonic lethality at a similar stage in development (Yang et al., 1993), indicating that cell adhesion to Fn is vital for development. Indeed, aberrant adhesion to ECM contributes to the progression of a number of pathologies, including cancer cell proliferation, migration and invasion by activation of signalling pathways downstream of integrin engagement (Ahmed et al., 2005). Increase in platelet aggregation leads to thrombosis (Phillips et al., 2005). Reduced expression of cartilage VII, a major component of the basement membrane (specialised ECM), results in Dystrophic Epidermolysis Bullosa, a severe skin disease causing tissue separation of the dermis, blistering and scarring of the skin (Sawamura et al., 2010).

It is important to note that although binding to Fn is responsible for the activation of many intracellular signalling pathways, substantial evidence suggests that Fn must first be
activated and form fibrils before it can elicit any response from the cell (Gao et al., 2003; Mao and Schwarzbauer, 2005). Atomic force microscopy and imaging approaches with fluorescently tagged ECM molecules or GFP-fusion constructs have demonstrated that ECM fibrillar networks such as Fn are subjected to stretching, contraction and reorganisation by the cell (Mao and Schwarzbauer, 2005; Ohashi et al., 1999). This would therefore suggest that the cell is not only capable of responding to the ECM but that it is also capable of initiating a signal to regulate ECM assembly. This unique ability is mediated by integrins, a class of receptors belonging to the immunoglobulin family (Hynes, 2002).

Fn is primarily composed of type I, type II and type III repeats, and combinations of these repeats constitute binding domains for other extracellular molecules and cells (Fig. 1.3). A large proportion of integrin receptors bind to matrix molecules, such as Fn and vitronectin, by coordinating an Arg-Gly-Asp (RGD) tripeptide, which functions as a core cell-binding sequence in many matrix proteins (Pierschbacher and Ruoslahti, 1984). Replacement of the RGD motif of Fn results in severe vascular defects that resemble the phenotype of α5-integrin-deficient mice and leads to embryonic lethality (Takahashi et al., 2007; Takahashi et al., 2009). Fn also includes a Phe-His-Arg-Ser-Asp (PHSRN) synergy site (type III9-10), adjacent to the RGD motif, which confers integrin specificity, increasing affinity for a specific Fn receptor, α5β1 integrin (Danen et al., 1995). The combination of RGD and PHSRN sites is often referred to as the cell-binding domain (CBD) of Fn and the major ligand for α5β1 integrin.

Cells plated on to a polypeptide encompassing the CBD of Fn spread and form integrin containing adhesions. However, α5β1 integrin signalling is insufficient to recruit cytoskeletal proteins (Bloom et al., 1999; Woods et al., 1986) and to promote cell survival (Jeong et al., 2001). In order to elicit a full adhesive response and prevent apoptotic pathways, cells require a second signal provided by one of the heparin-binding domains of Fn (Jeong et al., 2001; Woods et al., 1986). Fn contains one low-affinity heparin-binding site within its N-terminus and three heparin-binding sites located near the C-terminal region (Type III13, Type III14 and alternatively spliced IIICS domain (Fig. 1.3)) that facilitate binding of heparan sulphate proteoglycans (HSPGs) (Mostafavi-Pour et al., 2001). The syndecan family of transmembrane HSPGs are receptors for heparin-binding domains of ECM molecules and the ubiquitously expressed Fn receptor syndecan-4 has been shown to be the second Fn receptor required for propagation of complete adhesion
signalling in cooperation with \(\alpha_5\beta_1\) integrin (Bass et al., 2007b; Longley et al., 1999; Saoncella et al., 1999; Woods and Couchman, 1998; Woods and Couchman, 2000).

**Figure 1.3. Fibronectin contains binding sites for transmembrane receptors including \(\alpha_5\beta_1\) integrin and syndecan-4.** Fn, a prototypical ECM molecule, consists of Type I, Type II and Type III repeats and three alternatively spliced segments, EIIIA and EIIIB (not shown) and IIICS. The CBD of Fn consists of type III repeats 8-10 and facilitate binding of integrins such as \(\alpha_5\beta_1\) and \(\alpha_v\beta_3\). The LDV motif in IIICS region constitutes a binding site for \(\alpha_4\beta_1\) integrin. Fn fragments (50K and H/0) corresponding to \(\alpha_5\beta_1\) integrin and syndecan-4 binding sites, respectively, have been generated to isolate signalling pathways downstream of each receptor (Bass et al., 2007a; Makarem et al., 1994).

1.2 Integrins: receptors for ECM

Integrins are type I transmembrane glycoproteins, composed of non-covalently linked \(\alpha\) and \(\beta\) subunits. Integrins are present within all multicellular organisms and are the major receptors for ECM proteins, capable of signalling through the cell membrane in either direction (Hynes, 2002). Integrins were originally named to denote their role as integral membrane complexes linking the ECM to the actin cytoskeleton (Tamkun et al., 1986). However, it is now clear that integrins alone or in combination with other cell-surface receptors mediate many key intracellular signals to regulate cell survival, proliferation, polarity, migration and gene expression that are fundamental to physiological processes.
including morphogenesis, development and tissue repair (Bokel and Brown, 2002; Desgrosellier and Cheresh, 2010; Giancotti and Ruoslahti, 1999; Huveneers et al., 2007a).

Genetic ablation of integrins often results in embryonic lethality (e.g. α5 (Yang et al., 1993), αV (Bader et al., 1998), β1 (Fassler and Meyer, 1995) ) and therefore much of our in vivo understanding of integrin function has relied on studying integrins in disease, inducing tissue-specific deletion of integrins (Naylor et al., 2005) or antibody-dependent regulation of integrin function. Mutations of β2 integrins, which are exclusively expressed by leukocytes and participate in the immune response, lead to leukocyte adhesion deficiency (LAD) of varying severity (Hogg and Bates, 2000). Mutations of αIibβ3, a platelet integrin, results in Glanzmann thrombasthenia bleeding disorder, and αVβ3 has been implicated in tumour metastasis and angiogenesis (Eliceiri and Cheresh, 2000; Hood et al., 2002; Sloan et al., 2006). Integrin α5β1, a ubiquitously expressed Fn receptor, plays a fundamental role in inflammation and wound healing (Cavani et al., 1993; Giancotti and Ruoslahti, 1999; Hertle et al., 1992).

In addition to engaging ECM macromolecules, the extracellular domains of some integrins bind other receptors on adjacent cells and mediate cell-cell adhesion. The short cytoplasmic tail of integrins coordinate extracellular signals to determine cell survival, proliferation and cell migration in a process termed “outside-in” signalling. The integrin cytoplasmic domain lacks enzymatic activity and therefore integrin-mediated signalling is initiated by direct recruitment of intracellular proteins to form macromolecular complexes (FAs) at sites of cell-ECM contact. For example, direct binding of talin and α-actinin provides structural links to the actin cytoskeleton, thus integrating the extracellular environment with the contractile machinery of the cell. Moreover, a physical connection between the ECM and the actin cytoskeleton is crucial for generation of traction force during cell migration. Integrins also recruit scaffolding and signalling proteins, such as vinculin, paxillin and FAK, to transmit long range signals throughout the cell (Geiger et al., 2009).

The intracellular domains of integrins are also fundamental in controlling the affinity of integrin receptors for their extracellular ligands, via a process termed “inside-out” signalling (Hynes, 2002). Integrins can switch between an active (high affinity) and inactive (low affinity) conformation by a mechanism dependent on an intact β-integrin cytoplasmic tail. In this way, cells regulate their adhesiveness to the ECM and restrict
transmission of signals initiated at the cell surface. Integrin activation is mediated through protein interaction with the conserved NPxY motifs within β-integrin tails (Liu et al., 2000). The FERM (four point one, ezrin, radixin, moesin)-domain-containing protein talin has been shown to bind NPxY motifs and promote integrin activation in vitro and in vivo (Bouaouina et al., 2008; Calderwood et al., 2002; Wegener et al., 2007). Interestingly, recent work has demonstrated that the talin head domain is sufficient to induce conformational rearrangement and thus activate membrane-embedded integrins in the absence of integrin ligands, clustering or mechanical forces (Ye et al., 2010). In addition to talin, others have demonstrated a role for kindlins in regulating integrin activation that may occur in a β-subunit-specific manner (Harburger et al., 2009; Moser et al., 2009). Differential β-integrin activation is an important mechanism regulating heterodimer-specific integrin signalling and dynamic cell-ECM interaction during cell protrusion and migration.

1.2.1 Heterodimer-specific integrin function

In mammals, 8 β subunits and 18 α subunits have been identified, and combinations of these subunits results in 24 functionally distinct heterodimers that determine ligand specificity (Humphries et al., 2006; Plow et al., 2000). A comprehensive analysis of identified integrin heterodimers and their ligand interactions has been described elsewhere (Humphries et al., 2006). Figure 1.4 shows a simplified representation of integrin-ligand interactions. Although some integrin subunits appear in only a single heterodimer such as α5 in α5β1, 12 integrins contain β1 and 5 contain αV. Integrin α5β1 binds Fn only, whereas αVβ3 interacts with a diverse range of ligands, including fibrinogen, vitronectin, thrombospondin and Fn. ECM proteins in turn are able to bind several integrins; for example, Fn is a ligand for α3β1, α4β1, αVβ1, α8β1, α2β3 and αVβ6.

Although many ECM-integrin binding specificities overlap, it is clear that the biological function of each heterodimer can vary, as exemplified by Fn receptors, α5β1, αVβ3 and α4β1 integrins. Integrin α4β1 binds to an alternatively spliced region of Fn. Cells plated on α4β1 integrin ligand form FAs, organise actin cytoskeleton and promote cell survival without the need for additional Fn receptors. Therefore, α4β1 engagement offers a mechanism for regulating cell-ECM adhesion that is independent of α5β1 integrin-syndecan-4 signalling (Jeong et al., 2001; Liao et al., 2002).
Figure 1.4. Integrin structure and ECM ligand binding. Each integrin is composed of a large extracellular domain (700-1100 residues) with cation-binding motifs in the head region (not shown). Integrin cytoplasmic domains are short, approximately 30-50 amino acid residues in length (A) and promote intracellular recruitment of adaptors and signalling proteins to sites of ECM engagement. Integrins can form many different combinations of heterodimers that determine ECM ligand specificity (B).
Recently, a quantitative proteomic methodology was used to isolate integrin α4β1 and α5β1 complexes following binding to vascular cell-adhesion molecule (VCAM) and Fn, respectively, and identified both core receptor components and novel proteins not previously linked with integrin adhesion signalling. This revealed substantial differences in both the composition and scale of adhesion complexes associated with these different integrins. For example, regulator of chromosome condensation–2 (RCC2) was detected in the α5β1–Fn complex and implicated in GTPase activity, cell spreading and persistent cell migration (Humphries et al., 2009). Importantly, this study demonstrates the requirement for dissecting integrin heterodimer-ligand complexes in order to understand the complexity of adhesion signalling in the regulation of cell migration and disease.

Integrin α5β1 and αVβ3 bind to Fn RGD motifs; however, α5β1 requires the additional PHSRN synergy site within Fn (Danen et al., 1995). Binding of α5β1 and αVβ3 integrins to Fn elicits very different cellular responses and during cell migration the specific functions of these heterodimers must be precisely coordinated. Cells adhering to Fn via integrin α5β1 exhibit dynamic adhesion contacts that promote random cell motility, whereas Fn engagement by αVβ3 integrin stabilises adhesions and supports highly persistent cell migration. Interestingly, this switch in motility from random (α5β1) to persistent (αVβ3) is dependent on the mechanics of interaction between the extracellular domain of these integrins and Fn as swapping the cytoplasmic tails of α5β1 with αVβ3 fails to rescue persistent motility (Danen et al., 2002; Danen et al., 2005).

Alternative roles for α5β1 and αVβ3 integrins have also been demonstrated in mechanical sensing upon adhesion to Fn. Clustering of α5β1 integrin supports high matrix forces whereas the weaker αVβ3-ECM linkages initiate translation of mechanical forces into biochemical signals (mechanotransduction) that recruit cytoskeletal adaptors and reinforce integrin-cytoskeletal interactions (Roca-Cusachs et al., 2009). The differential biomechanical properties of these integrins could, in part, determine the differential adhesion dynamics mediated by αVβ3 and α5β1. However, recent advances have revealed a novel mechanism by which β3 integrins regulate β1-dependent adhesion and cell migration. β3-null cells exhibited increased binding of talin to β1 integrin tails. Thus, integrin β3 suppresses association of talin with the β1-integrin tail by promoting formation of a VASP-RIAM (vasodilator-stimulated phosphoprotein-Rap1-GTP-interacting adaptor molecule) complex (Worth et al., 2010). Consequently, αVβ3 engagement is an important
mechanism in suppressing β1 integrin activation and regulating membrane protrusion and cell migration (Worth et al., 2010).

α5β1 and αVβ3 integrins also exhibit heterodimer-specific roles during Fn matrix assembly. During Fn fibrillogenesis, αVβ3 integrin remains in FAs, while α5β1 undergoes centripetal translocation, along the cellular contractile machinery, to apply tension to the extracellular Fn and promote matrix assembly (Clark et al., 2005; Mao and Schwarzbauer, 2005). However, additional α5β1-independent Fn fibrillogenesis mechanisms exists. Thus, αVβ3-mediated Fn assembly can occur in the absence of a functional RGD motif via a mechanism involving αVβ3-Fn interaction at an iso-DGR motif in the Fn N-terminal domain (Takahashi et al., 2007).

Cross-talk between integrins and growth factor receptors is an important mechanism coordinating signalling pathways in response to environmental cues. Integrin heterodimers appear to be preferentially associated with specific growth factor receptors. Thus, αVβ3 integrin can be immunoprecipitated in complexes with insulin-like growth factor (Brooks et al., 1997), platelet-derived growth factor (PDGF) and VEGF receptors (Schneller et al., 1997; Soldi et al., 1999), whereas α5β1 or β1 integrins associate with the epidermal growth factor (EGF) receptor (Miyamoto et al., 1996; Moro et al., 2002). Integrin clustering and association with the cytoskeleton appears to give rise to integrin-growth factor receptor complexes (Miyamoto et al., 1996), potentially contributing to growth factor receptor activation and signalling (Moro et al., 2002). By contrast, stimulation of VEGF receptor with VEGF can promote activation of αVβ3 integrins (Byzova et al., 2000). Also, growth factor signalling has been shown to mediate differential delivery of integrin receptors back to the membrane (see section 1.6), thus regulating surface expression of integrins and integrin heterodimer-specific signals.

In addition to growth factor receptors, syndecans cooperate with integrins to regulate cell adhesion and cell behaviour. Importantly, α5β1 integrin signalling is critically dependent on engagement of syndecan-4 by Fn to form FAs and reorganise the actin cytoskeleton.

1.3 Syndecans: a family of receptors and co-receptors

Syndecans are type I transmembrane receptors, substituted with long, unbranched glycosaminoglycan (GAG) chains on their extracellular domain (Fig. 1.4). The syndecan
family comprises four members, substituted with heparan sulphate and chondroitin sulphate chains on their extracellular domains (Bass and Humphries, 2002b; Couchman et al., 2001; Xian et al., 2010; Zimmermann and David, 1999). Syndecan-1, 2 and 3 are developmentally regulated and exhibit tissue-specific expression patterns. In general, syndecan-1 is specific to epithelial cells (Kim et al., 1994), whereas syndecan-2 (also known as fibroglycan) and syndecan-3 (also known as N-syndecan) are expressed mainly in fibroblasts (David, 1990) and neuronal cells (Gould et al., 1992), respectively. Interestingly, syndecan-4 (also known as amphiglycan) is almost ubiquitously distributed as a minor component in a range of cell types including fibroblasts, epithelial, and smooth muscle cells (Woods and Couchman, 1994).

Heparan sulphate chains such as those found on syndecan receptors interact with ECM molecules, growth factors and growth factor receptors (e.g. syndecan-1 and FGF (Filla et al., 1998; Salmivirta et al., 1992)) (Tumova et al., 2000), ECM proteins, cell-cell adhesion molecules and pathogens. Therefore, as HSPGs, syndecans are implicated in cell adhesion, migration and infection (Bernfield et al., 1999). Syndecan-1 is upregulated in dermal endothelial cells and granulation tissue during tissue repair (Elenius et al., 2004). A role for syndecan-1 has also been identified in regulating αVβ3 and αVβ5 integrin activation in mammary carcinoma cells (Beauvais et al., 2004; Beauvais and Rapraeger, 2003). Moreover, the same group has demonstrated a direct interaction between αVβ3 and αVβ5 extracellular domain and syndecan-1 ectodomain core protein. Disrupting the interaction between syndecan-1 and these integrins using synstatin (a peptide derived from the active site in syndecan-1 ectodomain) inhibits angiogenesis and mammary tumour growth (Beauvais et al., 2009).

Syndecan-2 is essential for vascular development. Ablation of syndecan-2 gene in Zebrafish leads to defective angiogenesis (Chen et al., 2004b) and recombinant syndecan-2 ectodomain alters adhesive functions in colon carcinoma cells (Park et al., 2002). In accordance with a role for syndecan-2 in adhesion, overexpression of syndecan-2 in Chinese hamster ovary (CHO) cells increases cell spreading (Longley et al., 1999; Woods and Couchman, 2001). Furthermore, syndecan-2 appears to regulate matrix assembly and therefore may be critical in regulating integrin-ECM interaction (Klass et al., 2000).

A role of syndecan-3 in cell migration has been described for neuronal cell function. Syndecan-3-null mice exhibit reduced cell numbers in cerebral cortex resulting from
perturbed neuronal cell migration (Hienola et al., 2006). Together, these data strongly suggest a role for syndecans in the control of cell migration, particularly through altering cell adhesiveness to ECM.

Syndecan-4 is the only member of the syndecan family reported to be enriched in focal adhesions (Woods and Couchman, 1994), although syndecan-1 has been shown to co-localise with microfilaments in basal membrane of epithelial cells (Rapraeger et al., 1986). Increasing evidence suggests a specific role of syndecan-4 transmembrane proteoglycan in cell migration especially during tissue repair. For example, syndecan-4 is upregulated in fibroblasts following incisional wounding of the skin (Gallo et al., 1996) and in the carotid artery tissue after balloon-catheter injury of the vascular smooth muscle cells (Cizmeci-Smith et al., 1997). Syndecan-4 is also strongly expressed in many tumour types, regardless of the site of origin (Beauvais and Rapraeger, 2004). In addition, syndecan-4 knockout mice have impaired skin wound healing and defective angiogenesis in granulation tissue (Echtermeyer et al., 2001). Muscle progenitor cells derived from syndecan-4-null mice fail to reconstitute damaged muscle, suggesting that the syndecan-4 receptor is required for migration of these cells and effective wound healing (Echtermeyer et al., 2001). Syndecan-4 has further been demonstrated to regulate protease activity and matrix degradation in cartilage tissue (Echtermeyer et al., 2009). Thus, all syndecan family members are critical components of processes involved in cell migration.

Perhaps the best evidence for a specific role of syndecan-4 in integrin-mediated cell adhesion comes from studying FA formation in response to Fn engagement. Syndecan-4 controls a number of signalling pathways in co-operation with α5β1 integrin to regulate FA assembly and cytoskeletal dynamics. Overexpression of syndecan-4 leads to enhanced FA and stress fibre formation (Bass et al., 2007b; Bass and Humphries, 2002b). Furthermore, glycosylation-deficient CHO cells overexpressing syndecan-4 are able to form vinculin- and syndecan-4-containing adhesions, albeit to a lesser extent than wild-type cells, demonstrating that the core protein of syndecan-4 is sufficient for this process (Echtermeyer et al., 1999). However, the mechanism by which syndecan-4 functions in synergy with α5β1 integrin to regulate ECM signals is largely unknown. Understanding and identifying new interactions at the cytoplasmic domain of syndecan-4 will no doubt be vital for decoding the integrin-syndecan-4 signalling axis.
1.3.1 Syndecan structure

Syndecans are composed of large extracellular domains, a single transmembrane span and a short cytoplasmic region. Syndecan extracellular domains exhibit very low levels of conservation between family members that may reflect differences in ligand-binding properties (Caccavari et al., 2010; Couchman et al., 2001; Morgan et al., 2007; Multhaupt et al., 2009; Tumova et al., 2000). In addition to GAG chains, the syndecan ectodomain includes a membrane proximal cleavage site that is responsible for extracellular domain shedding. The precise role of syndecan ectodomain cleavage is unclear; however, it is thought to modulate cell signalling by competing for ECM/growth factor binding (Bass et al., 2009; Morgan et al., 2007). Regulated shedding of this domain following thrombin, plasmin or EGF stimulation suggests a role for the soluble proteoglycan ectodomain in wound healing (Subramanian et al., 1997).

Unlike the extracellular domain, the transmembrane and cytoplasmic domains of the syndecans are highly conserved. The transmembrane domain contains GxxxG motif that strongly promote self-association and formation of detergent-resistant dimers (Dews and Mackenzie, 2007) and are crucial for syndecan function (Choi et al., 2005). The short, cytoplasmic domain of syndecan contains a variable domain unique to each syndecan member and highly conserved membrane proximal (C1) and membrane distal (C2) regions (Fig. 1.5). As such, the variable domain is thought to dictate functions specific to each syndecan member. Interactions identified at the C1 and C2 domain, however, offer potential overlap in all syndecans (Zimmermann and David, 1999). The following section will discuss some of these cytoplasmic interactions with specific focus on syndecan-4 binding partners.
### Figure 1.5. Syndecan structure and binding partners.

Syndecan, depicted as a dimer, contains a large HS-substituted extracellular domain and a highly conserved transmembrane and intracellular region. The sequence homology of the cytoplasmic domain of the syndecan family members and some of the important binding partners identified are shown. Syndecan-4 binding partners are depicted in black and interactions of other syndecan members in light grey.

<table>
<thead>
<tr>
<th>Syndecan</th>
<th>Sequence</th>
<th>Binding Partners</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>RMKKKDEGSY TLEEPKQA-SVTYQK-PDKQE EFYA</td>
<td>Microfilaments (syndecan-1,-3)? Syntenin</td>
</tr>
<tr>
<td>2</td>
<td>RMRRKKDEGSY DLGERK-PSSAAYQKA PTK--EFYA</td>
<td>CASK Synectin/GIPC1 Synbindin?</td>
</tr>
<tr>
<td>4</td>
<td>RMKKKDEGSY DLG-KK-P1--YKKA Pt--N EFYA</td>
<td>PKCα &amp;PIP2 (syndecan-4) Syntenin</td>
</tr>
</tbody>
</table>
1.3.2 Syndecan cytoplasmic interactions

The cytoplasmic domains of syndecans have several notable features that may influence syndecan-dependent signalling and protein-protein interaction (Fig. 1.5). The EFYA motif in the membrane distal C2 region constitutes a Postsynaptic density 95, Disk large, Zona occludens (PDZ) binding motif vital for recruitment of scaffolding proteins. The syndecan variable domain confers specific functions to each syndecan family member, and the existence of three conserved tyrosines, one conserved threonine and one conserved serine residue offers potential targets for phosphorylation and thus a mechanism for regulating syndecan signalling (Bass and Humphries, 2002a; Woods and Couchman, 2001; Zimmermann, 2006; Zimmermann and David, 1999).

1.3.2.1 Conserved region-1 (C1)-domain interactions

A variety of proteins including cytoskeletal proteins, adaptors and protein kinases have been shown to interact with C1 domain of syndecans. Ezrin, an ERM (ezrin/radixin/moesin) family member, has been shown to bind DEGSYD sequence in syndecan-2 C1 domain (Granes et al., 2003). ERM proteins link actin filaments to the membrane by direct interactions with transmembrane receptors or recruitment of scaffolding proteins. Therefore, ezrin potentially links syndecan-2 to the actin cytoskeleton (Granes et al., 2000). Furthermore, ERM proteins interact with signalling molecules such as phosphatidylinisitol-3 kinase and RhoGDI and this strengthens a role for syndecans as important regulators of GTPase activity and cell morphology (Niggli and Rossy, 2008).

The DEGSYD sequence in C1 domain of syndecans constitutes a potential tyrosine phosphorylation site. EphB2, a tyrosine kinase receptor, has been implicated in phosphorylation of the conserved tyrosine residue in the syndecan-2 C1 domain (Y180 equivalent in syndecan-4) to regulate neuronal outgrowth. Members of Src family kinases (SFKs) have also been implicated in phosphorylation of other syndecan family members. SFKs (including Yes, Fyn and Src) are tyrosine kinases that are involved in many processes that are influenced by syndecans, including FA formation, cell spreading and modulation of FGF signalling (Thomas and Brugge, 1997). Tyrosine phosphorylation of syndecan-1 and syndecan-4 has been described in fibroblasts (Ott and Rapraeger, 1998) where the endogenous phosphorylation was blocked with tyrosine kinase inhibitors gentistene, herbimycin A and staurosporine. This inhibition profile could be indicative of a
role for SFKs in syndecan phosphorylation, although the family member responsible and the site of tyrosine phosphorylation was never identified (Ott and Rapraeger, 1998).

Further evidence for SFK interaction with syndecans was obtained through affinity isolation columns in which either full length syndecan-3 cytoplasmic domain or membrane proximal C1 domain (which contains one tyrosine residue and is conserved within all syndecans) were used as bait. The proteins identified included Src, Fyn, cortactin and tubulin and an unidentified 30KDa protein. However, it was unclear whether these proteins bound directly to syndecan cytoplasmic domain or indirectly through formation of a protein-protein complex (Kinnunen et al., 1998; Zimmermann and David, 1999). The role of Src, the prototypical member of SFKs, in adhesion formation will be discussed in later chapters. The interaction of syndecan with SFKs offers a mechanism for syndecan regulation of many biological events. For example, cortactin, one of the proteins isolated with syndecan-3, is a major substrate for Src and its phosphorylation by Src decreases its ability to cross-link actin filaments (Kinnunen et al., 1998). Importantly, recent work in this laboratory has demonstrated a fundamental defect in adhesion turnover and cell migration in fibroblast expressing a phosphomimetic mutant of syndecan-4 receptor (Syn4Y180E).

In addition to tyrosine phosphorylation, serine phosphorylation of the conserved serine residue (S179 in human and S183 in rat) in C1 domain has been demonstrated with syndecan-4. In vitro kinase assays initially implicated two members of the PKC family (serine/threonine kinases) in S179 phosphorylation, PKCα and PKCδ. However, only cells expressing dominant negative PKCδ and not PKCα exhibited reduced levels of total syndecan-4 phosphorylation (Murakami et al., 2002). The implication for PKCδ-mediated phosphorylation of syndecans has only been examined in regulating syndecan-4-specific binding and activation of PKCα (see section 1.3.2.2). However, as the PKCδ-phosphorylation site is conserved within all syndecans, PKCδ-mediated phosphorylation of syndecans may play a more general role in syndecan function as yet not identified.

1.3.2.2 Syndecan-4 variable domain specific interactions

Syndecan-4 variable domain interactions are crucial in regulating syndecan-4-mediated FA formation and cell migration (Bass and Humphries, 2002a; Bass et al., 2007b; Dovas et al., 2006). The variable domain of syndecan-4 constitutes a unique PKCα-binding site and an
ability to associate with the phospholipid phosphatidylinositol-4, 5-bisphosphate (PIP$_2$) (Oh et al., 1998). As mentioned earlier, syndecan-4 engagement provides the second signal required for FA formation and stress fibre assembly in cells pre-spread on an α5β1 integrin ligand (Bass and Humphries, 2002a; Woods and Couchman, 1998; Woods et al., 1986). This second signal can also be emulated by direct activation of PKC using phorbol esters, indicating that perhaps PKC activation lies downstream of syndecan-4 signalling. PKCs are also among a number of kinases activated downstream of ECM engagement. PKCα, which localises to FAs in normal but not transformed fibroblasts (Hyatt et al., 1990), has been shown to directly associate with syndecan-4 variable region, via PKCα catalytic domain. The association between syndecan-4 and PKCα increases PKCα localisation to FAs (Lim et al., 2003) and has been shown to be dependent on syndecan-4 oligomerisation (Oh et al., 1997a; Oh et al., 1997b).

NMR studies revealed that syndecan-4 cytoplasmic domain oligomerisation is stabilised by the association of PIP$_2$ with the variable domain of syndecan-4 (Lee et al., 1998). Although all syndecans are able to form higher order oligomers, via their transmembrane domain, the ability of the syndecan-4 receptor to oligomerise via its cytoplasmic domain is unique to this family member (Lee et al., 1998). PIP$_2$ is an important component of the plasma membrane and a precursor for second messengers (Lee and Rhee, 1995) that activate downstream signals such as release of Ca$^{2+}$ and diacylglycerol to activate PKCα. However, the formation of a syndecan-4/PIP$_2$/PKCα tertiary complex appears to override the need for secondary messengers and leads to superactivation of PKCα in vitro. Intriguingly, the PKCδ-mediated phosphorylation of S179 in the C1 domain of rat syndecan-4 has been implicated in regulating the formation of this ternary complex and therefore syndecan-4-mediated PKCα activity (Murakami et al., 2002).

In vitro studies suggested that PKCδ-mediated phosphorylation of S179 inhibits PIP$_2$ binding to the lysine-rich syndecan-4 variable domain and abrogates the capacity of the tail to multimerise and activate PKCα (Horowitz and Simons, 1998a; Horowitz and Simons, 1998b). Consistent with this, the expression of a dominant negative PKCδ enhances syndecan-4/PIP$_2$-mediated PKCα activation (Murakami et al., 2002).

A functional role for syndecan-4 in cytoskeletal regulation became apparent when α-actinin was identified as another binding partner of syndecan-4 variable domain. α-actinin
is responsible for cross-linking actin stress fibres. α-actinin also associates with integrins, vinculin and zyxin within FAs (Belkin and Koteliiansky, 1987; Otey et al., 1993; Reinhard et al., 1999). There may also be potential signalling roles as reports indicate α-actinin interacts with PIP₂ and FAK (Greene et al., 2003). Others have implicated syndecan-4 variable and C1 domain as a binding site for a scaffolding protein, syndesmos. When overexpressed in fibroblastic cells, syndesmos accelerated cell spreading and the assembly of actin stress fibres (Baciu et al., 2000). Further investigation revealed paxillin as a cytoplasmic binding partner for syndesmos (Denhez et al., 2002). Paxillin is capable of binding both structural (α-tubulin) as well as signalling molecules (e.g. FAK, Csk, and c-Abl), allowing it to play an intimate role in the dynamics of the FA. The connection of syndecan-4 with paxillin and possible downstream signalling events through syndesmos may reflect a mechanism in which syndecan-4 signalling works synergistically with α5β1 in the formation of FAs (Baciu et al., 2000; Denhez et al., 2002). However, as will be discussed later (section 1.4) the most compelling evidence suggests that syndecan-4-α5β1 synergy is predominantly regulated by coordination of GTPase activity and localisation.

A link between syndecan-4 and dynamin II was recently identified, although whether this interaction is specific to syndecan-4 or requires binding of other proteins is unclear (Yoo et al., 2005). Dynamin is a GTPase involved in membrane constriction and vesicle formation important for clathrin- and caveolin-dependent endocytosis (Henley et al., 1999). These data are intriguing as several reports and unpublished data from our laboratory have highlighted the importance of syndecan-4 in receptor trafficking and cell migration (Bass et al., 2007b; Fuki et al., 1997; Gao et al., 2000; Tkachenko et al., 2006; Zimmermann et al., 2005).

1.3.2.3 Conserved region-2 (C2) is a putative PDZ-binding motif

The C-terminal domain of syndecans constitutes a class II PDZ-binding motif (EFYA) (Grootjans et al., 1997; Songyang et al., 1997; Zimmermann, 2006) that facilitates interaction with PDZ-domain containing proteins such as syntenin (Grootjans et al., 1997; Zimmermann et al., 2001), GIPC1 (Gao et al., 2000) and CASK (calcium/calmodulin-dependent serine/threonine kinase) (Cohen et al., 1998). PDZ domains are highly conserved protein-binding modules that are present within many intracellular proteins and function as scaffolding and targeting components to establish dynamic multi-protein complexes at the membrane. Several PDZ-domain-containing proteins bind to receptors
and ion channels and appear to be critical for the formation and stability of adhesion structures and establishing polarity (Fanning and Anderson, 1999). As syndecans do not display intrinsic enzymatic activity, the ability to bind to PDZ domains and recruit potential scaffolding and signalling proteins may be essential for their role in signalling cascades. Indeed, deletion of the C-terminal PDZ-ligand motif in syndecan-4 abrogates cell migration (Tkachenko et al., 2006).

Syntenin is an example of a PDZ-domain-containing protein originally identified in yeast two-hybrid screens as a syndecan binding partner (Grootjans et al., 1997). Syntenin contains two tandem PDZ domains which are capable of binding either syndecan receptor or PIP$_2$ (Zimmermann et al., 2002). It is believed that syntenin is recruited to the membrane by engaging PIP$_2$ via its PDZ-1 domain and syndecan via its PDZ-2 domain and the highest affinity binding is achieved when both PDZ domains are occupied (Zimmermann et al., 2002). In epithelial cells, endogenous syntenin is enriched in areas of cell-cell contact, co-localising with syndecan-1. In fibroblastic cells, syntenin staining is seen in cell-substratum contacts. Nuclear staining of syntenin has also been observed in many cell lines (Zimmermann et al., 2001).

The function of syntenin-syndecan interaction has been analysed using PDZ-binding mutants of syntenin. Coexpression of a syntenin mutant defective in PIP$_2$ binding and syndecan-2 results in perinuclear accumulation of syndecan/syntenin complexes in Rab11-positive recycling endosomes. Syntenin was shown to support recycling of syndecan-1 back to the membrane in an Arf6-dependent manner. The principles of endocytosis and recycling pathways will be described later and are integral to regulation of cell-ECM function (Zimmermann et al., 2005).

Proteins such as synbindin and CASK have also been shown to bind to the EFYA motif of syndecans via their PDZ domains (Cohen et al., 1998). CASK is a member of the membrane-associated guanylate kinase (MAGUK) protein family (Cohen et al., 1998). The single PDZ domain of CASK has been shown to bind to other transmembrane receptors, including junctional adhesion molecules in epithelial cells. It is believed that CASK may act as a scaffolding protein like other MAGUK members and link a number of transmembrane receptors in an adhesion complex (Bass and Humphries, 2002a).

GIPC1, also known as synectin, is another PDZ-domain protein and a potential syndecan binding partner. However, sequence alignments reveal that the ligand-binding pocket of
GIPC1 PDZ domain is not compatible with the EFYA motif in syndecan-4 (Bass and Humphries, 2002a). Despite this, studies using PDZ mutants of syndecan-4 have implicated a role for GIPC1 in cell migration. GIPC1 has also been reported to localise to lamellipodia of migrating cells and regulate syndecan-4-specific signalling (Elfenbein et al., 2009; Gao et al., 2000), but whether this is as a result of direct interactions with the syndecan tail is not clear. Importantly GIPC1 has been implicated in endocytosis of several receptors (Lanahan et al., 2010; Valdembri et al., 2009).

It is impossible that all the above-mentioned proteins simultaneously interact with the 28 amino-acid cytoplasmic domain of syndecan-4. Therefore, the ability to dynamically recruit different proteins and form different protein complexes offers a way by which syndecans can modulate intracellular signals in response to environmental changes. It is clear that the diversity of interactions at syndecan cytoplasmic domain is important in the role of this receptor in regulating Rho GTPase signalling and cell migration.

1.4 Rho GTPases

GTPases are monomeric guanine nucleotide-binding proteins (G proteins) that constitute a superfamily of small GTPases (20-40KDa) – comprised of more than 100 members - and large GTPases – consisting of dynamin and dynamin-related proteins (Praefcke and McMahon, 2004; Takai et al., 2001). All GTPases share a simple enzymatic property; they bind and hydrolyse guanosine triphosphate (GTP). In this way, GTPases act as molecular switches, cycling between an active GTP-bound form capable of associating with effectors and an inactive guanosine diphosphate (GDP)-bound conformation. Dynamins are distinguished from other GTPases by having large GTPase domains and relying on oligomerisation for GTPase activation. Dynamins are crucial regulators of membrane scission and therefore essential for many endocytic pathways (Praefcke and McMahon, 2004).

The small GTPases are structurally classified into at least five subfamilies, Ras, Rho, Rab, Arf and Ran (Takai et al., 2001), and regulate a diverse range of cellular functions including mitogenesis, cytoskeletal organization, vesicle traffic, nuclear transport and cellular migration (Bamba et al., 2002; D'Souza-Schorey and Chavrier, 2006; Parsons et al., 2010; Stenmark, 2009; Takai et al., 2001). The main interest of our laboratory is regulation of processes that mediate cell migration including adhesion contact dynamics,
cytoskeletal reorganisation and adhesion receptor trafficking. It is clear that syndecan-4 and α5β1 synergistic signalling is critical in coordinating these events. Initially, the role of Rho GTPases in modulating actin cytoskeleton dynamics will be discussed, and later the role of Rabs, Arfs and other GTPases involved in receptor trafficking will be considered (see section 1.6).

Members of the Rho GTPase family have been shown to regulate many aspects of intracellular actin dynamics and are found in all eukaryotic organisms including yeast (Burridge and Wennerberg, 2004; Nobes and Hall, 1995a; Parsons et al., 2010). The formation of adhesion contacts and regulation of the actin dynamic are mediated primarily by three members of the Rho GTPase family: RhoA, Rac1 and Cdc42.

Initial studies using fluorescence resonance energy transfer (FRET) probes to detect GTPase activities in live cells, demonstrated the local distribution of GTP-bound Rac1 and Cdc42 at the leading edge (points of protrusion) of migrating cells (Itoh et al., 2002), while active RhoA was found at both the leading edge and at points of retraction in the cell rear (Kurokawa et al., 2005). Intriguingly, a recent study used “computational multiplexing” to show that RhoA is actually activated at the leading edge of migrating cells in a region of leading edge advancement and in front of the zone of Rac1 and Cdc42 activity (Machacek et al., 2009). Thus, in migrating cells, activity of Rac1, RhoA and Cdc42 is precisely regulated both spatially and temporally. Therefore, it is important to understand how environmental stimuli restrict and coordinate the activity of GTPases during cell migration.

In order to study the role of adhesion receptors in GTPase activity, several studies have examined Rac1 and RhoA activity profiles during the early stages of cell spreading. Adhesion to Fn induces a transient wave of Rac1 activation, a process that is blocked by mutations in the cytoplasmic tail of β integrins (Berrier et al., 2002; Schwartz et al., 1998). Antibody clustering of integrin β1 tails leads to redistribution of Rac1 to lipid microdomains (del Pozo et al., 2004; Del Pozo et al., 2002). Importantly, a fundamental role for syndecan-4 in Rac1 activation has been identified. Engagement of both integrin α5β1 and syndecan-4 was found to be necessary for Rac1 activation in response to Fn engagement (Bass et al., 2007b). Mouse embryonic fibroblasts (MEFs) plated on an α5β1 ligand alone fail to activate Rac1 during cell spreading or form vinculin-containing adhesions. MEFs deficient of the syndecan-4 gene (Syn4-/-) are also unable to induce the wave of Rac1 activation when plated on Fn demonstrating that syndecan-4 engagement is
an absolute requirement for Rac1 activation. Moreover, stimulation of pre-spread MEFs with a soluble syndecan-4 ligand is sufficient to induce Rac1 activity (Bass et al., 2007b).

Intriguingly, Syn4-/- cells display constitutively high levels of active Rac1, suggesting that syndecan-4 may in fact be acting as a negative regulator of Rac1 activity and that the ligation of syndecan-4 releases the inhibitory signals leading to PKCα-dependent Rac1 activation (Bass et al., 2007b).

Research by Pankov et al. has demonstrated how control of Rho GTPase activity, specifically that of Rac1, is essential for determining mode of cell migration (Pankov et al., 2005) (Fig. 1.6A). High Rac1 activity promotes formation of peripheral lamella (see section 1.4.1) and random migration. By contrast, a decrease in Rac1 activity promotes formation of a single leading lamella and switches mode of migration from random to directionally persistent (Fig. 1.6A). On 3D matrices, where cells migrate in a directionally persistent manner, Rac1 activity is localised to the leading edge (Fig. 1.6B & C). Intriguingly, persistence of migration is also dependent on matrix architecture rather than composition as mechanical flattening of the fibrillar 3D matrix restores random migration (Pankov et al., 2005).

Figure 1.6. Rac1 activity, syndecan-4 and directional cell migration. Rac1 activity dictates mode of cell migration (A). High Rac1 activity (e.g. on 2D substrates) supports formation of multiple lamella that mediate random cell motility. Localised and regulated Rac1 activity (e.g. on 3D matrices) promotes formation of a leading lamella and directional persistence (A). Syndecan-4-dependent spatiotemporal control of Rac1 activity is required for directional cell migration on 3D matrices (B) (migration tracks depicted in C). (Part A was adapted from Pankov et al. (2005)).
Syn4-/− cells, which exhibit high Rac1 activity, extend several off-axial lamella and thus migrate randomly on fibrillar CDMs (Fig. 1.6B & C). Therefore, syndecan-4 expression is necessary to restrict Rac1 activity and consequently to drive directional cell migration. Furthermore, the PKCα-binding motif of syndecan-4 is critical for syndecan-4-dependent Rac1 regulation and consequently controls the ability of cells to sense changes in their local environment and regulate Rac1-dependent changes of direction (cell turning) (Bass et al., 2007b).

In contrast to Rac1, RhoA activity is rapidly and transiently suppressed during cell spreading on Fn, followed by reactivation at later time points (Arthur and Burridge, 2001; Bass et al., 2007b; Ren et al., 1999). Sustained Rac1 activation by stimulation with PDGF or expression of constitutively activated Rac1 downregulates Rho activity (Ohta et al., 2006; Sander et al., 1999). Syndecan-4-ECM engagement has also been shown to exert opposing effects on Rac1 and RhoA activity (Bass et al., 2008; Bass et al., 2007b). Rac1 and Rho are thus described as being mutually antagonistic (Burridge and Wennerberg, 2004; Meili and Firtel, 2003) and mounting evidence suggests the existence of several shared mechanisms acting downstream of cell-ECM engagement that regulate Rac1 and Rho activity in a reciprocal manner. However, studies have also demonstrated that constitutively active or dominant negative Rho has no effect on endogenous Rac1 activity (Sander et al., 1999). Furthermore, the engagement of different integrin heterodimers differentially regulates RhoA activity. Expression of β1 in β1-deficient cells increases formation of actin stress fibres and cell-matrix adhesions specifically at membrane protrusions, whereas increased expression of β3 results in a random distribution of adhesions over the entire basal surface of the cell. Engagement of either α5β1 or αvβ3 is sufficient to cause the initial suppression of RhoA activity observed during cell spreading. However, RhoA reactivation is only observed in cells expressing α5β1 integrin and not αVβ3. In agreement with RhoA activity promoting Fn remodelling, fibrillogenesis is only observed in cells expressing α5β1 which exhibit high RhoA activity (Danen et al., 2002; Danen et al., 2005).

Syn-4-/− cells exhibit lower levels of activated Rho. However, syndecan-4-dependent stress fibres and FA formation can be bypassed by direct activation of either Rho, with lysophosphatidic acid (LPA), or PKC, using the phorbol ester PMA (Dovas et al., 2006). These data suggest that the relationship between Rac1 and RhoA activity is more
complicated than our current understanding and dynamic regulation of these GTPases requires receptor cross-talk.

1.4.1 Coordination of cell migration by Rho GTPases

Cell migration is a dynamic process involving extension of lamellipodia, formation of new adhesions, contraction and detachment of adhesions at the cell rear to allow tail retraction (Fig. 1.2). Coordination of these events by adhesion receptor-dependent regulation of GTPase activity is central to the regulation of directed cell migration. Consequently, intracellular trafficking of adhesion receptors is also essential for cell migration as will be discussed later (see section 1.6) and this is also coordinated by GTPases including Rab, Arf and dynamin family members. In this section, the control of cell migration by the Rho GTPases Rac1, RhoA and Cdc42 will be discussed.

During directed cell migration, it is vital that membrane protrusion is restricted to the front of a cell and retraction occurs at the rear. The current theory suggests that these opposing activities are mediated by Rac1 and RhoA. Pioneering work by Nobes and Hall demonstrated that Rac1 and Cdc42 induce membrane ruffles (lamellipodia and filopodia, respectively) and RhoA promotes FA formation and tail retraction (Nobes and Hall, 1995a; Nobes and Hall, 1999). However, it is now becoming clear that RhoA may play a role in membrane protrusion (Machacek et al., 2009).

Yeast two-hybrid screens, affinity chromatography and co-immunoprecipitation techniques have identified over 50 potential targets for Rac1, RhoA and Cdc42, many of which directly affect actin cytoskeletal reorganisation and play roles in establishing a polarised cell morphology. Many of the Rho GTPase effectors contain a consensus sequence, the CRIB (Cdc42/Rac1 interactive binding) or RBD (Rho-binding domain) that constitutes the binding sites for Rho GTPases (Etienne-Manneville, 2004; Hall, 1998; Nobes and Hall, 1999).

1.4.1.1 Membrane protrusion

Formation of membrane protrusions is achieved through polymerisation of a branching filamentous actin network behind the leading edge of the cell. Inhibition of actin polymerisation with cytochalasin blocks the formation of cell-surface protrusions and inhibits cell migration. Similarly, inhibition of Rac1 prevents lamellipodia formation and...
cell migration observed in scratch wound assays (Nobes and Hall, 1999). Rac1 stimulates formation of lamellipodia by acting on members of the Wiskott-Aldrich syndrome protein (WASP) family (Abou-Kheir et al., 2008; Eden et al., 2002). Rac1 interacts with verprolin homologous protein (WAVE, a WASP family member), leading to the recruitment of actin-related protein 2/3 (Arp2/3), a complex involved in actin polymerisation (Burridge and Wennerberg, 2004; Millard et al., 2004; Takenawa and Itoh, 2001). The local activation of Arp2/3 induces formation of new actin filaments on an existing actin branch producing a dendritic network of actin filaments. Signalling downstream of Cdc42 has also been linked to Arp2/3 activation (Lim et al., 2008). Intriguingly, activation of Arp2/3 by Rac1 and Cdc42 leads to morphologically distinct structures, i.e. lamellipodia and filopodia, respectively. The reason for this is not entirely clear but may involve other effectors of Rac1 and Cdc42 and tight regulation of GTPase localisation. In addition to promoting actin polymerisation, Rac1 inhibits actin depolymerisation thus reinforcing actin growth and lamellipodia formation.

The role of RhoA in cell protrusion is unclear. Recent work investigating the role of RhoA activity at the leading edge suggested that RhoA may in fact initiate membrane protrusions, while Rac1 and Cdc42 stabilise protrusions (Machacek et al., 2009). RhoA activity has also been associated with both membrane extension of T-cells and formation of uropods (protrusive structures at rear of motile T-cells). Coordinated RhoA activity at these structures is believed to be required for T-cell crawling and transendothelial migration (Heasman et al., 2010). These data are intriguing as it was previously believed that Rac1 and Cdc42 alone were responsible for membrane protrusion.

1.4.1.2 Adhesion formation

Rac1 activity is required for the formation of focal complexes (Allen et al., 1997; Nobes and Hall, 1995b). Generation of tensile force through the actin and myosin machinery triggers the maturation of focal complexes into FAs and this is dependent on RhoA. Inactivation of RhoA using botulinum C3 toxin leads to complete loss of FAs and response to chemotactic factors (Burridge and Wennerberg, 2004).

Once formed, cell-ECM adhesions are subject to two fates: maturation and turnover. The major trigger for maturation of adhesions as described above is the application of actomyosin tensile forces under the control of RhoA. FA turnover requires dynamic assembly and disassembly of adhesion complexes at cell periphery and is fundamental for
cell migration; disassembly of adhesions at the rear allows tail retraction and turnover of adhesions at the leading edge promotes membrane protrusion and formation of new cell-matrix adhesions. Numerous mechanisms have been suggested for disassembly of cell-ECM adhesions, and reversing the signals leading to adhesion maturation, i.e. decreasing tensile force by inhibiting RhoA or myosin activity, appears to be an important contributor to adhesion disassembly (Broussard et al., 2008; Webb et al., 2002).

1.4.1.3 Cell body contraction

While membrane protrusions of the cell serve as a means to extend forward, the contractile machinery enables transmission of force to a substrate at sites of adhesion, therefore allowing the cell body to translocate. One of the most prominent cytoskeletal structures, present in most cultured cells, are bundles of actin filaments (stress fibres) (Hall, 1998; Nobes and Hall, 1995a; Pellegrin and Mellor, 2007). These actin bundles together with myosin II, α-actinin and various other proteins constitute the contractile actomyosin machinery in non-muscle cells. Myosin II is a major cytoskeletal protein that is responsible for converting the chemical energy of ATP into mechanical energy for cell contraction, an activity which has been implicated in processes such as cell spreading, migration and stress fibre formation (Vicente-Manzanares et al., 2009b).

RhoA regulates actomyosin contractility and therefore mediates stress fibre formation, maturation of nascent adhesions into FAs and is essential for tail retraction. Furthermore, stimulation of RhoA with lysophosphatidic acid or expression of constitutive active RhoA stimulates Fn matrix assembly that is dependent on RhoA-induced cell contractility (Zhang et al., 1997a; Zhong et al., 1998). Inhibiting RhoA activity in migrating leukocytes induces protrusion at inappropriate sites (Burridge and Wennerberg, 2004). Y-27632, a specific inhibitor of RhoA effector Rho-associated coiled-coil kinase (ROCK) has revealed a role for RhoA in monocyte and neurite tail retractions during migration (Wylie and Chantler, 2003).

1.4.2 Regulation of Rho GTPase activity

The regulation of Rho GTPase activity is essential for control of many processes during migration. Although it is evident that integrins and syndecan-4 regulate the activity of Rho GTPases, a direct link from receptor activation to the reorganisation of the actin and microtubule cytoskeletons has remained elusive. It is believed that syndecan-4 and
integrins mediate their regulation of Rho GTPases by acting on the Rho guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine dissociation inhibitors (GDIs). Regulation of GTPase activity can be achieved by either controlling the guanine nucleotide loading or subcellular localisation and association with the membrane. GEFs positively regulate GTPases by catalysing the exchange of GDP to GTP and GAPs negatively regulate activity by increasing the rate of GTP hydrolysis (Fig. 1.7). GDIs sequester GTPases within the cytoplasm and prevent translocation to the membrane required for GTPase activation. Here, examples of each form of GTPase regulation by GEFs, GAPs and GDIs will be highlighted.

Figure 1.7. GTPase molecular switch: GTPase activity is regulated by GEFs, GAPs and GDIs. GEFs and GAPs allow the cycling of GTPases between active GTP-bound and inactive GDP-bound forms, respectively. GDIs retain GTPases in the cytosol. (Stacked triangles represent prenyl groups).

1.4.2.1 Tiam1: an example of a Rho GEF

GTPases have a high affinity for GDP and GTP nucleotides and therefore require the activity of GEFs to disrupt interactions between nucleotides. GEFs decrease nucleotide-binding affinity leading to GDP dissociation and subsequent GTP association (Bos et al., 2007). T-cell lymphoma invasion and metastasis-1 protein (Tiam1) is one of the best
studied GEFs, known to be activated through adhesion to the ECM (Minard et al., 2004). It is believed that Tiam1 is recruited to the membrane by interactions with PIP$_2$. PDGF also stimulates PKC$\alpha$-mediated phosphorylation of Tiam1 and relocalisation to the membrane (Buchanan et al., 2000). Once activated, Tiam1 specifically activates Rac1. However, as with many GEFs, Tiam1 exhibits activity towards other Rho GTPases including Cdc42 and to a lesser extent RhoA (Mertens et al., 2003). The link between PIP$_2$ and Tiam1 and recent work demonstrating association of Tiam1 PDZ domain to syndecan-1 (Shepherd et al., 2010) may be indicative of a direct role for syndecan-4 in activation of this GEF. Furthermore, Tiam1-mediated Rac1 activity has been implicated downstream of several integrin heterodimers and mediates cell spreading, migration and tumour cell invasion (Cruz-Monserrate and O'Connor, 2008; Hamelers et al., 2005).

1.4.2.2 p190RhoGAP: an example of a Rho GAP

The intrinsic ability of GTPases to hydrolyse GTP is accelerated by GAP regulatory proteins and results in termination of GTPase signalling events. As described previously, initial cell spreading induces a rapid and transient suppression of RhoA activity and this ECM-dependent suppression has been largely attributed to the action of p190RhoGAP. Src-mediated phosphorylation of p190RhoGAP, at tyrosine 1105, enables association of p120RasGAP, an interaction necessary for p190RhoGAP-dependent suppression of RhoA activity. Constitutive Src activity has been shown to decrease RhoA activity in a p190RhoGAP-dependent manner (Grande-García et al., 2007). Defects in p190RhoGAP-mediated regulation of RhoA activity effects cell spreading, FA formation and cell migration (Arthur and Burridge, 2001; Bass et al., 2008; Grande-García et al., 2007).

The mechanisms leading to the activation of p190RhoGAP are a prime example demonstrating synergy between $\alpha$5$\beta$1 integrin and syndecan-4 to regulate GTPase activity. Tyrosine phosphorylation of p190RhoGAP is stimulated by engagement of $\alpha$5$\beta$1 integrin independently of syndecan-4 stimulation. However, engagement of syndecan-4 and activation of PKC$\alpha$ is necessary for serine/threonine phosphorylation of p190RhoGAP, its redistribution to the membrane and inhibition of RhoA activity (Bass et al., 2008).

Rac1 is believed to play an important role in activation of p190RhoGAP through inhibition of low-molecular-weight protein tyrosine phosphatase (LMW-PTP), thereby increasing phosphorylation of its downstream target, p190RhoGAP (Nimnual et al., 2003). Cdc42 and
Rac1 activation of PAK1 has also been demonstrated to downregulate activity of the RhoA-specific GEF NET1 (Alberts et al., 2005). These data highlight the complex level of interplay between the different GTPases and their regulators.

1.4.2.3 Membrane localisation of Rho GTPases: role of RhoGDI

In addition to nucleotide exchange, association with the plasma membrane is crucial for Rho GTPase activity. FRET probes that report binding of Rac to downstream effector PAK1 have demonstrated specific binding of constitutively active Rac1 (V12 Rac) to PAK1 in lamellipodia only, despite the uniform cellular distribution of V12 Rac1. The binding of Rho GTPases to the plasma membrane is inhibited by guanine dissociation inhibitors (GDIs) (Fig. 1.6). GDIs mask the hydrophobic prenyl moiety of Rho GTPases from the aqueous cytoplasm and thus retain the GTPase within the cytosol and prevent association with the plasma membrane, GAPs and GEFs (Fig. 1.7) (Jaffe and Hall, 2005). The mechanisms leading to GDI-mediated dissociation and subsequent targeting of Rho GTPases to the membrane are not clearly understood; however, integrin-mediated anchorage of cells is believed to be a critical requirement, as detachment from ECM impairs Rac1-dependent PAK activation (Del Pozo et al., 2002). Furthermore, Rho coupling to effector mDia1 is stabilised by integrin adhesion and FAK signalling (Palazzo et al., 2004). It has been suggested that integrin-mediated adhesion promotes localised Rho GTPase activity at specific membrane compartments, termed lipid rafts or cholesterol-enriched membrane microdomains (CEMMs). CEMMs are highly ordered membrane domains, enriched for cholesterol, sphigolipids and glycosylphosphatidylinositol- (GPI) linked proteins, that serve to spatially compartmentalise transmembrane signal transduction. CEMMs are often concentrated within lamellipodia where active GTPases RhoA and Rac1 are localised (del Pozo and Schwartz, 2007; Guan, 2004). Rac1 has been shown to co-localise with the CEMM marker ganglioside GM1, and cell detachment causes the internalisation of CEMMs and loss of Rac effector activation (del Pozo et al., 2004). Together, these data implicate integrin adhesion in localised and targeted Rac1 activation, which is important for directed cell migration.

1.5 Modulation of adhesion dynamics in regulating cell migration

Adhesions continuously form, mature and disassemble at the leading edge of migrating cells in a process termed adhesion turnover. The integration and coordination of processes
that regulate FA dynamics at specific regions within the cell (i.e. at sites of protrusion and retraction) is critical for efficient cell migration and is highly dependent on tight regulation of Rho GTPase activity as discussed in section 1.4. FA disassembly and turnover have been shown to be regulated via several mechanisms, including microtubule targeting, protease cleavage and dynamic protein phosphorylation (Broussard et al., 2008; Webb et al., 2002).

Phosphorylation is a significant regulatory mechanism controlling a diverse range of biological processes (Blume-Jensen and Hunter, 2001; Koch et al., 1991; Manning et al., 2002a; Manning et al., 2002b). Protein kinases constitute 1.7% of the human genome and it is estimated that 30% of all proteins exist in phosphorylated forms (Manning et al., 2002b). It is clear that regulation of phosphorylation is essential for coordination of adhesion dynamics.

1.5.1 Tyrosine kinases in adhesion dynamics

Tyrosine kinases and Src homology-2 (SH2) (phosphotyrosine recognition sites) domain-containing proteins comprise a significant proportion of the integrin adhesome (Zaidel-Bar and Geiger, 2010; Zaidel-Bar et al., 2007a). Furthermore, phosphotyrosines are enriched at FAs (Maher et al., 1985) demonstrating the significance of tyrosine phosphorylation in control of cellular signalling. Indeed, reversible tyrosine phosphorylation is an important mechanism for FA formation and stability and reorganisation of the actin cytoskeleton. Treatment of cells with herbimycin A, an SFK inhibitor, or genistein, a broad spectrum tyrosine kinase inhibitor, prevents redistribution of a number of cytoskeletal proteins (paxillin, F-actin and filamin) in response to integrin clustering and ligation (Miyamoto et al., 1995). Although recruitment of other molecules, such as talin, α-actinin and vinculin, to ligand-bound integrins remains unaffected, it is believed that tyrosine kinase activity together with integrin-ligand interaction and cytoskeletal integrity is fundamental for recruitment of other FA components and propagation of signalling downstream of integrin-ECM interaction (Miyamoto et al., 1995). Here, the role of Src and FAK, two important FA tyrosine kinases, in regulating FA turnover will be discussed.

1.5.1.1 Src tyrosine kinase
Src is the prototypical member of a family of non-receptor tyrosine kinases and has a critical role in mediating signal transduction through interaction with multiple protein-protein complexes. Src is vital in many physiological processes such as growth, differentiation, adhesion, transcription and migration and was the first proto-oncogene to be identified in vertebrates (Kim et al., 2009; Mitra and Schlaepfer, 2006). Overexpression of Src and mutations that lead to hyperactivation of Src are associated with cancer metastasis. Evidence for Src-dependent adhesion disassembly has been obtained by observations made in Src-deficient cells (Src-/-). Src-/- cells display enhanced number and size of FAs and reduced rates of cell spreading (Fincham and Frame, 1998; Klinghoffer et al., 1999), an effect that is recapitulated by expressing kinase-defective mutants of Src and is indicative of an inability to turn over adhesions.

Src can associate with, and its activity is modulated by, a range of different receptors including integrins (Huveneers et al., 2007b) and VEGF receptor (Brunton et al., 2004; Streuli and Akhtar, 2009). Src activation requires a change in protein conformation achieved in three steps: 1) dephosphorylation of the C-terminal tyrosine residue disrupts intramolecular binding of this residue to SH2 domain, 2) further disruption of SH3 interaction with prolines in the linker region leads to an unfolded or “primed” Src conformation and 3) Src attains full enzymatic activity through transphosphorylation of primed Src at another tyrosine residue in Src kinase domain (Huveneers et al., 2007a) (Fig. 1.8). Src activation downstream of integrin adhesions has been proposed to occur via different mechanisms and in most instances requires the formation of a FAK/Src complex.
FAK is a non-receptor tyrosine kinase that binds directly to β integrin tails and is enriched in FAs. The link between FAK and Src was originally observed in v-Src transformed cells in which FAK was identified as a protein exhibiting high tyrosine phosphorylation levels (Kanner et al., 1990; Playford and Schaller, 2004). Similarly to Src-/- cells, FAK-deficient cells (FAK-/-) exhibit enhanced FA formation, an inability to turnover adhesions, decreased cell spreading and reduced rates of migration and constitutive RhoA activation (Ilic et al., 1995; Richardson et al., 1997). By contrast, FAK overexpression in CHO cells results in increased migration rates (Cary et al., 1996). Moreover, increased FAK expression in melanoma cell lines and breast tumours correlates with increased cell motility and invasiveness, respectively (Akasaka et al., 1995; Weiner et al., 1993). These data demonstrate a fundamental role for Src-FAK co-signalling in cell adhesion and motility.

**Figure 1.8. Src structure and kinase activation.** Src is composed of four Src homology domains: SH1 kinase domain; SH2 phosphotyrosine recognition and substrate-binding domain; SH3 proline rich target domain for protein binding; SH4 myristoylated N-terminal for membrane binding. Dephosphorylation of C-terminal Y530 (human Src) leads to Src unfolding into an open conformation. Phosphorylation of Y419 (human Src) in the kinase domain leads to fully activated Src and Src-mediated phosphorylation of substrate (adapted from Guarino (2010)).

### 1.5.1.2 FAK and activation of Src tyrosine kinase

FAK is a non-receptor tyrosine kinase that binds directly to β integrin tails and is enriched in FAs. The link between FAK and Src was originally observed in v-Src transformed cells in which FAK was identified as a protein exhibiting high tyrosine phosphorylation levels (Kanner et al., 1990; Playford and Schaller, 2004). Similarly to Src-/- cells, FAK-deficient cells (FAK-/-) exhibit enhanced FA formation, an inability to turnover adhesions, decreased cell spreading and reduced rates of migration and constitutive RhoA activation (Ilic et al., 1995; Richardson et al., 1997). By contrast, FAK overexpression in CHO cells results in increased migration rates (Cary et al., 1996). Moreover, increased FAK expression in melanoma cell lines and breast tumours correlates with increased cell motility and invasiveness, respectively (Akasaka et al., 1995; Weiner et al., 1993). These data demonstrate a fundamental role for Src-FAK co-signalling in cell adhesion and motility.
FAK is activated upon clustering of β integrin tails via a mechanism involving autophosphorylation of FAK tyrosine 397 (Schaller et al., 1994). Importantly, cells spread on the CDB of Fn exhibit limited FAK phosphorylation until stimulated with a heparin binding fragment of Fn and Syn-4/-/- fibroblasts have similar defects in FAK activation (Jeong et al., 2001; Wilcox-Adelman et al., 2002). Therefore, cooperative syndecan-4 and integrin signalling is central to regulation of FAK activity.

Adhesion-stimulated FAK phosphorylation creates a high-affinity binding site for the SH2 domain of Src leading to formation of a transient FAK/Src complex (Schlaepfer et al., 2004). Binding of Src to FAK is believed to promote conformational changes required for Src activation, thus promoting Src-induced phosphorylation. While FAK appears to be important for α5β1 integrin-mediated Src activation, α4β1 integrin stimulates Src independently of FAK by an unknown mechanism (Mitra and Schlaepfer, 2006).

An alternative mechanism regulating Src activation is as a direct consequence of Src binding to β3 integrin cytoplasmic tail (Arias-Salgado et al., 2003; Shattil, 2005). The requirement for SFKs has been demonstrated in a number of αVβ3-mediated processes such as cytoskeletal reorganization, bone remodelling by osteoclasts (Duong et al., 2000; Faccio et al., 2003; Shattil, 2005) and metastasis of melanoma cells (Chellaiah et al., 1996); however, direct Src-β3 interaction has only recently been identified (Arias-Salgado et al., 2003; Arias-Salgado et al., 2005). Src was shown to immunoprecipitate with αIIbβ3 and αvβ3 but not with β1 or β2 containing integrins in cell suspension. Src interaction with β3 was dependent on an intact Src SH3 domain and was independent of Src kinase activity or Src phosphorylation targets within the β3 tail. Moreover, clustering of β3 tails was sufficient to increase Src activation. The authors suggested that Src/β3 interaction acts to suppress Src activity in the absence of a matrix ligand. The ligation and clustering of β3, in response to the matrix, increases local concentration of Src and promotes Src autophosphorylation and activation (Arias-Salgado et al., 2003; Arias-Salgado et al., 2005). The role of αVβ3-mediated regulation of Src activity has not been fully elucidated. However, direct interaction between Src and β3 integrin may induce a faster response to extracellular stimuli, target Src activity to specific sites within the cell, or regulate other Src activation mechanisms.
1.5.1.3 Src/FAK signalling regulates adhesion turnover

Once activated, Src kinase can interact with and phosphorylate many binding partners including FAK (Mitra and Schlaepfer, 2006), p130Cas (Crk associated substrate) (Nasertorabi et al., 2006), paxillin (Playford and Schaller, 2004; Turner et al., 1990), cortactin (Wu and Parsons, 1993), dynamin (Ahn et al., 1999) and integrins (Playford and Schaller, 2004); each of these pathways can impact on Rho GTPase signalling and adhesion turnover (Playford and Schaller, 2004) (Fig. 1.9). Tyrosine phosphorylation of paxillin has been suggested to act as a switch regulating assembly and formation of cell-ECM adhesions (Zaidel-Bar et al., 2007a). Cortactin is an actin-binding protein that interacts with actin-nucleating protein Arp2/3 (Martinez-Quiles et al., 2004). In osteoclasts, Src-mediated phosphorylation of cortactin has been associated with podosome maturation and turnover (Luxenburg et al., 2006). Importantly, SFKs have been implicated in tyrosine phosphorylation of syndecan-1 and syndecan-4 cytoplasmic domains (see section 1.3.2), although direct interactions and the functional significance of this phosphorylation was not established (Ott and Rapraeger, 1998).

It is believed that Src-mediated signalling influences FA formation/turnover via transphosphorylation of FAK by Src, to facilitate docking of other protein targets. Src/FAK phosphorylation cascades include extracellular signal-regulated kinase (ERK) and myosin light chain kinase (MLCK). Phosphorylation of myosin light chains by MLCK reduces myosin-generated contractility at the leading edge (Webb et al., 2004). Consequently, FAK-mediated FA disassembly is triggered by a local decrease in myosin contractility. Src/FAK also mediates suppression of RhoA activity (Ren et al., 2000) through phosphorylation of FA-associated adaptor protein paxillin. Phosphorylation of specific tyrosine residues within paxillin provides docking sites for Crk and p120RasGAP. Crk is required for Rac1 activation, whereas p120RasGAP leads to RhoA inhibition through association with, and activation of, p190RhoGAP. Furthermore, Src-dependent phosphorylation of p190RhoGAP has been shown to regulate cells spreading, migration and cancer metastasis (Arthur and Burridge, 2001; Bass et al., 2008; Grande-García et al., 2007).

Recently, tyrosine phosphorylation of paxillin was proposed to regulate the type of cell-ECM adhesion formed. While non-phosphorylated paxillin was essential for fibrillar adhesion formation, phosphorylated paxillin regulated both the assembly and turnover of
nascent adhesions (Zaidel-Bar et al., 2007b). Thus, Src/FAK signalling acts as a coordinating centre, regulating adhesion turnover, actin cytoskeleton dynamics and cell shape.

Figure 1.9. Downstream targets of Src tyrosine kinase. Key players of integrin-mediated signalling include SFKs and FAK. Tyrosine phosphorylation of downstream targets such as p130Cas, ERK and paxillin impinge on activity of Rho GTPases and adhesion turnover (adapted from Playford et al (2004)).

1.5.2 Serine/threonine kinases in adhesion dynamics

In addition to tyrosine phosphorylation of FA components, serine/threonine phosphorylation is important in regulating FA dynamics. For example, phosphorylation of paxillin by PAK plays a role in FA targeting of paxillin and cell spreading. Paxillin phosphorylation has been shown to regulate adhesion assembly via recruitment of a Rac1/Cdc42 specific GEF (PAK-interactive exchange factor (PIX)) and effector PAK to the leading edge (Nayal et al., 2006). Thus, dual phosphorylation of paxillin by Src and PAK demonstrates dynamic cooperation between tyrosine and serine/threonine kinases to regulate FA dynamics by tilting the Rac1/RhoA balance.
Other serine/threonine kinases activated downstream of adhesion receptor engagement include members of the PKC family. PKCs are divided into distinct groups according to their phospholipid or calcium requirement for activation: conventional PKCs (α, β and γ) require phospholipids, DAG and phosphatidylserine (PS) and calcium; novel PKCs (δ, ε, η and θ) are calcium-independent but require phospholipids; atypical PKC (γ and λ) activity occurs independently of both DAG and calcium (Mackay and Twelves, 2007). PKCs are implicated in tumourogenesis, and inhibition of PKC activity reduces tumour invasion and cell motility (Brenner et al., 2008; Mackay and Twelves, 2007; Morse-Gaudio et al., 1998). However, the complex interplay between different PKC family members means that the mechanisms by which PKCs regulate adhesion dynamics is not clear. For example, stable expression of PKCα increases PKCβ but reduces PKCδ expression in cancer cell lines. Cell adhesion to ECM molecules, Fn and collagen, increases activation of several PKC isoforms (α, δ, ε and γ) in an integrin-dependent manner (Disatnik et al., 2002; Disatnik et al., 2004; Lam et al., 2001). In addition, growth factor signalling has also been shown to regulate PKC activity. In osteoclasts, enhancement of Fn fibrillogenesis and α5β1 integrin clustering following bFGF-stimulation is mediated by an increase in membrane localisation of PKCs (Tang et al., 2004). Furthermore, PKCδ has been shown to phosphorylate β2 integrins (Fagerholm et al., 2002) and syndecan-4 (Murakami et al., 2002). These data demonstrate a direct involvement for PKCs in adhesion receptor signalling as exemplified by PKCδ-mediated syndecan-4 phosphorylation. Syndecan phosphorylation by PKCδ is suggested to regulate syndecan-4-PKCα interaction and PKCα activation (Murakami et al., 2002). Importantly, syndecan-4-dependent PKCα activity is crucial for FA formation, regulation of GTPase activity and cell migration (Bass et al., 2007a). Thus, while the mechanisms regulating activity and function of PKCs in adhesion are complex, it is clear that the precise coordination of PKC-mediated phosphorylation is fundamental to regulating cell migration.
**1.5.3 Role of phosphatases in adhesion turnover**

As described above, post-translational modification of proteins by addition of a phosphate group can determine activation state (e.g. Src), localisation of proteins within a signalling network (e.g. paxillin) and modulate protein-protein interaction (e.g. FAK). Such phosphorylation events have a profound effect on cell-matrix interactions and cell migration. Dynamic regulation of adhesion signalling, however, requires reversible protein phosphorylation and this is mediated by the action of protein phosphatases. Unlike protein tyrosine kinases, information for the role of protein tyrosine phosphatases (PTPs) in regulation of cell migration and adhesion turnover is limited. However, it has been shown that both receptor and non-receptor PTPs influence the earliest phases of FA assembly (Petrone and Sap, 2000) and that strengthening of integrin–cytoskeleton linkages is blocked by a tyrosine phosphatase inhibitor, phenylarsine oxide (Choquet et al., 1997). These studies are indicative of PTPs involvement in force-dependent signal transduction. Moreover, PTP-dependent modulation of FAK and Src activity appears to be a key mechanism regulating adhesion dynamics. Inhibition of PTPs using vanadate causes an increase in cellular pools of phosphorylated FAK and paxillin (Retta et al., 1996). However, Src activation is decreased in breast tumour cells grown in the presence of the same inhibitor (Egan et al., 1999). These data are indicative of the complex nature of FA regulation by protein kinases and phosphatases.

Genetic deletion or expression of specific PTP mutants has provided more insight into the relationship between individual PTPs and their targets in regulation of integrin-mediated signals. Cells deficient in SH2-domain-containing PTP-2 (Shp2) display severe defects in spreading, hyperphosphorylation of FAK and have increased number of FAs (Manes et al., 1999; Saxton et al., 1997; Yu et al., 1998). Furthermore, increased RhoA activation has been reported in Shp2-/- cells, leading to enhanced stress fibre formation (Kodama et al., 2000; Schoenwaelder et al., 2000; Yu et al., 1998). Therefore, Shp2-dependent regulation of integrin function has been shown to occur via inhibition of FAK and subsequent RhoA-dependent maturation of FAs (von Wichert et al., 2003a). Further insights into Shp2 activity have highlighted a role for Shp2 in regulation of Src activity. Initially, Shp2 was thought to activate Src by dephosphorylation of Src Y530; however, later studies implicated Shp2 in regulation of another protein tyrosine kinase, Csk, that phosphorylates Src at carboxy-terminal tyrosine thereby inhibiting Src activation (Zhang et al., 2004).
Importantly, Shp2 regulation of Src activity can occur downstream of EGF receptor and integrin crosstalk (Ren et al., 2004).

Another phosphatase implicated in regulation of adhesion is PTPα, a receptor-like tyrosine phosphatase. Genetic inactivation of PTPα delays spreading on Fn and downregulates activation of SFKs (Pallen, 2003; Ponniah et al., 1999; Su et al., 1999). By contrast, PTPα overexpression has been shown to activate Src in vivo and induce cellular transformation (den Hertog et al., 1993; Zheng et al., 2000). Importantly PTPα association with αVβ3 integrins during initial cell spreading is required for activation of SFKs, the assembly of focal complexes and the strengthening of integrin–cytoskeleton bonds on both Fn and vitronectin (von Wichert et al., 2003b). Together, these data demonstrate the importance of dynamic phosphorylation and dephosphorylation in regulating adhesion dynamics and ultimately cell migration.

1.5.4 Microtubules and protease activity

In addition to tyrosine kinase and phosphatase signalling, microtubules and proteases have been implicated in adhesion turnover. Talin proteolysis by calpain (a Ca^{2+}-dependent protease) leads to dissociation of paxillin, zyxin and vinculin from adhesions. Microtubule targeting of FAs leads to adhesion disassembly at both the leading edge and retracting tails (Ezratty et al., 2005; Kaverina et al., 1999). It has been proposed that microtubules deliver relaxing factors to sites of cell-ECM contacts, thus facilitating adhesion turnover. However, the nature of this relaxing factor remains unknown. Arg kinase delivery to adhesions is mediated by microtubules and its activity can inhibit RhoA activation (Peacock et al., 2007). Others have suggested microtubule-induced FA disassembly by direct endocytosis of α5β1 integrin mediated by clathrin and clathrin adaptors. Clathrin accumulated at subsets of adhesions that were susceptible to microtubule-induced disassembly, and total internal reflection microscopy (TIRF) revealed the simultaneous disappearance of β1 integrin and clathrin during adhesion disassembly (Chao and Kunz, 2009; Ezratty et al., 2009; Ezratty et al., 2005). Whether endocytosis of α5β1 integrin occurs directly via a clathrin-dependent route and whether this microtubule-induced disassembly applies to other integrin heterodimers remains to be determined. Nonetheless, integrin endocytosis and trafficking is central to regulation of integrin-mediated intracellular signalling pathways during processes such as cell division and migration (Caswell et al., 2009) and is likely to have a profound effect on FA dynamics.
1.6 Receptor trafficking - regulating cell-ECM signalling

The endocytic pathway (internalisation of membrane components for degradation or redelivery to the membrane) is now recognised as an important mechanism to regulate receptor function, cell migration and tumour cell invasion (Caswell and Norman, 2008; Muller et al., 2009; Ramsay et al., 2007b). Indeed, intracellular trafficking is a key mechanism to control receptor signalling. For example, delayed trafficking of the VEGF receptor leads to a reduction in arterial morphogenesis. VEGF receptor signalling is mediated by phosphorylation of its cytoplasmic domain. Internalised VEGF receptor is exposed to protein phosphatases, such as PTPb1, and VEGF receptor internalisation is dependent on association with a GIPC1-myosin-VI complex (Lanahan et al., 2010). Moreover, there is increasing evidence that integrin trafficking is important in cell migration and disease progression (Caswell and Norman, 2006; Caswell et al., 2007; Muller et al., 2009; Ramsay et al., 2007a; Ramsay et al., 2007b).

The role for integrins in endocytosis of specific membrane microdomains and regulation of Rho GTPase activity, whereby loss of adhesive signals following cell detachment causes the internalisation of CEMMs and Rac1 inhibition (del Pozo et al., 2004), was briefly mentioned previously (section 1.4.2.3). Furthermore, the differential expression of integrin heterodimers on the cell surface has been demonstrated to dictate GTPase activity and cell morphology. α5β1 and not αVβ3 engagement leads to RhoA activation in response to Fn engagement, dynamic adhesions and random cell migration (Danen et al., 2002; Danen et al., 2005). Thus, the expression of specific heterodimers on the cell surface determines migratory characteristics of a cell; expression of α5β1 promotes adhesion turnover and random cell motility, whereas αVβ3 expression leads to stable adhesions and persistent cell migration. Integrin endocytosis and redelivery back to the membrane, termed integrin recycling, offers an efficient system to control heterodimer availability on the cell surface and is therefore an important mechanism controlling differential integrin engagement and signalling. Several key interactions with syndecan-4, namely PIP2, PKCα and dynamin, could potentially be involved in integrin recycling. Here, the mechanism outlining the endocytosis and recycling of integrin receptors will be discussed.
**1.6.1 Integrin endocytosis**

Integrin receptor endocytosis has been proposed to occur via two main pathways (see Table. 1.1; Caswell et al., 2009) involving either the formation of clathrin-coated vesicles (clathrin-dependent pathway) or via invagination of the plasma membrane into morphologically identifiable caveolae, rich in sphingolipids, cholesterol and caveolin proteins. In most cases, integrin heterodimers can be internalised via more than one mechanism, and the mode of endocytosis is regulated by association with different endocytic adaptor proteins within the same pathway. β1-containing integrins in combination with different α-integrin subunits can enter the cell via clathrin-dependent and caveolar routes. In some instances however the α-integrin subunit of the heterodimer remains unidentified. Understanding the nature of the different pathways of integrin endocytosis and the conditions under which each pathway is activated is critical to appreciate fully heterodimer specific signalling in cell migration.

<table>
<thead>
<tr>
<th>Clathrin-dependent</th>
<th>Clathrin-independent</th>
<th>Caveolin-dependent</th>
</tr>
</thead>
<tbody>
<tr>
<td>α5β1 (Numb) or (NPXY proteins) or (Nrp1*, GIPC1* and myosin VI)</td>
<td>α5β1 (Rab21 overexpression)</td>
<td>α5β1 (adaptors or associated proteins unknown)</td>
</tr>
<tr>
<td>αVβ5 (Clathrin)</td>
<td>Unknown mechanism</td>
<td>α?β1 (PKCα*)</td>
</tr>
<tr>
<td>αVβ3 (Numb)</td>
<td>α?β1 (JAMA*)</td>
<td>αVβ3 (adaptors or associated proteins unknown)</td>
</tr>
<tr>
<td>α?β1 (DAB2*/AP2*)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.1. Integrin endocytosis occurs via clathrin-dependent and clathrin-independent pathways.** Integrin endocytosis occurs mainly via two mechanisms: clathrin- and caveolin-dependent pathways. Adaptors or associated proteins required for endocytosis of each integrin are indicated in brackets. Table was adapted from Caswell et al. (2009) and highlights specific endocytic pathways of β1-, β3-, α5- and αV-containing integrins.

(*Nrp1: neuropilin-1; GIPC1: GAIP carboxy terminus-interacting protein, also known as synectin; DAB2: disabled homologue 2; AP2: activating protein 2; JAMA: junctional adhesion molecule A).
1.6.1.1 Clathrin-dependent endocytosis

Clathrin-mediated endocytosis is a well characterised endocytic pathway for uptake of nutrients, pathogens and internalisation of membrane receptors (Le Roy and Wrana, 2005). In addition to clathrin, various accessory/bridging proteins such as disabled-2 (Dab2) and adaptor protein-2 (AP2) are required for cargo recruitment into clathrin-coated vesicles, and the GTPase dynamin is responsible for vesicle scission.

The highly conserved NxxY motifs (in which x denotes any amino acid) are found in the cytoplasmic tail of β-integrins and these motifs are known to mediate clathrin-dependent endocytosis of other receptors such as low-density lipoprotein. Evidence of direct association between integrins and clathrin accessory molecules has been demonstrated for αVβ6 and a clathrin adaptor, HCLS1-associated protein X1 (HAX1) (Ramsay et al., 2007a) and occurs independently of NxxY motif. Numb, another clathrin adaptor contains a phosphotyrosine binding (PTB) domain and binds directly to β1 and β3 NPxY motif to facilitates α5β1 and αVβ3 endocytosis (Nishimura and Kaibuchi, 2007). RNA interference (RNAi)-mediated knockdown of Numb reduces integrin internalisation and impedes cell migration towards integrin substrates. Others have identified Dab2 and AP2 as necessary components in microtubule-initiated disassembly of FAs that coincides with loss of β1 integrin and clathrin from the cell membrane (Ezratty et al., 2009). The authors analysed integrins in their active conformation by using an antibody specifically recognising ligand-bound β1 integrins. Whether integrins need to be in an active state to be endocytosed is still under debate; however, the idea that alternative pathways could exist for internalisation of active and inactive conformations of integrins is intriguing.

Recently, neuropilin-1 (Nrp-1) was found to support endothelial cell adhesion to Fn by promoting fast vesicular transport of active α5β1 integrin in a complex with GIPC1 and myosin VI, thus linking α5β1 endocytosis to the actomyosin machinery (Valdembri et al., 2009). Intriguingly, GIPC1 is a binding partner for syndecan PDZ motifs, although a role for syndecans in Nrp-1 endocytosis of β1 integrin has not been explored. Nrp-1 is a transmembrane glycoprotein that mediates neuronal guidance (Shimizu et al., 2000), acts as a co-receptor for angiogenic factors (Olsson et al., 2006; Soker et al., 1998) and is expressed by numerous tumour cell types. Nrp-1-mediated endocytosis of active integrin was independent of its co-receptor function and, importantly, inactive integrin was internalised via an alternative Nrp-1-independent route (Valdembri et al., 2009).
Interestingly, clathrin-mediated endocytosis of α5β1 can be overcome by overexpression of a Rab5-related GTPase, Rab21 (Pellinen et al., 2008), presumably via caveolar-dependent mechanisms.

1.6.1.2 Clathrin-independent endocytosis

Disruption of NxxY motifs in β3-integrin tails or inhibition of clathrin-mediated endocytosis does not impede the rapid internalisation of αVβ3 or α5β1, respectively, indicating existence of alternative integrin endocytic pathways (Altankov and Grinnell, 1995; Ylanne et al., 1995). The mechanisms of clathrin-independent integrin endocytosis are not as clearly defined as those regulated by clathrin. Caveolins are integral membrane proteins, proposed to regulate clathrin-independent internalisation of receptors. Caveolins induce formation of caveolae structures at the plasma membrane that are highly sensitive to cholesterol depletion. Trafficking of cargo through caveolae is dependent on dynamin (Nabi and Le, 2003) and PKCα activity (Mineo et al., 1998; Smart et al., 1995; Upla et al., 2004).

Using fluorescence life-time imaging (FLIM), direct binding of GFP-PKCα to the cytoplasmic tail of active β1 integrin in the cell periphery has been demonstrated. Expression of PKCα enhanced cell-surface integrin expression (Ng et al., 1999). 12-O-tetradecanoylphorbol-13-acetate (TPA) stimulation of PKC activity led to increased association between PKCα and β1 and redistribution of active β1 integrins from cell surface to perinuclear recycling compartments (PNRCs). The authors showed that physical interaction of β1 integrin with PKCα, though the PKCα regulatory domain, is required for targeting active β1 integrin to the surface. However, full kinase activity promotes β1 integrin internalisation as evidenced by Fn uptake upon TPA stimulation. Introduction of dominant-negative dynamin I inhibited both the uptake of Fn and the PKCα-dependent cell migration towards β1 integrin ligands (Ng et al., 1999). In addition, recent work has shown αVβ3 and α5β1 localisation to caveolae in endothelial cells (Galvez et al., 2004) and myofibroblasts (Shi and Sottile, 2008). These data, together with caveolae dependence on PKCα activity and dynamin, imply that caveolae are involved in integrin endocytosis and that the regulation of PKCα is fundamental to the regulation of integrin trafficking.

In addition to being endocytosed, integrins have been implicated in the direct regulation of endosomal trafficking via caveolin-dependent pathways (Caswell et al., 2009). In adherent
cells, integrins inhibit endocytosis of CEMMs by retaining phosphorylated caveolin in FAs. In this way, integrin-ECM interaction maintains localised Rac1 activity at the cell surface (del Pozo et al., 2004; del Pozo et al., 2005).

1.6.2 Integrin recycling: the return journey

Following endocytosis, receptors are transported to Rab5-positive early endosomes located near the leading edge of migrating cells (Pierini et al., 2000; Rappoport and Simon, 2003). Rab proteins constitute the largest family of Ras-related small GTPase molecules that facilitate docking and fusion of transport vesicles (Zerial and McBride, 2001). Studies using GFP-tagged Rab4, Rab5 and Rab11 have revealed compartmentalisation of Rab members into specific endosomal membranes suggesting a unique function for each GTPase in recycling pathways (Sonnichsen et al., 2000). Rab5, found in early endosomes, is involved in fusion of the plasma membrane to early endosomes as well as consolidation of early endosome vesicles (Lanzetti et al., 2000; Rybin et al., 1996). In these Rab5 subcellular compartments, proteins destined for degradation are transported to late endosomes and lysosomes. However, most integrin receptors appear not to be degraded; instead, integrins are recycled back to the membrane in one of two spatially and temporally distinct mechanisms originally described for the transferrin receptor (Caswell and Norman, 2006; Morgan et al., 2009; Scita and Di Fiore, 2010; Widera et al., 2003).

Rab5 is also found in conjunction with Rab4 in particular subdomains of early endosomes (Sonnichsen et al., 2000). These recycling endosomes promote rapid delivery of receptors back to the membrane and as such this Rab4-dependent pathway is often referred to as short-loop recycling (Caswell and Norman, 2006). Alternatively, receptors can enter a long-loop pathway by relocating to Rab11-positive PNRC, clustered around the microtubule organising centre (Sonnichsen et al., 2000), prior to returning to the cell surface (Caswell and Norman, 2006).

1.6.2.1 Long-loop recycling

The diverse range of receptors that accumulate in Rab11-PNRC include a large number of integrin receptors (α5β1 integrin and αVβ3 (Roberts et al., 2001; Roberts et al., 2004; Skalski and Coppolino, 2005) and other β1 integrins (Ng et al., 1999; Powelka et al., 2004)), transferrin receptor (Hopkins et al., 1994; Ren et al., 1998), syndecan family
members and fibroblastic growth factor receptor (Ren et al., 1999; Tkachenko et al., 2004; Tkachenko and Simons, 2002; Zimmermann et al., 2005).

Selective Rab11-mediated transport of integrins has been demonstrated in recent years (Fig. 1.10). Inhibition of protein kinase B (PKB/Akt) activity or expression suppresses integrin α5β1 and αVβ3 recycling and leads to accumulation of these integrins within the cell. Importantly, the transport of transferrin receptor remains unaffected, suggesting a specific role for PKB/Akt in integrin trafficking (Roberts et al., 2004). Intriguingly, in the same study, PDGF stimulation of αVβ3 appeared to overcome the inhibition of PKB, suggesting an alternative route (short-loop) for αVβ3 recycling and not α5β1 in the presence of growth factor stimulation (Fig. 1.11). Indeed, the potential for αVβ3 to cycle independently of α5β1 may be critical in determining cell-surface expression and localised engagement of these integrins and therefore Rho GTPase signalling in cells.

**Figure 1.10. Long-loop integrin α5β1 and αvβ3 recycling in response to matrix engagement.** Long-loop recycling of integrins occurs through PNRC associated with Rab11 GTPase. Integrin recycling is separated from other receptors through specific pathways such as PKB/Akt phosphorylation and inhibition of glycogen-synthase kinase 3β (adapted from Morgan et al. (2009)).
As described earlier, expression of α5β1 and not αVβ3 supports RhoA reactivation during cell spreading (Danen et al., 2002) and suppresses αVβ3-dependent Rac-mediated lamellipodia formation (Danen et al., 2005).

A recent study has highlighted an intriguing mechanism whereby a functioning αVβ3 integrin suppresses recycling of α5β1 integrin. In ovarian carcinoma cell lines expressing both αVβ3 and α5β1, a Rab11 effector, Rab-coupling protein (RCP) also known as Rab11 family interacting protein-1 (Rab11FIP1), is associated with αVβ3 integrin. Disruption of αVβ3 function by incubation with αVβ3 inhibitors such as cilengitide (a cyclic RGD peptide) or the soluble αVβ3 ligand osteopontin (a matrix protein associated with range of malignancies (Rittling and Chambers, 2004)) releases β3-associated RCP. RCP then binds to and promotes recycling of α5β1 integrin to the membrane and fast random cell migration.

Further selectivity of integrin recycling has been supported by the novel isoform of the PKC family, PKC epsilon (nPKCe) (Ivaska et al., 2005; Ivaska et al., 2002). On activation, PKCe translocates to the cell membrane and co-localise with β1-integrin in membrane ruffles. Inhibition of PKC activity leads to accumulation of β1 in vesicles due to defects in recycling and not the initial endocytic steps (Ivaska et al., 2002).

Another GTPase, ADP-ribosylation factor 6 (Arf6), has been implicated in long-loop receptor recycling. Arf6 is present at Rab11-positive PNRC and regulates the recruitment of coat proteins during the formation of transport vesicles (D'Souza-Schorey and Chavrier, 2006). Arf6 is critical for trafficking of lipid rafts (Balasubramanian et al., 2007) and coordinates localised Rac1 activity that is dependent on integrin-adhesion signalling (Dunphy et al., 2006; Palamidessi et al., 2008; Radhakrishna et al., 1999). Consistent with these observations, Arf6 has been implicated in the stimulated recycling of β1 integrins and accumulates with β1 in membrane ruffles upon stimulation with serum, epidermal growth factor (EGF) or TPA (Powelka et al., 2004). Adhesion to Fn induces a wave of Arf6 activity indicating a role for Fn receptors in regulating its activity (Balasubramanian et al., 2007; Humphries et al., 2009). Furthermore, abrogating syndecan recycling by modulating the ability of syntenin, a syndecan binding partner, to engage PIP2 results in accumulation of β1 integrin and syndecan in Arf6-positive PNRC. However, the specific integrin heterodimer regulated by Arf6-dependent recycling pathways has yet to be identified. Differential integrin heterodimer recycling is an important mechanism whereby surface
expression of integrins is regulated, and this consequently determines integrin-heterodimer specific engagement and signalling to control cell behaviour and migration. It is now important to understand the mechanism that coordinate these pathways in a migrating cell, particularly, how microenvironmental cues are detected and interpreted to regulate integrin recycling.

1.6.2.2 Short-loop recycling

Under basal conditions, internalised α5β1 and αVβ3 enter PNRC and are recycled back to the membrane in a Rab11 and PKB/Akt-dependent manner. However, following stimulation with PDGF, αVβ3, but not α5β1, exits the early endosomes and is trafficked directly back to the membrane without entering Rab11-positive compartments and independently of Arf6 activity. This fast short-loop recycling requires Rab4 and the association of αVβ3 with a PKC-related kinase PKD1 (Woods et al., 2004) (Fig. 1.11). The perturbation of Rab4-mediated αVβ3 recycling via suppressing PKD1 expression leads to a marked increases in α5β1 recycling (White et al., 2007). Thus, it is suggested that αVβ3 antagonises α5β1 recycling and in this way contributes to directed cell migration under PDGF stimulation. The role of PDGF and other growth factors mentioned above highlights the fundamental role of other receptors in regulating integrin activity and therefore cell-ECM signalling.
Short-loop integrin αvβ3 recycling in response to PDGF. Short-loop recycling of αvβ3 integrin occurs through Rab4-positive recycling compartments and antagonises α5β1 long-loop recycling (adapted from Morgan et al. (2009)).
1.6.3 Syndecans in receptor trafficking

Syndecans work synergistically with integrin receptors to regulate cell adhesion, Rho GTPase activity and cell migration upon matrix engagement. It is now becoming clear that syndecans can also modulate pathways implicated in receptor trafficking. For example, syndecans have been suggested to mediate internalisation of lipoproteins (Fuki et al., 1997; Fuki et al., 2000), and recently syndecan-1 cytoplasmic domain has been shown to associate with early endosomal marker Rab5 (Hayashida et al., 2008). This association between Rab5 and syndecan-1 was suggested to trigger shedding of syndecan-1 ectodomain, therefore providing a mechanism to regulate syndecan signalling (Bass et al., 2009; Hayashida et al., 2008). The most compelling evidence of a role for syndecans in receptor trafficking was highlighted when syndecan receptor recycling was disrupted. In this study, mutations of the syndecan-PDZ-binding partner, syntenin, led to co-accumulation of syndecans, FGF receptor and β1 integrins in Rab11-positive PNRCs (Zimmermann et al., 2005). Moreover, syndecan recycling back to the membrane was dependent on Arf6, another GTPase involved in integrin recycling (Powelka et al., 2004; Zimmermann et al., 2005). These data suggest that perhaps recycling of syndecans and integrins occurs via common pathways. Moreover, other syndecan binding partners, GIPC1 (Syndecan-PDZ interaction) and PKCa (syndecan-4 specific interaction), have been implicated in endocytosis of integrin receptors, although a direct role for syndecans was not investigated (Ng et al., 1999; Valdembri et al., 2009).

Recent work in this laboratory has revealed a direct role for syndecan-4 in activation of Arf6 upon matrix engagement (Mark Morgan, unpublished data). Furthermore, Arf6 activation was dependent on the integrity of the Y180 residue in the C1 domain of syndecan-4. Importantly, modulation of syndecan-4-dependent Arf6 activity led to defects in adhesion dynamics, integrin heterodimer recycling and cell migration (Mark Morgan, unpublished data).

As discussed throughout this introduction, precise regulation of FA dynamics is critical for the orchestration of membrane protrusion, tail retraction and the generation of tensile forces required for cell movement (Petrie et al., 2009; Ridley et al., 2003). The ability of syndecan-4 to regulate FA formation, GTPase activity and integrin recycling, means that syndecan-4 is central in integrating these migratory processes.
Aims of PhD

Spatiotemporal control of adhesion dynamics is crucial for efficient cell migration. Rho GTPase activity, protein phosphorylation and integrin surface expression are key processes involved in dynamic adhesion turnover and signalling downstream of cell-ECM engagement. Coordination of all these processes is critical for cell migration and is mediated by synergistic signalling between extracellular receptors, integrins and syndecans (Morgan et al., 2007).

The focus of this project is to investigate the role of phosphorylation in modulating syndecan-4-dependent signalling. PKCδ-mediated phosphorylation of syndecan-4 has been identified but the biological functions of this phosphorylation event are unclear. SFKs have been implicated in syndecan phosphorylation; however, direct phosphorylation of syndecans by SFKs has not been proven and the tyrosine residues within syndecan cytoplasmic domain that could serve as SFK substrates have not been identified. Therefore, the aims of this project are:

1) To determine whether Src directly phosphorylates syndecan-4 and elucidate the regulatory mechanism of Src-mediated syndecan-4 phosphorylation.

2) To examine the functional role of PKCδ-mediated phosphorylation of syndecan-4 with specific focus on cross-talk between different phosphorylation sites within syndecan-4 cytoplasmic domain.

3) To examine the consequences of differential phosphorylation of syndecan-4 on processes associated with migration, e.g. GTPase activity, FA formation, regulation of integrin traffic and cell migration.
2. Materials and Methods

2.1 Materials

All reagents were obtained from Sigma-Aldrich (Poole, UK) unless otherwise stated.

2.1.1 General buffers

**Coomassie stain**: 10% (v/v) Methanol, 7% acetic acid (Thermo Fisher, Loughborough, UK), 250 µg/ml Coomassie Brilliant Blue

**Luria Bertani (LB) broth**: 1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl

**LB agar**: as above with the addition of 1.5% (w/v) bacto-agar

**PBS**: Dulbecco’s phosphate buffered saline without divalent cations.

**PBS-T**: PBS containing 0.1% (v/v) Tween-20.

**PBS**: phosphate buffered saline solution with divalent cations Mg$^{2+}$ and Ca$^{2+}$

**SDS sample buffer**: 2x buffer – 0.1 M Tris-HCl pH 6.8, 5% (w/v) glycerol, 2% Sodium dodecyl-sulfate (SDS), 0.01% Bromophenol Blue and 5% (v/v) 2-β-mercaptoethanol; 5x buffer – 0.2 M Tris-HCl pH 6.8, 30% (v/v) glycerol, 7% (w/v) SDS, 0.01% Bromophenol Blue, 10% (v/v) 2-β-mercaptoethanol

**TAE**: 50x buffer – 2 M Tris-acetate, 50 mM EDTA pH 8.0

**TBS**: 10 mM Tris-HCl pH 7.4, 150mM NaCl

**TBS-T**: TBS containing 0.1% (v/v) Tween-20

**Western blotting buffer**: 25 mM Tris (Thermo Fisher) pH 8, 192 mM glycine (Thermo Fisher) and 10% (v/v) Methanol (Thermo Fisher)

2.1.2 cDNA and vectors

- Thrombin-cleavable GST-syndecan-4 cytoplasmic domain in pGEX4T1 vector for expression in *E. coli* (GE Healthcare Life Sciences, Buckinghamshire, UK).
2.1.3 Bacterial strains

- DH5α maximum efficiency competent cells (Invitrogen, Paisley, UK): (F- φ80lacZΔM15 Δ(lacZYA-argF) U169, recA1, endA1, hsdR17 (r+, m-, k+), phoA, supE44, λ- thi-1, gyrA96, relA1) were used for general cloning processes.

- JM109 competent cells (Stratagene, La Jolla, USA): (e14–(McrA–) recA1 endA1 gyrA96 thi-1 hsdR17 (rK- mK+) supE44 relA1 Δ(lac-proAB) [F’ traD36 proAB lacIqZΔM15] were used for general protein expression.

- XL10-Gold ultracompetent cells (Stratagene): (Tet (mcrA)183 (mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F proAB lacI 9ZM15 Tn10 (Tet')] Amy Cam']) were used for transforming mutagenesis reactions.

2.1.4 Oligonucleotides

All synthetic oligonucleotide primers for sequencing and mutagenesis were obtained from MWG Biotech (Milton Keynes, UK) and are outlined in appendices 1A and 1B.

siRNA duplexes with ON TARGET modifications for enhanced specificity were obtained from Dharmacon (Thermo Fisher). siRNA targeting mouse PKCδ are detailed below:

PKCδ Oligo 5: CGGAAUAUACCAGGGAUUU
PKCδ Oligo 6: GCAAUUCCUGGACAAUUA
PKCδ Oligo 7: GUUCGACGCCAUCUAA
PKCδ Oligo 8: GCAAACAGUCUAUGCGUAG

siRNA targeting mouse Arf6 is detailed below:

Arf6 Oligo 10: CUGACAUUUUGACACGAAUA

ON-TARGETplus Non-Targeting Pool siRNA (Dharmacon) was used as negative control for all siRNA knockdown.
2.1.5 Extracellular matrix adhesion proteins

Bovine plasma Fn was purchased from Sigma. Recombinant polypeptides encompassing type III repeats 6-10 (50K) and 12-15 (H/0) of Fn were expressed as described previously (Makarem et al., 1994) and used to engage the Fn receptors, α5β1 integrin and syndecan-4, respectively. ECM proteins, 50K and Fn, were prepared in PBS+ and H/0 was prepared in serum free media.

2.1.6 Antibodies

**Immunofluorescence antibodies** were diluted in 3% bovine serum albumin (BSA) unless otherwise stated. Anti-vinculin mouse (clone hVIN-1, 1:400 dilution) and rabbit anti-Fn antibody (clone F3648, 1:800 dilution) were obtained from Sigma. All fluorophore-conjugated secondary antibodies (FITC/TRITC/Cy2/Cy5/Cy3) were purchased from Jackson ImunoResearch (Suffolk, UK) and Texas-red conjugated Phalloidin from Molecular Probes Invitrogen.

**Western blot antibodies** were diluted in 1x casein Sigma blocking buffer containing 0.1% (v/v) Tween-20. Primary antibodies are listed in table 2.1 below. Alexa Fluor 680- (Invitrogen) and IRDye 800- (LI-COR Biotechnology, Cambridge, UK) conjugated anti-mouse, anti-rabbit, anti-rat and anti-goat were diluted 1:5000 and used as secondary antibodies.
<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Clone</th>
<th>Host species</th>
<th>Supplier</th>
<th>Dilution</th>
<th>MW (KDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Src</td>
<td>----</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>56</td>
</tr>
<tr>
<td>Anti-Src(p416)</td>
<td>----</td>
<td>Rabbit</td>
<td>Cell signalling</td>
<td>1:1000</td>
<td>56</td>
</tr>
<tr>
<td>Anti-GST</td>
<td>Z-5 sc-459</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>1:200</td>
<td>26</td>
</tr>
<tr>
<td>Anti-Arf6</td>
<td>ARFAG</td>
<td>Mouse</td>
<td>abcam</td>
<td>1:1000</td>
<td>18</td>
</tr>
<tr>
<td>Anti-actin</td>
<td>AC40</td>
<td>Mouse</td>
<td>Sigma</td>
<td>1:2000</td>
<td>42</td>
</tr>
<tr>
<td>Anti-phosphotyrosine</td>
<td>PY20</td>
<td>Rabbit</td>
<td>Millipore</td>
<td>1:1000</td>
<td>N/A</td>
</tr>
<tr>
<td>Anti-phosphotyrosine</td>
<td>4G10</td>
<td>Mouse</td>
<td>Millipore</td>
<td>1:1000</td>
<td>N/A</td>
</tr>
<tr>
<td>Anti-SDCBP (syntenin)</td>
<td>----</td>
<td>Mouse</td>
<td>Sigma</td>
<td>1:1000</td>
<td>30</td>
</tr>
<tr>
<td>Anti-GIPC1</td>
<td>----</td>
<td>Goat</td>
<td>abcam</td>
<td>1:1000</td>
<td>38/25</td>
</tr>
<tr>
<td>Anti-PKCδ</td>
<td>14</td>
<td>Mouse</td>
<td>BD Biosciences</td>
<td>1:500</td>
<td>78</td>
</tr>
<tr>
<td>Anti-HSP70</td>
<td>JG1</td>
<td>Mouse</td>
<td>Affinity BioReagents</td>
<td>1:1000</td>
<td>70</td>
</tr>
<tr>
<td>Anti-Rac1</td>
<td>102</td>
<td>Mouse</td>
<td>BD Biosciences</td>
<td>1:1000</td>
<td>21</td>
</tr>
<tr>
<td>Anti-vinculin</td>
<td>hVIN-1</td>
<td>Mouse</td>
<td>Sigma</td>
<td>1:1000</td>
<td>116</td>
</tr>
<tr>
<td>Anti-PKCα</td>
<td>MC5</td>
<td>Mouse</td>
<td>abcam</td>
<td>1:1000</td>
<td>82</td>
</tr>
<tr>
<td>Anti-PKCα</td>
<td>3/PKC</td>
<td>Mouse</td>
<td>BD Biosciences</td>
<td>1:1000</td>
<td>82</td>
</tr>
</tbody>
</table>

Table 2.1. List of primary antibodies used for western blotting

1Cell Signaling Technologies (Massachusetts, USA), 2Santa Cruz Biotechnologies (Middlesex, UK) 3abcam (Cambridge, UK), 4Millipore (Hampshire, UK), 5BD Biosciences (Oxford, UK), 6Affinity BioReagenst (now supplied by Thermo Fisher)

For flow cytometry and fluorescence-activated cell sorting (FACS) primary antibodies were used at 10 µg/ml and included: mouse monoclonal raised against human syndecan-4 (clone 5G9) from Santa Cruz Biotechnologies; rat anti-mouse β1 (clone KM16) and anti-mouse αV integrin (clone RMV7); hamster anti-mouse β3 (2C9.G2) all from BD Biosciences and hamster anti-mouse α5 integrin (HMα5-1, Santa Cruz). Rat anti-mouse α4 hybridoma (clone PS2) was obtained from ECACC (Porton Down, UK)
FITC-conjugated rabbit anti-mouse IgG (9B), rabbit anti-rat IgG (17B) (Serotec, Oxford, UK, 1:200 dilution) and FITC-conjugated goat anti-armenian hamster IgG (Jackson ImunoResearch) were used as secondary antibodies in flow cytometry.

All integrin recycling antibodies were used at 5 µg/ml and are detailed in table 2.2.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Clone</th>
<th>Host species</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse α5</td>
<td>5H10-27</td>
<td>Rat</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Anti-mouse αV</td>
<td>RMV7</td>
<td>Rat</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Anti-mouse β3</td>
<td>2C9.G2</td>
<td>Hamster</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Anti-HA</td>
<td>12CA5</td>
<td>Mouse</td>
<td>Roche(^1)</td>
</tr>
<tr>
<td>Anti-human α5</td>
<td>VC5</td>
<td>Mouse</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Anti-human αV</td>
<td>L230</td>
<td>Mouse</td>
<td>Enzo Life Sciences(^2)</td>
</tr>
<tr>
<td>Ant- human β3</td>
<td>B3A</td>
<td>Mouse</td>
<td>Millipore</td>
</tr>
<tr>
<td>Anti-human syndecan-4</td>
<td>KY8.2</td>
<td>Mouse</td>
<td>BD Biosciences</td>
</tr>
</tbody>
</table>

Table 2.2. List of antibodies used in recycling assays

\(^1\)Roche (Sussex, UK), \(^2\)Enzo Life Sciences (Exeter, UK)
2.2 Mammalian cell culture

Immortalised Mouse embryonic fibroblasts (Im\(^+\) MEFs) were obtained from Dr T. Muramatsu, Nagoya University. Briefly, primary fibroblasts, isolated from 13.5-day-old wild-type and syndecan-4 homozygous mutant embryos were crossed with the Immorto mouse carrying the simian virus 40 large T antigen (SV40) under the control of the temperature-sensitive H-2Kb-tsA58 promoter as described previously (Bass et al., 2007b; Ishiguro et al., 2000; Jat et al., 1991). Immortalisation was achieved by approximately 10 passages at the permissive temperature for large T expression (33°C). Syndecan-4-null MEFs (Syn4\(-/-\)) expressing the human syndecan-4 wild-type (Syn4WT), syndecan-4 S179A (Syn4S179A), syndecan-4 S179D (Syn4S179D) and syndecan-4 ΔPDZ (Syn4ΔPDZ) mutant cDNAs were generated by retroviral transduction by Dr M. Morgan, University of Manchester (as described in Bass et al., 2007).

Im\(^+\) MEFs were maintained in Dulbecco’s Modified Eagles Medium (DMEM) (high glucose), supplemented with 10% (v/v) fetal calf serum (FCS) (Lonza, Slough, UK), 2 mM L-glutamine, and 20 U/ml IFN-\(\gamma\), at 33°C, 5% CO\(_2\).

Primary human foreskin fibroblasts (HFF) (a gift from Professor Karl Kadler, University of Manchester) were maintained in DMEM (high glucose) supplemented with 15% FCS and 2 mM glutamine at 37°C, 5% CO\(_2\).

2.2.1 Routine cell culture

Confluent fibroblasts were washed once with PBS\(^-\) and dissociated from culture surface using 1x trypsin-EDTA (5 min at 37°C). Cells were centrifuged at 375x g for 4 min, resuspended in media and split appropriately into culture flasks containing fresh media.

2.2.2 Preparation of cells for use in assays

Cells were passaged the day before an experiment and used when 60-80% confluent. Where appropriate, cells were treated with 25 µg/ml cyclohexamide for 2 hrs prior to assay, to prevent de novo synthesis of matrix proteins.
2.2.3 Freezing cells

Cells were dissociated from culture flasks and centrifuged at 375x g for 4 min. Cell pellets were resuspended in FCS containing 10% (v/v) DMSO, allowed to freeze at -80°C for 24Hrs and transferred to liquid nitrogen for long-term storage.

2.2.4 Transfection of mammalian cells with siRNA oligos

Cells were grown to approximately 90-95% confluence and RNAi-mediated knockdown of proteins carried out using Lipofectamine 2000 reagent (invitrogen) and specific siRNA oligonucleotides (see 2.1.4) in the presence of opti-MEM according to manufacturers’ instructions. Solutions A and B were prepared as described in the following table and incubated at room temperature (RT) for 5 min, combined and further incubated at RT for approximately 40 min. Medium was removed from cells and the transfection mixture was added in a 1:1 ratio with complete cell culture media.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Transfection size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T25 flask</td>
</tr>
<tr>
<td>A) Lipofectamin 2000</td>
<td>10 µl + 0.5 ml opti-MEM</td>
</tr>
<tr>
<td>B) siRNA oligo</td>
<td>8 µl of 20 µM stock + 0.5 ml opti-MEM</td>
</tr>
</tbody>
</table>

2.3 Flow cytometry and fluorescently activated cell sorting (FACS)

For standard flow cytometric analysis, MEFs were washed with PBS- and detached from culture surfaces with either 1x trypsin-EDTA or 1x cell dissociation buffer (Invitrogen) at 37°C. The dissociated cells were harvested by centrifugation at 375 g, 4 min. The cell pellet was resuspended in 0.1% (w/v) BSA/0.1% (w/v) sodium azide in PBS (0.1/0.1 solution). Cells were then incubated with primary antibody, diluted in 0.1/0.1 solution, at 4°C for 30 min. Following 2 washes with 0.1/0.1 solution and centrifugation steps, cells were incubated with appropriate species-specific FITC-conjugated secondary antibody at
4°C for 30 min. Cells were then washed three times with 0.1/0.1, resuspended in PBS and analysed on a Dako CYAN, FACS machine.

For FACS, levels of cell-surface expression were determined using the same method described above with the following exceptions: all steps were performed under sterile conditions and 0.1/0.1 solution was replaced with serum-free DMEM. Cells expressing the receptor of interest at the cell surface were sorted using BD Biosciences ARIA, FACS machine and the selected cells were placed in T25 flasks containing complete MEF media.

2.4 RNA extraction and reverse transcription (RT) PCR

2.4.1 RNA extraction

MEFs were grown on 6-well plates and lysed in 1 ml TRI reagent (a protein denaturant that inactivates RNases) at RT for 5 min and samples were homogenised by pipetting. Chloroform (200 µl) was added, samples shaken for 15 sec and incubated for 5 min at RT. RNA was separated from cell debris and DNA by centrifugation at 12,000x g at 4°C for 15 min. The upper aqueous layer, containing RNA, was transferred to a separate tube and RNA precipitated by addition of 500 µl of isopropanol, 15 sec shaking and centrifugation as before. The pellet was washed twice with 70% ethanol and air-dried for 5 min. RNA pellets were resuspended in RNase-free H2O and incubated at 55°C for 10 min.

2.4.2 RT-PCR

SuperScript® III One-Step RT-PCR System (Invitrogen) was used according to manufacturer’s instructions to assess levels of human syndecan-4 cDNA. Briefly, 1 µg of extracted cellular RNA was mixed with 25 µl of reaction buffer, 1 µl of sense (ATGGCCCCCGCCGTCT) and anti-sense (AGGCACCAAGGGATGGAC) (10 µM) primers against human syndecan-4 and 2 µl of SuperScript™ III RT/ Platinum® Taq/Taq HiFi Mix, made up to 50 µl with sterile RNase-free H2O and subjected to the following thermal cycles using a 2720 Thermal Cycler (Applied Biosystems, Warrington, UK):
Human syndecan-4 cDNA was visualised on DNA agarose gels as described below.

### 2.4.3 DNA agarose gel electrophoresis

Agarose (Invitrogen) was dissolved in 1x TAE buffer, containing SYBR® Safe DNA gel stain (1:10,000 dilution, (Invitrogen)), to achieve a 1% (w/v) agarose solution. Agarose was heated in a microwave and allowed to set in a GNA 100 eletrophoresis frame (Amersham Pharmacia, Little Chalfront, UK). DNA samples were mixed with 5x DNA loading dye (BioLine, London, UK) and loaded into the gel alongside Hyperladder I/IV marker (BioLine). Gels were run at 90V for 2 hrs and visualised at 460nm using a BioDoc-It Imaging System UV transilluminator (UVP, Cambridge, UK) fitted with a Visi-Blue convertor plate.

### 2.5 Protein analysis

#### 2.5.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

All samples were preheated in SDS-sample buffer and loaded on a precast, 4-12% Bis-Tris NuPAGE gel (Invitrogen) with 1x MES running buffer (Invitrogen). Precision Plus protein marker (Biorad, Hertfordshire, UK) was used as molecular weight standard. Samples were run at 200 V for 50 min.

#### 2.5.2 Western Blotting

Following SDS-PAGE separation, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell), using a Biorad Trans Blot Cell apparatus and western blotting
buffer at 30 V for 1-1.5 hrs. The nitrocellulose membrane was blocked in casein Sigma blocking buffer for 1 hour, with agitation at RT. The blocked membrane was incubated with appropriate primary antibody, diluted in 1x Sigma blocking buffer containing 0.1% Tween-20, overnight at 4°C with agitation. Membrane were then washed five times with PBS-T over a 25 min period and incubated with an appropriate Alexa 680 or IRDye 800 Fluorophore-conjugated secondary antibody, for 1 hr at RT with agitation. Following four washes with PBS-T, immunoblotted proteins were detected using the Odyssey infrared imaging system (LI-COR, Biosciences, UK Ltd (700 nm and 800 nm channels, 169 µm resolution)) and band intensity was determined by digital-densitometric analysis using Odyssey software version 2.1.

2.5.3 Standard coomassie Staining

Following SDS-PAGE separation, total protein levels were visualised by incubation of gel with coomassie or Instant Blue gel stains (Expedeon, Cambridgeshire, UK ) at RT for 1 hr. Gels were destained in either 10% methanol and 7% acetic acid for coomassie gels, or MilliQ H₂O for Instant Blue-stained gels. Proteins were detected using the Odyssey infrared imaging system (700 nm channel, 169 µm resolution, 0.5 mm focus offset) and band intensity was determined by digital-densitometric analysis using Odyssey software version 2.1.

2.5.4 Colloidal coomassie Staining

For mass spectrometric analysis, following SDS-PAGE separation of proteins, gels were stained with a colloidal coomassie gel stain (10% (w/v) ammonium sulphate, 3% (v/v) orpho-phosphoric acid, 20% (v/v) ethanol and 0.1% (w/v) coomassie G-250) made up in MilliQ H₂O) for 2 hrs at RT. Following several washes in MilliQ H₂O, gels were visualised with the Odyssey infrared imaging system and band intensity was determined by digital-densitometric analysis using Odyssey software version 2.1.

2.5.5 Phos-tag staining

Following SDS-PAGE separation, gels were stained with Phos-tag 540 (PerkinElmar) stain according to manufacturer’s instructions, to visualise phosphorylated proteins. Briefly, Phos-tag Dye Concentrate was diluted 1:100 in stain buffer and incubated with the gel for
1.5 hrs at RT on an orbital shaker. Gels were washed four times with the wash buffer provided over a 1.5 hr period. Wash buffer was then discarded and gels washed thrice with MilliQ H$_2$O and scanned on a Bio-Rad Phosphorimager allowing analysis using Quantity One - 4.5.0 Molecular Imager FX software (532 nm excitation filter, 555 nm long-pass emission filter and 100 µm scan resolution). The volume/count (pixel intensity x mm$^2$) of bands was determined.

2.6 Generation of recombinant peptides

2.6.1 Growth of bacterial cultures

*E. coli* strains were routinely grown in LB broth or on agar plates containing 100 µg/ml ampicillin for selection of cells transformed with vector. Cultures were grown overnight, at 37°C with shaking at 225 rpm, and plates were incubated overnight in a static 37°C oven.

2.6.2 Transformation of competent cells

Competent *E.coli* cells were thawed on ice and 50 µl transferred to pre-cooled 14 ml polypropylene tubes (Falcon 2059). DNA (50 ng) was added and incubated on ice for 30 min with gentle agitation. Cells were heat shocked for 40 seconds at 42°C, incubated on ice for a further 2 min and allowed to express the antibiotic resistant gene by incubation in LB broth at 37°C, 220 rpm for 1 hour. The cells were then plated on appropriate LB agar plates and incubated at 37°C overnight.

2.6.3 Isolation of Plasmid DNA

Plasmid DNA was extracted from 5 ml overnight bacterial cultures using QIAprep miniprep spin kit (Qiagen, Surrey, UK) according to manufacturer’s instructions and resuspended in 10 mM Tris, pH 8.5. DNA yield was determined using a NanaDrop ND 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA).

2.6.4 Site-directed mutagenesis

Mutagenesis was performed using QuickChange Lightning Site-directed Mutagenesis Kit (Stratagene) according to manufacturer’s instructions. Double-stranded DNA template (10
ng) was mixed with 125 ng of each sense and antisense mutagenic primers, 1 µl of dNTP mix, 1.5 µl of Quicksolution reagent, 5 µl of 10x reaction buffer and made up to 50 µl with MilliQ H₂O. QuickChange Lightning Enzyme (1 µl) was added to each reaction and the mixture subjected to the following cycle pattern using a 2720 Thermal Cycler:

\[
\begin{align*}
95^\circ C & \quad - \quad 2 \text{ min} \\
95^\circ C & \quad - \quad 20 \text{ sec} \\
64^\circ C & \quad - \quad 10 \text{ sec} \\
68^\circ C & \quad - \quad 30 \text{ sec/kb of plasmid length} \\
68^\circ C & \quad - \quad 5 \text{ min}
\end{align*}
\]

Non-mutated template DNA was digested by addition of 2 µl of \textit{Dpn I} reaction enzyme to the PCR product, at 37°C for 30 min. XL10-Gold ultracompetent cells were transformed with 2 µl of \textit{DpnI}-treated reaction mixture and the presence of desired mutation was confirmed by DNA sequencing.

\subsection*{2.6.5 DNA sequencing}
A mixture of DNA template (400 ng), forward and reverse sequencing primers (4 pmol) was prepared and made up to 10 µl with milliQ H₂O and sent for analysis by the University of Manchester, Faculty of Medicine, Sequencing Services.

\subsection*{2.6.6 Expression of GST-tagged recombinant proteins}
JM109 \textit{E.coli} cells expressing ampicillin-resistant DNA of interest were grown in 5 ml LB broth for 8 hrs at 37°C, 220 rpm.

Autoinduction medium (10% (w/v) tryptone, 5% (w/v) yeast extract) was prepared in MilliQ H₂O, autoclaved and supplemented with sterile filtered 25 mM Na₂HPO₄, 25 mM KH₂PO₄, 50 mM NH₄Cl, 5 mM Na₂SO₄, 2 mM MgSO₄, 2 mM CaCl₂, 0.5% (v/v) glycerol, 0.05% (w/v) glucose and 0.2% (w/v) lactose and 25 mg/ml ampicillin. 0.5 ml of JM109 8 hr culture was incubated in autoinduction medium overnight at 37°C, 220 rpm. Cells were harvested by centrifugation (Beckman centrifuge, JLA10.5 rotor, 5000 rpm), resuspended
in TBS containing complete protease inhibitor cocktail tablet (Roche) and lysed for 30 min at RT, with the addition of 1x bug buster (Novagen, Nottingham, UK), 0.05 mg/ml RNase and 0.05 mg/ml DNase. The soluble cell lysate was collected by centrifugation (Beckman centrifuge, JA25.5 rotor, 20,000 rpm) and incubated with pre-washed Glutathione Sepharose beads (GE Healthcare, Slough UK) for 1 hr at RT, to allow protein binding.

Following one wash in TBS-T and two washes in TBS the beads were resuspended in either:

1) PBS in a 1:1 ratio, ready for use (i.e. p21 activated kinase (GST-PAK), Golgi associated, gamma adaptin ear containing, Arf binding protein-3 (GST-GGA3) and GST-syndecan-4 cytoplasmic domain constructs)

2) Elution buffer (50 mM Tris pH 8.0, 0.5 M NaCl, 7.5 mM reduced glutathione, and 5 mM DTT) to elute GST-tagged proteins from the beads (i.e. GST-syndecan-4 cytoplasmic domain constructs).

3) TBS containing 2.5 mM CaCl₂, 20 mM thrombin, to cleave off the GST-tag from the protein following purification (i.e. Fn fragment H/0)

Protein levels were detected by SDS-PAGE and coomassie staining as described previously.

### 2.7 Src phosphorylation of recombinant syndecan-4 cytoplasmic domain

GST-tagged syndecan-4 cytoplasmic domains were purified from JM109 *E.coli*, as described earlier, and diluted in reaction buffer (25 mM MOPS pH 7.2, 125 mM β-glycerophosphate, 20 mM MgCl₂, 12.5 mM MnCl₂, 5 mM EGTA, 0.25 mM DTT). Samples were incubated with either 1.8 ng/µl or 5.4 ng/µl recombinant active Src kinase (Stressgen) and 50 µM ATP as phosphate donor, at 30°C. Kinase reactions were stopped after 15 min or 1 hr incubation, by addition of 3% phosphoric acid and 5x SDS sample buffer and proteins resolved by SDS-PAGE. For radio-labelling assays, the 50 µM ATP solution was spiked with 5 µCi of [γ-³²P ATP] (Perkin Elmer). Phosphorylation of GST-syndecan-4 constructs was analysed by three independent methods: immunoblotting, radio-labelling and mass spectrometry.
2.7.1 Immunoblotting:
Following SDS-PAGE, proteins were transferred to nitrocellulose membrane as previously described and blotted for phosphotyrosine using anti-phosphotyrosine antibody (pY20) and total GST-syndecan-4 anti-GST antibody. Odyssey infrared imaging system was used to quantify band intensity and phospho-tyrosine levels were normalised to total GST-syndecan-4 protein in each sample.

2.7.2 Radio-labelling
Following SDS-PAGE, total GST-syndecan-4 protein levels were determined by coomassie staining as previously described and quantified using Odyssey infrared imaging system. Gels were then dried and exposed to Fuji film BAS Cassette 2040 overnight and scanned on Bio-Rad Phosphorimager using Quantity One – 4.5.0 Molecular Imager FX software (radioisotope channel, 532nm excitation filter and 100 µm scan resolution). Volume (pixel intensity x mm²) was determined and analysed relative to total GST-syndecan-4 protein levels as determined by coomassie staining.

2.7.3 Mass Spectrometry
Following SDS-PAGE separation, phosphorylated proteins were visualised by Phos-Tag 540 gel stain, followed by colloidal coomassie for analysis of total protein levels. Gels were then sent to the Mass Spectrometry Facility (Faculty of Life Sciences, University of Manchester) where coomassie bands of interest were excised and subjected to in-gel trypsin digestion and analysis on Q-TRAP and LTQ-Velos mass spectrometers.

Briefly, samples underwent desalting, concentration on a pre-column and separation by reverse phase liquid chromatography, followed by instant ionisation and mass spectrometry analysis. The full protocol for band excision and in-gel trypsin digestion - provided by the Mass Spectrometry Facility (Faculty of Life Sciences, University of Manchester) - is detailed in appendix 2. A brief description of mass spectrometric analysis of samples is given below.

2.7.3.1 Q-TRAP mass spectrometer
Precursor ion scanning was performed in negative ion mode to identify phosphopeptides by loss of a −79 Da signature ion (PO3-). Precursor m/z values indicating evidence of
phosphorylation were subsequently targeted in positive ion mode to obtain product ion data. Spectra were then manually inspected and validated. Extracted ion chromatograms were obtained for specific precursor m/z values over retention time and area under the curve was calculated as a direct quantification of amount of peptide present.

2.7.3.2 LTQ-Velos mass spectrometer

Samples were analysed using tandem mass spectrometry, m/z and retention times recorded and spectra manually inspected and validated as with Q-TRAP mass spectrometer.

2.8 Recombinant syndecan-4 cytoplasmic domain pull-down

Confluent MEFs in T225 culture flasks were lysed in RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 5 mM EGTA, 1% (v/v) Triton X-100, 10 mM sodium fluoride, 5 mM sodium orthovanadate, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 0.5 mM) and centrifuged at 20,000 g, 4°C for 2 min. Clarified lysates were precleared with GST-coated beads at 4°C for 30 min and then incubated with GST-syndecan-4 cytoplasmic domain beads (see section 2.6.6) for 2 hrs at 4°C. Bound proteins were separated on SDS-PAGE gel and detected by western blotting.

2.9 GTPase activity assays

2.9.1 Arf6 effector pull-down assay:

15 cm culture dishes were coated with 10 µg/ml Fn (in PBS⁺) for 2 hrs at RT, washed three times with PBS⁻ and blocked with 10 mg/ml heat-denatured BSA for 30 min at RT. To determine steady-state Arf6 activity, MEF cells were spread on Fn-coated plates (5 x 10⁶ cells/plate) in the presence of 2% FCS-containing DMEM for 4 hrs at 37°C, 8% CO₂.

To determine the ability of cells to induce Arf6 activity in response to matrix-engagement, MEF cells were kept in suspension for 30 min, following trypsinisation. Arf6 activity was measured in suspension cells and following 1 hr spreading on Fn-coated plates. Cells were lysed in Arf6 lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1% (v/v) Triton X-100, 0.5% (v/v) sodium deoxycholate, 0.1% (w/v) SDS, 10% (v/v) glycerol, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 0.5 mM AEBSF) and centrifuged at 20,000x g
and 4°C for 2 min. Clarified lysates were incubated for 1 hr with GST-GGA3-bound sepharose beads (see section 2.6.6) at 4°C. Beads were washed three times in wash buffer (50 mM Tris pH 7.5, 100 mM NaCl, 2 mM MgCl₂, 1% (v/v) IGEPAL and 10% (v/v) glycerol) harvested at 4000x g, 4°C for 1 min. Bead samples were boiled and eluted in 2x SDS sample buffer. Levels of bound active Arf6 were detected by western blotting with anti-Arf6 monoclonal antibody. Total cell lysates were used to determine total Arf6 levels.

2.9.2 Rac1 effector pull-down assay

10 cm culture dishes were coated with 10 µg/ml 50K for 2 hrs, washed three times with PBS and blocked with 10 mg/ml heat-denatured BSA for 30 min at RT.

MEFs pretreated with cyclohexamide (described in section 2.2.2) were plated at a density of 7 x 10⁵ cells/plate and allowed to attach and spread for 2 hrs at 37°C, 8% CO₂. Where appropriate, cells were stimulated with 10 µg/ml H/0 for 10, 30, 60, 90 and 120 min. Cells were transferred to ice, washed with ice-cold PBS and lysed in HIPPIY buffer (20 mM HEPES pH 7.4, 1% (v/v) IGEPAL, 0.5% (w/v) sodium-deoxycholate, 140 mM NaCl, 10 mM sodium vanadate, 4 mM EGTA, 4 mM EDTA, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 0.5 mM AEBSF). Lysates were clarified by centrifugation at 20,000x g and 4°C for 2 min. The pellet was discarded and the clarified lysate was added to GST-PAK-coated G-sepharose beads (see section 2.6.6), retaining 20 µl of total cell lysate as a loading control. Active Rac1 was precipitated by incubating the lysate with PAK beads for 1 hr, at 4°C. The beads were washed three times with HIPPIY buffer and harvested at 4000x g, 4°C for 1 min. Bead samples were boiled and eluted in 2x SDS sample buffer. Levels of bound active Rac1 were detected by western blotting with anti-Rac1 antibody (BD transduction). Total cell lysates were used to determine total Rac1/Hsp70 levels.

2.9.3 G-LISA RhoA activation assay

RhoA activation was assessed using G-LISA™ RhoA Activation Assay kit (Cytoskeleton, Inc., tebu-bio, Peterborough, UK) according to the manufacturer’s instructions.

Briefly, to monitor steady-state RhoA activity, MEFs were spread on 35 mm dishes - precoated with 10 µg/ml 50K - at 37°C, 8% CO₂, for 3 hrs. Cells were lysed on ice with 50 µl of ice-cold lysis buffer and centrifuged at 20,000x g and 4°C for 2 min. Clarified lysate (30 µl) was mixed with equal volume of ice-cold binding buffer and 50 µl added to a well
of the microplate containing the Rho GTP-binding protein and incubated at 4°C on an
orbital microplate shaker (400 rpm) for 30 min. The wells were washed twice with wash
buffer and incubated with Antigen Presenting Buffer for 2 min. Following three washes,
anti-RhoA primary antibody was added for 45 min at RT on an orbital microplate shaker.
The primary antibody was removed; wells washed three times with wash buffer and
incubated with secondary antibody for 45 min at RT on an orbital shaker. Following one
final wash, HRP detection reagent was added for 15 min at 37°C, followed by stop buffer.
The levels of RhoA activity were measured at 490 nm absorbance wavelength using a
microplate spectrophotometer. Lysis buffer only was used as assay blank and cells treated
with C3 toxin (Rho inhibitor) were used as negative control. Total protein levels were
determined from the remaining cell lysates using protein assay reagents provided in the kit.

2.10 Integrin recycling

2.10.1 Preparation of ELISA plates

Costar EIA/RIA 96-well plates (Life Technologies, Inc.) were coated overnight at 4°C with
5 µg/ml anti-integrin, anti-HA or anti-syndecan-4 antibodies (detailed in section 2.1.6 and
prepared in 50 mM Na₂CO₃ pH 9.6) and blocked with 5% BSA in PBS-T. ELISA plates
were washed twice with PBS-T prior to addition of cell lysates.

2.10.2 Cell-surface labelling

Cells were grown on 10 cm culture plates for 48 hrs until 80% confluent, washed twice
with cold PBS and surface labelled with 0.13 mg/ml sulfo-NHS-SS-Biotin (Thermo
Fisher) at 4°C for 30 min, with gentle agitation. Excess biotin was removed with 2 washes
of chilled Krebs buffer (25 mM NaHCO₃, 4.8 mM KCl, 1.2 mM KH₂PO₄ 118 mM NaCl,
1.2 mM MgSO₄, 11 mM Glucose, 1.5 mM CaCl₂.2H₂O and 1.5 mM sodium pyruvate).

2.10.3 Internalisation of receptors

Internalisation of biotinylated cell-surface receptors was triggered with addition of serum-
free media at 37°C for 30 min. Following internalisation, plates were washed once with
Krebs buffer and once with surface reduction buffer (50 mM Tris pH 7.5, 1 M NaCl, pH
8.6). Cells were then treated with 3.75 mg/ml 2-Mercaptoethane sulfonate sodium (MesNa)
in surface reduction buffer (4 ml/plate) at 4°C for 30 min, to remove any remaining biotinyl label from the cell surface.

To allow analysis of total levels of receptor internalisation, cells were washed twice with PBS and lysed in buffer containing 75 mM Tris pH 7.5, 200 mM NaCl, 15 mM NaF, 1.5 mM Na3VO4, 7.5 mM EDTA, 7.5 mM EGTA, 1.5% Triton X-100, 0.75% Igepal CA-630, 50 µg/ml leupeptin, 50 µg/ml aprotinin and 1 mM AEBSF. Lysates were clarified by centrifugation at 20,000x g, 4°C for 10 min and 50 µl of lysate added to ELISA plates precoated with appropriate antibodies and incubated overnight at 4°C. Additional plates, for analysis of rates of receptor recycling, were not lysed immediately after the internalisation period but treated as described in section 2.10.4.

N.B. Several plates were excluded from the internalisation step and were either kept in Krebs buffer at 4°C until lysis - to measure total biotinylated cell-surface receptor or subjected to MesNA treatment to remove biotinyl label from the cell surface and used as negative control for the ELISA assay.

2.10.4 Receptor recycling

Cells, surface-labelled with biotin and allowed to internalise their receptors as described above, were washed twice with Krebs buffer and recycling of internalised receptors was triggered by addition of pre-warmed media. Delivery of receptors back to the membrane was monitored in triplicate at 7 min, 15 min, 30 min and 45 min post stimulation. At appropriate times, plates were removed from the incubator, placed on ice and washed with ice-cold Krebs buffer, treated with MesNa as before and the reaction quenched by addition of 3.4 mg/ml iodacetamide for 10 min. Cells were lysed as described in 2.10.3, centrifuged and 50 µl of clarified lysate added to antibody-coated ELISA plates and incubated overnight at 4°C.

2.10.5 ELISA development

ELISA plates incubated with lysates were washed four times with PBS-T and incubated with 50 µl of extravidin peroxidise (1:500 dilution in PBS-T, 1% BSA) for 1 hr at 4°C. Following extensive washing with PBS-T, plates were incubated with detection reagent ABTS buffer (2 mM ABTS, 25 mM H2O2, 0.1 M NaAcetate, 0.05 M NaH2PO4 pH 5) and absorbance recorded at 405 nm on a BioTek Power Wave plate reader.
2.11 PKCα activity assay

MEFs, pre-treated with cyclohexamide (described in section 2.2.2), were plated on 10 µg/ml 50K- or Fn-coated 150 mm dishes, at a final density of 10⁷ cells/dish and allowed to grow for 2 hrs. Cells were then stimulated with either H/0, 12-O-tetradecanoylphorbol-13-acetate (TPA) (200 nM) or bisindolylmaleimide (BIM) (200 nM). Cells were lysed in Tris-based lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% (v/v) IGEPAL, 0.25% (w/v) sodium-deoxycholate, 2 mM EGTA, 10 mM sodium fluoride, 5 mM sodium orthovanadate, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 0.5 mM (AEBSF) containing Phosphotase Inhibitor Cocktail 1 (1:1000 dilution) and okadaic acid (30 nM), centrifuged at 20,000x g for 2 min and clarified lysate precleared with recombinant protein-G sepharose beads (Invitrogen) for 30 min. Precleared lysate was added to protein-G beads precoated with 1 µg of anti-PKCα monoclonal antibody MC5, and incubated at 4°C for 2 hrs. Beads were washed three times with 25 mM Hepes and resuspended in 25 mM Hepes, 15 mM MgCl₂, 0.75 mM CaCl₂, 1x lipid activator (Millipore), 0.4 mg/ml myelin basic protein (MBP) and 0.6 mM ATP spiked with 0.5 µCi of [γ-³³P ATP] and incubated at 30°C for 30 min. Reactions were stopped by addition of 3% phosphoric acid and 5x SDS sample buffer and proteins resolved by SDS-PAGE. Following fixation and coommassie staining, gels were dried and exposed to Fujifilm BAS Cassette 2040 overnight, scanned on Bio-Rad Phosphorimager and analysed using Quantity One - 4.5.0 Molecular Imager FX software (radioisotope channel, 532nm excitation filter and 100 µm scan resolution). Volume (pixel intensity x mm²) was determined and analysed relative to total PKCα/actin levels as determined by western blotting of total cell lysates.

2.12. Generation of cell-derived matrices

2.12.1 Cell preparation

HFF cells were plated at a density of 5 x 10⁴ cells/ml on 24-well culture plates, for migration assays, or glass bottom poly-D-lysine coated MatTek dishes (MatTek Corporation, Massachusetts, USA), for immunofluorescence. Cells were maintained at 37°C, 5% CO₂ and grown to confluence. Cells were kept in culture for a further 8 days and media replaced every 2 day with HFF media supplemented with 50 µg/ml ascorbic acid in order to enhance collagen deposition and to strengthen the matrix produced by the cells.
2.12.2 Denudation of HFF cells

Following 8 days of growth, HFF cells were washed with PBS− and incubated with a pre-warmed solution of extraction buffer (20 mM ammonium hydroxide and 0.5% (v/v) Triton X-100) until no intact cells were observed (3-5 min). Extraction buffer was gently and partially aspirated to avoid removal of the HFF-derived matrix. Matrices were washed in PBS+ and incubated with 10 µg/ml DNase (Roche) at 37°C, 5% CO₂ for 30 min. Following three washes with PBS+, cell-derived matrices (CDMs) were stored in 1% (v/v) penicillin/streptomycine and 1% (v/v) fungizone at 4°C until needed.

2.12.3 Preparation of cell-derived matrices for cell plating

CDMs were washed 2x with PBS− and blocked with serum containing media at 37°C for 30 min. MEFs were plated on CDMs at a density of 5 x 10³ cells/ml and allowed to integrate into the matrix for 4 hrs prior to immunofluorescence or analysis of migration.

2.13 Cell migration

Cell migration was assessed on 10 µg/ml Fn (2-dimensional (2D) migration) and on fibrillar CDMs (3D migration). Cells were plated at a density of 5x10⁵ cells/ml and time-lapse brightfield images were acquired on an AS MDW live-cell imaging system (Leica) using a 5x/NA0.15 Plan Fluotar objective and 1.5x magnification. Cells were maintained at 37°C and 5% CO₂ and images were collected every 10 min over 18 hrs using a Coolsnap HQ camera (Photometrics). Cell migration was tracked manually using the MTrackJ plugin for ImageJ. Migration speed and persistence of migration (total distance divided by linear distance) were calculated and migration tracks were plotted using the Chemotaxis Tool and Manual Tracking ImageJ plugins.

Average number of membrane protrusions was measured by manually counting protrusions for each cell at three separate time frames within migration movies.

2.14 Immunofluorescence

Cells plated on MatTek dishes or glass coverslips were fixed with 4% paraformaldehyde (8 min, RT), permeabilised with PBS− containing 0.5% (v/v) Triton X-100 (4 min, RT) and blocked in 3% (w/v) BSA overnight. Cells were incubated with primary antibodies for 1 hr
at RT, washed three times with PBS− and incubated with appropriate secondary antibodies (1 hr at RT). Cells were then washed twice with PBS− and once with MilliQH₂O. Cells on MatTek dishes were kept in PBS− whereas glass coverslips were mounted on slides using ProlongGold anti-fade reagent (Invitrogen). Immunofluorescent images were acquired on an Olympus inverted microscope (IX71) under the control of DeltaVisionRT software (Applied Precision) using 60x/NA1.40 Plan Apo or 40x/NA0.85 Uplan Apo objectives and CoolSnap HQ (Photometrics) camera. The same microscope settings were used to acquire all images within each experiment.

2.15. Focal adhesion formation

2.15.1 Preparation of coverslips and ligand coating

Glass coverslips (13 mm diameter) were derivatised with 1mM sulfo-3-maleimidobenzoyl-N-hydroxysulphosuccinimide ester (Sulfo-MBS) (Thermo Fisher) for approximately 30 min. Sulfo-MBS-coated coverslips were washed three times with PBS+ and subsequently coated with 10 µg/ml 50K (in PBS+) at 4°C, overnight. Coverslips were then washed three times with PBS- and blocked with 10 mg/ml sterile-filtered heat denatured BSA.

2.15.2 Cell preparation

To test the integrin- and syndecan-dependence during FA formation, cyclohexamide treated MEFs (see 2.2.2) were plated on 10 µg/ml 50K-coated coverslips at a density of 2x10⁴ cells/coverslip for 2 hr at 37°C and 8% CO₂. Spread cells were then stimulated with media containing 10 µg/ml H/0 for 30 min, fixed with 4% (w/v) paraformaldehyde and stained (as described in section 2.14) with an anti-vinculin antibody (hVin1) and Texas-red phalloidin.

Focal adhesion area was quantified using ImageJ software. Briefly, background was subtracted from vinculin-stained images using a rolling ball of size 15. The threshold was set to select all focal adhesions within the cell with minimum background. The regions selected by the applied threshold were then used to measure focal adhesion area for each cell.
2.16. Statistical analysis

All error bars represent standard error of the mean (SEM). For statistical analyses, one-way analysis of variance (one-way ANOVA) was applied to compare means of more than two samples within the same experiment. Student’s $t$-test was used, where appropriate, to directly compare between two samples.
3. Results

3.1 Syndecan-4 cytoplasmic domain as a target for protein kinases: Tyrosine phosphorylation by Src

Aims and background:

Cell adhesion to ECM is vital for cell survival, proliferation and cell migration. FAs function to integrate the ECM with the actin cytoskeleton, allowing the application of tractional forces and the transmission of dynamic intracellular signals to modulate cell morphology, fate and migration (Bershadsky et al., 2003; Chen et al., 2004a; Geiger et al., 2001; Ridley et al., 2003; Sastry and Burridge, 2000). Syndecan-4 is a transmembrane HSPG that interacts with a range of ECM molecules to regulate cell behaviour. Engagement of syndecan-4 by Fn regulates Rho family GTPase activity in order to promote dynamic FA formation, reorganisation of the actin cytoskeleton and cell migration (Bass et al., 2008; Bass et al., 2007b). Understanding the mechanism by which syndecan-4-ECM engagement triggers downstream signalling is therefore central to understanding the process of cell migration.

Phosphorylation is a key post-translational modification, occurring in both prokaryotic and eukaryotic organisms, which plays a regulatory role in almost all signalling networks. Phosphorylation of proteins serves multiple roles including enzyme activation/inhibition, exposure of protein recognition motifs to promote protein-protein interactions and generation of ubiquitination/degradation signals (Blume-Jensen and Hunter, 2001; Koch et al., 1991; Manning et al., 2002a). Thus, phosphorylation is pivotal to the regulation of intracellular signalling and the role and regulatory mechanisms of syndecan-4 phosphorylation were therefore investigated.

The cytoplasmic domain of syndecan-4 contains three tyrosines residues (Y180, Y188 and Y197), one threonine (T193) and one serine residue (S179) that are potential targets of protein kinases (Fig. 3.1). Phosphorylation of S179 by PKCδ has been well characterised (Murakami et al., 2002) although the biological consequences of syndecan-4 phosphorylation by PKCδ remain unclear. Surprisingly, very little is known about the regulatory mechanisms and consequences of syndecan-4 tyrosine phosphorylation. However, endogenous tyrosine phosphorylation of syndecans has been demonstrated in
fibroblasts and is inhibited by herbimycin and staurosporine suggesting involvement of Src family kinases (Ott et al., 1998). Recent work in the Humphries laboratory has suggested a role for Src, a non-receptor tyrosine kinase, in modulating syndecan-4 function. Src was isolated from cell lysates using GST-tagged syndecan-4 cytoplasmic domain, suggesting an interaction between Src and syndecan-4 (Fig. 3.1 and Mark Morgan, unpublished data). Moreover, introduction of constitutively active Src in cells expressing a chimeric receptor, comprising IL2Rα extracellular domain and syndecan-4 transmembrane and cytoplasmic domain, increased tyrosine phosphorylation of the chimeric receptor, indicating a role for Src in tyrosine phosphorylation of syndecan-4 (Mark Morgan, unpublished data).

SFKs are central mediators of adhesion signalling, cell migration and cell survival and aberrant Src activation promotes tumour metastasis (Kim et al., 2009). The syndecan-4 receptor has been implicated in wound healing and angiogenesis and is over-expressed in many tumours (Beauvais and Rapraeger, 2004; Echtermeyer et al., 2001). Therefore, exploring the potential role of Src in regulating syndecan-4 signalling may be vital for the discovery of new therapeutic targets for many diseases. In this chapter, the aim was to determine whether Src directly phosphorylated syndecan-4, and if so, to identify the specific tyrosine target(s) for Src in the cytoplasmic domain of syndecan-4. Finally, potential mechanisms regulating Src-mediated phosphorylation of syndecan-4 were investigated.

### 3.2 Tyrosine phosphorylation of syndecan-4 by Src: in vitro kinase assay

It has been shown previously that c-Src promotes phosphorylation of syndecan-4 cytoplasmic domain in cells. However, it was unclear whether Src directly phosphorylated syndecan-4. Therefore, GST-fusion peptides of syndecan-4 cytoplasmic domain were generated and incubated with recombinant active Src kinase in an in vitro kinase assay.

Tyrosine phosphorylation of syndecan-4 cytoplasmic domain (GST-Syn4WT) by Src was assessed by two independent techniques – radio-labelling with $^{33}$P-[$\gamma$-ATP] as a phosphate donor (Fig. 3.2A) and immunoblotting with an anti-phosphotyrosine antibody (Fig. 3.2B). In the absence of Src, GST-Syn4WT was not phosphorylated; however, incubation with Src promoted tyrosine phosphorylation of syndecan-4. Moreover, Src phosphorylation of GST protein was not observed (Fig. 3.2A). Detection by immunoblotting demonstrated that Src specifically promoted tyrosine phosphorylation of syndecan-4 (Fig. 3.2B). Together,
these data demonstrated that syndecan-4 cytoplasmic domain (GST-Syn4WT) is directly phosphorylated by Src.

As syndecan-4 is directly phosphorylated by Src, it was important next to identify specifically which tyrosine residues were Src phosphorylation targets. In order to address this question it was necessary to use a mutagenesis approach whereby tyrosine residues were ablated and Src-mediated phosphorylation assessed.
Figure 3.1 Src tyrosine kinase isolated in pull-down with GST-Syn4WT cytoplasmic domain.

Syndecan-4 cytoplasmic domain was fused to GST protein (A). The PKCδ-phosphorylation site, S179 (red), and the three tyrosine residues of interest Y180 (green), Y188 (blue) and Y197 (purple) are highlighted.

Active Src (Src<sup>p</sup>Y416) was isolated from MEF lysates in pull-down with GST-tagged syndecan-4 cytoplasmic domain (GST-Syn4WT) but not GST-alone (left hand side) (B). In the absence of lysate no Src band was detected (right hand side). Dashed line in B denotes exclusion of additional lanes in the same blot.
Figure 3.2. Src tyrosine kinase phosphorylates syndecan-4 cytoplasmic domain. GST-tagged syndecan-4 cytoplasmic domain (GST-Syn4WT) (A) was incubated with 1.8 ng/µl recombinant active Src kinase at 30°C for 15 min. Phosphorylation of syndecan-4 was detected by radio-labelling using $^{33}$p-ATP as phosphate donor (B) and by immunoblotting (IB) using a phospho-tyrosine specific antibody (pY20) (C).
3.3 Identification of tyrosine phosphorylation sites within syndecan-4 cytoplasmic domain

Comparison of the syndecan-4 cytoplasmic domain with the optimal peptide sequence for Src-dependent phosphorylation (EEEIYGEFD (Fig. 3.3A)) (Songyang et al., 1995) revealed high levels of homology with the sequence surrounding the Y180 residue in the syndecan-4 cytoplasmic domain. Furthermore, NetPhorest, an online atlas of consensus sequence motifs covering 179 kinases (Miller et al., 2008), highlighted Src-family kinases as one of the main candidates capable of phosphorylating syndecan-4 at Y180. The posterior probability of Src phosphorylating Y180 was calculated as 0.085 compared to 0.19 for the Src optimal peptide (Miller et al., 2008 and Fig. 3.3B).

To test whether Y180 was a Src phosphorylation site, GST-syndecan-4 cytoplasmic domains were constructed in which tyrosine phosphorylation was disrupted by mutation to leucine (Fig. 3.4 and 3.5). In this way the relative contribution made by a single tyrosine to the total phosphorylation signal was assessed. Disruption of the Y180 residue (GST-Syn4Y180L) significantly reduced Src-mediated phosphorylation of syndecan-4 compared to WT sequence (to 53% that of GST-Syn4WT levels) whereas the control tyrosine mutation, GST-Syn4Y188L, had no significant effect (Fig. 3.6). These findings demonstrated that Syn4 Y180 is a direct phosphorylation target for Src kinase activity. However disruption of Y180 did not completely abolish Src-dependent phosphorylation of syndecan-4 to basal levels, suggesting that an alternative phosphorylation site must be present in the syndecan-4 cytoplasmic domain. As the Y188L mutant had no significant effect on levels of syndecan-4 phosphorylation, this suggested that the second site for Src-mediated tyrosine phosphorylation could be Y197, found in the C2 (PDZ-binding) domain of syndecan-4.

Disruption of Syn4 Y197 residue (GST-SynY197L) consistently reduced Src-mediated phosphorylation to 70% of WT levels (Fig. 3.7A and B). Moreover, perturbation of both Y180 and Y197 (GST-Syn4Y180197L) further decreased Src-mediated phosphorylation of syndecan-4 to 10% of WT levels. By contrast, phosphorylation of Y180L and Y188L (GST-Syn4Y180188L) or Y197L and Y188L (GST-Syn4Y188197L) double mutants was not significantly different from GST-Syn4Y180L or GST-Syn4Y197L, respectively (Fig. 3.7A and B).
Together, the results showed that Src specifically and directly phosphorylates syndecan-4 on the Y180 and Y197, but not Y188, residues.

To determine the effects of tyrosine phosphorylation on Src-dependent phosphorylation of other tyrosine residues, tyrosines were mutated to glutamic acid to mimic phosphorylation. Similar to previous results, mutation of Y180 (GST-Syn4Y180E) reduced phosphorylation levels significantly as compared to all other mutants (Fig. 3.7C). However, a phosphomimetic Y188 (GST-Syn4Y188E) also suppressed phosphorylation, compared to GST-Syn4WT and GST-Syn4Y188L. Since previous experiments had eliminated Y188 as a Src phosphorylation site, the fact that Y188E suppressed Src-induced phosphorylation may illustrate an interplay between phosphorylation sites, where the phospho-state of one residue may affect phosphorylation of another. Thus, in future it will be important to identify any kinases that phosphorylate the Y188 residue.

The existence of two Src phosphorylation sites, one in the C1 domain and the other in the C2 domain of syndecan-4 may be indicative of different signalling roles for each phosphorylation event and will be discussed in later chapters. Having established a role for Src tyrosine kinase in phosphorylating syndecan-4 it was then important to investigate the mechanisms regulating differential Src-mediated syndecan-4 phosphorylation.
Figure 3.3. Comparison of syndecan-4 cytoplasmic domain sequence with optimal peptides for Src phosphorylation.

Optimal peptide sequences for Src are depicted in table A in descending order. Overlay of the sequences surrounding Y180 in syndecan-4, and Src optimal peptides reveals a high degree of similarity (red circles in table A). The peptide sequence of syndecan-4 cytoplasmic domain surrounding Y180 and the Src optimal peptide sequence were entered into Netphorest, an online database of 125 sequence-based classifiers for linear motifs in phosphorylation-dependent signalling. Src was identified as a potential candidate in phosphorylation of Y180 residue (table B).

(KIN: Kinase; Tec: a T-cell specific kinase; MAPK: mitogen activated kinase; FLT3: Fms-like tyrosine kinase.)
Figure 3.4. GST-fusion syndecan-4 cytoplasmic domain constructs: tyrosine mutants.

Potential Src phosphorylation sites Y180 (green), Y188 (blue) and Y197 (purple) are highlighted (A). To explore Src phosphorylation of syndecan-4 cytoplasmic domain, single tyrosine residues were mutated to leucine L or glutamic acid E (B) or combinations of tyrosine mutants were created (C). A tyrosine-null syndecan-4 construct (GST-Syn4YYYLLL) was also generated as negative control to test the specificity of Src-dependent phosphorylation of syndecan-4 cytoplasmic domain.
Figure 3.5. GST-fusion syndecan-4 cytoplasmic domain constructs resolved by SDS-PAGE.

GST-syndecan-4 constructs separated by SDS-PAGE present dominant bands of approximately 28KDa (blue arrows), whereas GST alone (red arrows) runs at approximately 26KDa (A & B). GST-syndecan-4 constructs bound to glutathione sepharose beads (coomassie gel A) were used in pull-down assays to isolate syndecan binding partners. GST-syndecan-4 constructs were also eluted off beads (coomassie gel B) and used as substrates in Src phosphorylation assays.
Figure 3.6. Src phosphorylates Y180 in syndecan-4 cytoplasmic domain.

GST-tagged syndecan-4 cytoplasmic domain constructs with mutations at Y180 (GST-Syn4Y180L) and Y188 (GST-Syn4Y188L) were incubated with 1.8 ng/µl recombinant active Src kinase at 30°C for 15 min. Phosphorylation of syndecan-4 was detected by radio-labelling using $^{33}$p-ATP as phosphate donor and normalised to total GST-syndecan-4 protein in coomassie gel. Src-mediated phosphorylation of syndecan-4 cytoplasmic domain was significantly reduced in GST-Syn4Y180L mutants (53% of GST-Syn4WT, whereas Y188 mutation had no significant effect. No tyrosine phosphorylation of GST-alone was observed (n=11). (**p<0.01, ****p< 0.0001).
Figure 3.7. A second Src phosphorylation site exists within syndecan-4 cytoplasmic domain.

GST-tagged syndecan-4 constructs containing tyrosine mutations were incubated with 1.8 ng/µl recombinant active Src kinase at 30°C for 15 min. Phosphorylation of syndecan-4 was detected by radio-labelling using $^{33}$p-ATP as phosphate donor and normalised to total GST-syndecan-4 protein in coomassie gel. Perturbation of Y197 in syndecan-4 cytoplasmic domain resulted in decreased phosphorylation of syndecan-4 by Src (A-C). Double mutations of Y180 and Y197 significantly reduced tyrosine phosphorylation (B). Unmutated tyrosine residues remaining after double mutation are shown above each bar. Dashed lines in A denote exclusion of additional lanes in the same blot. (**p<0.001, ****p<0.0001).
3.4 PKCδ-mediated phosphorylation of syndecan-4 regulates tyrosine phosphorylation by Src

The results in this chapter so far demonstrate the presence of two Src phosphorylation sites, Y180 and Y197, within the C1 and C2 domains of syndecan-4, respectively. However, the regulatory mechanism controlling Src-mediated syndecan-4 phosphorylation is unclear. As both Src and syndecan-4 are important for regulation of adhesion dynamics it was necessary to identify the factors that modulate syndecan-4 phosphorylation and to determine whether residue Y180 and Y197 are differentially phosphorylated by Src.

The PKCδ-phosphorylation site, S179, lies adjacent to the Y180 residue in the C1 domain of syndecan-4 (Fig. 3.1). NMR analysis of the cytoplasmic domain of syndecan-4 showed substantial structural rearrangement in syndecan-4 at C1 and C2 domains, as a consequence of S179 phosphorylation (Lee et al., 1998). The proximity of the PKCδ-phosphorylation site to Y180 and the conformational change induced by S179 phosphorylation raises a key question: Does PKCδ-mediated phosphorylation of syndecan-4 modulate phosphorylation of Y180 and Y197 by Src?

To address this question, GST-syndecan-4 cytoplasmic constructs were generated in which the PKCδ-phosphorylation site (S179) was removed by mutation to alanine (GST-Syn4S179A) or changed to a phosphomimetic residue (GST-Syn4S179D) and the ability of Src to phosphorylate these peptides was assessed using in vitro kinase assays. Total Src-dependent phosphorylation of GST-Syn4S179D was enhanced, by a factor of 1.9, relative to WT (Fig. 3.8). Custom-made syndecan-4 cytoplasmic domain peptides with and without an added phosphate group on S179 (Syn4P-S179WT and Syn4WT, respectively) confirmed that the enhanced Src-mediated phosphorylation of syndecan-4 was as a result of S179 phosphorylation rather than due to mutation of the S179 residue (Fig. 3.9).

The increase in phosphorylation resulting from perturbation of the PKCδ-phosphorylation site, implies that under basal conditions i.e. in the absence of S179 phosphorylation, tyrosine phosphorylation by Src is not maximal. Furthermore, phosphorylation of syndecan-4 by PKCδ may be a mechanism to prime syndecan-4 for Src-mediated phosphorylation.
Figure 3.8. PKCδ-phosphorylation site, S179, regulates phosphorylation of syndecan-4 cytoplasmic domain by Src.

GST-tagged syndecan-4 constructs with phosphomimetic PKCδ-phosphorylation site (GST-Syn4S179D), or disrupted PKCδ-phosphorylation site (GST-Syn4S179A) (A) were incubated with 1.8 ng/μl recombinant active Src kinase at 30°C for 15 min. Phosphorylation of syndecan-4 was detected by radio-labelling using ^33^p-ATP as phosphate donor and normalised to total GST-syndecan-4 protein in coomassie gel. Mutation of PKCδ-phosphorylation site significantly enhanced Src phosphorylation of syndecan-4 cytoplasmic domain by a factor of 2.2 for GST-Syn4S179A and 1.9 for GST-Syn4S179D (B) (n=13) (**p<0.01, ****p<0.0001).
Figure 3.9. Specific phosphorylation of S179 in syndecan-4 cytoplasmic domain enhances tyrosine phosphorylation of syndecan-4 by Src.

To investigate the direct effect of PKCδ phosphorylation of syndecan-4 on Src-mediated phosphorylation of syndecan-4, synthetic peptides of WT syndecan-4 cytoplasmic domain (Syn4WT) were custom-made with and without addition of a phosphate group on S179 (Syn4WTpS179 and Syn4WT, respectively). The synthetic peptides were incubated with Src kinase as before and phosphorylation assessed by radio-labelling. Enhanced tyrosine phosphorylation of syndecan-4 cytoplasmic domain was detected in peptides containing phosphorylated S179.
As phosphorylation of S179 promotes Src-mediated phosphorylation of syndecan-4, it was important to determine the site(s) of enhanced Src-dependent tyrosine phosphorylation. Therefore a panel of peptides was generated containing a range of tyrosine mutations and a phosphomimetic S179D mutation to mimic phosphorylation of S179 by PKCδ (Fig. 3.10).

Abolition of Y180, but not Y188 or Y197, in combination with the phosphomimetic mutation of the PKCδ-phosphorylation site (GST-SDY180L) suppressed the increased levels of Src-mediated tyrosine phosphorylation to levels seen in the presence of wild-type S179 (GST-Syn4WT) (Fig. 3.11). This suggested that the increase in phosphorylation induced by phosphomimetic S179D mutation was on Y180. In previous experiments, disruption of Y180 alone (GST-Syn4Y180L) reduced phosphorylation below GST-Syn4WT levels, whereas here GST-SDY180L only suppressed phosphorylation to GST-Syn4WT levels. Also a decrease in phosphorylation was also observed in double tyrosine mutants (GST-SD8897L), suggesting that either Y188 or Y197 phosphorylation may also be affected by a phosphomimetic PKCδ-phosphorylation site (Fig. 3.11). Together, these data suggest that phosphorylation of S179 promotes Src-mediated phosphorylation of Syn4 Y180. However, the exact influence of S179 phosphorylation on Src-mediated phosphorylation of Syn4 Y197 is unclear. In addition, the possibility that mutating one tyrosine residue may inhibit or promote the phosphorylation of another residue cannot be ruled out. Indeed, work with the GST-Syn4S179A mutant of the PKCδ-phosphorylation site (Fig. 3.12), suggested that the mechanism of PKCδ-mediated regulation of Src phosphorylation of syndecan-4 may be even more complex. Src-mediated phosphorylation of syndecan-4 in GST-Syn4S179A mutants was enhanced similarly to phosphomimetic Syn4S179D; however, the abolition of Y197 in GST-S179A (GST-SAY197L) substantially increased phosphorylation of syndecan-4 beyond that of GST-S179A alone (4.1- and 2.2-fold more than GST-Syn4WT, respectively) (Fig. 3.12). While these data rely on the use of mutagenesis (S179A) to perturb the PKCδ-phosphorylation site, they still highlight the important role that PKCδ-phosphorylation site (S179) plays in differentially regulating Src-mediated phosphorylation of Y180 and Y197. Moreover, the use of custom-made peptides demonstrated that differential phosphorylation of syndecan-4 mediated by Src is specifically dependent on S179 phosphorylation rather than as a consequence of mutagenesis (Fig. 3.9).
Figure 3.10. GST-fusion syndecan-4 cytoplasmic domains: PKCδ-phosphorylation site mutants.

GST-tagged syndecan-4 cytoplasmic domain constructs were generated with mutations of the PKCδ-phosphorylation site (GST-Syn4S179D and GST-Syn4S179A) (A) in combination with single and double tyrosine mutations (B & C).
Figure 3.11. Introduction of a phosphomimetic mutation of PKCδ-phosphorylation site (S179D) increases Src-mediated phosphorylation of syndecan-4 on Y180.

GST-tagged syndecan-4 constructs with phosphomimetic PKCδ-site (GST-Syn4S179D) in combination with different tyrosine mutations were incubated with 1.8 ng/µl recombinant active Src kinase at 30°C for 15 min. Phosphorylation of syndecan-4 was detected by radio-labelling and normalised to total GST-syndecan-4 protein in coomassie gel. Mutation of both Y180 together with phosphomimetic PKCδ-phosphorylation site, S179D, (GST-SD-Y180L) restored phosphorylation levels to GST-Syn4WT levels (**p<0.005). No significant effect was seen with other single tyrosine mutants. (n=7). Unmutated tyrosine residues remaining after double mutation are shown above each bar. (*p<0.05, **p<0.01, ***p<0.001).
Figure 3.12. Disruption of the PKCδ-phosphorylation site (S179A) increases Src-mediated phosphorylation of syndecan-4 on Y180.

GST-tagged syndecan-4 cytoplasmic domain constructs with phosphonull PKCδ-site (GST-Syn4S179A) in combination with different tyrosine mutations were incubated with 1.8 ng/µl recombinant active Src kinase at 30°C for 15 min. Phosphorylation of syndecan-4 was detected by radio-labelling and normalised to total GST-syndecan-4 protein in coomassie gel. Disruption of Y180 (GST-SA-Y180L) significantly decreased phosphorylation levels below WT (**p<0.005), whereas mutation of Y197 (GST-SA-Y197L) enhanced Src-mediated syndecan-4 phosphorylation above that seen in GST-Syn4S179A alone (*p<0.05) (n=7). Unmutated tyrosine residues remaining after double mutation are shown above each bar. (*p<0.05, **p<0.001, ***p<0.0001).
3.5 Mass Spectrometric analysis and phosphomapping of Src phosphorylation sites

To further analyse Src-mediated phosphorylation of syndecan-4 tyrosine residues a method that does not rely on tyrosine mutations was required. Therefore mass spectrometry was used in an attempt to map the Src phosphorylation sites and to evaluate the contribution made to tyrosine phosphorylation of syndecan-4 by the PKCδ-phosphorylation site.

3.5.1 Tryptic digestion of syndecan-4 cytoplasmic domain

GST-Syn4WT, GST-Syn4S179A and GST-Syn4S179D samples (Fig. 3.13A), either in an unphosphorylated native form or following phosphorylation by Src, were prepared for analysis by mass spectrometry. The lysine-specific activity of trypsin coupled with the high lysine content of syndecan-4 cytoplasmic domain dictated that, prior to quantification of any phosphopeptides, an analysis of the digestion products arising from trypsinisation was required. Tryptic digestion of syndecan-4 cytoplasmic domain resulted in three cleavage products encompassing Y180 (1 fully cleaved peptide (a), and 2 miscleaved peptides (b and c) and two containing Y197 (1 fully cleaved peptide (d) and 1 miscleaved peptide (e)) (Fig.3.13B). The reproducibility of tryptic digestion was tested by comparing the level of detection of each peptide variant in 3 biological repeats and comparing ratios of the miscleaved peptides to the fully cleaved peptide (Fig. 3.13C). All 3 repeats yielded the same pattern of digestion, providing confidence in the reproducibility of trypsin digestion for future experiments. More miscleaved peptides, containing extra lysine residues, were detected compared to fully cleaved peptides (e.g. the ratio of fully cleaved peptide d to miscleaved peptide e encompassing Y197 was approximately 1:20) (Fig. 3.13C). However, the rate of miscleavage was comparable for GST-Syn4WT and GST-Syn4S179A/D mutants (data not shown), suggesting that the efficiency of tryptic digestion was reproducible for the different mutations.

Although providing valuable information regarding peptide detection, the initial MS approach was unable to detect any phosphorylated peptides. The inability to detect phosphopeptides was attributed to only a small proportion of syndecan-4 being phosphorylated and the presence of highly abundant unphosphorylated GST-syndecan-4 protein.
Figure 3.13. Proteolytic peptides of syndecan-4 cytoplasmic domain following tryptic digest.

GST-Syn4WT and PKCδ-phosphorylation site mutants (GST-Syn4S179D, GST-Syn4S179A) (A) were incubated with 1.8 ng/µl Src for 15 min at 30°C and analysed on a Q-TRAP mass spectrometer. The tryptic peptides detected (B) included three peptides containing Y180 residue (green residue, peptides a-c) and two containing Y197 residue (purple residue, peptides d & e) (B & C). In three biological repeats, the level of detection was reproducible for each peptide (B). The fold difference in detection of each miscleaved peptide was compared to the corresponding fully cleaved peptide (C). GST-Syn4WT peptides shown are representative of GST-Syn4WT, GST-Syn4S179D and GST-Syn4S179A samples.
3.5.2 Phospho-specific gel stains and detection of phosphopeptides by Q-TRAP

To facilitate detection of phosphorylation by MS analysis, it was necessary to increase the stoichiometry of Src-mediated phosphorylation of syndecan-4. To achieve higher levels of syndecan-4 phosphorylation, the *in vitro* Src kinase assay was optimised by varying the amount of recombinant active Src and the incubation time of the GST-syndecan-4 construct with active Src kinase. Previous assays used 1.8 ng/µl of active Src (1 x Src) with a reaction time of 15 min at 30°C. Increasing Src to 5.4 ng/µl (3 x Src) and reaction time to 1 hr substantially enhanced tyrosine phosphorylation of syndecan-4 detected by immunoblotting and radio-labelling (Fig. 3.14). A further increase of Src to 18 ng/µl (10 x Src) did not significantly increase levels of phosphorylation (data not shown). Therefore 3x Src and 1 hr reaction conditions were used to prepare samples for future mass spectrometric analyses.

In addition to optimising kinase assay conditions, phospho-specific gel stains were used to visualise phosphorylated proteins prior to Coomassie Blue staining and loading on the mass spectrometer. Phospho-specific stains detected 2.4-fold higher phosphorylation of GST-Syn4S179A compared to GST-Syn4WT confirming previous results obtained with radio-labelling and immunoblotting (Fig. 3.15). Samples were then analysed using a Q-TRAP mass spectrometer instrument. Precursor ion scanning in negative ion mode detected phosphorylated Y197 in both GST-Syn4WT and GST-Syn4S179A samples (Fig. 3.16) whereas Y180 phosphorylation was only detected in the GST-Syn4S179A sample (Fig. 3.17). The inability to detect phosphorylation of Y180 in GST-Syn4WT may suggest that in the absence of S179 modification, Src-mediated Y180 phosphorylation has a low stoichiometry and that this balance is altered in the presence of PKCδ site modifications. This conclusion is consistent with modulation of syndecan-4 S179 promoting Src-mediated phosphorylation of syndecan-4 Y180 (Figs. 3.8, 3.9, 3.11 and 3.12). However, direct comparisons of Y180 phosphorylation between GST-Syn4WT and GST-Syn4S179A/D using this technique may not be ideal as Y180 is in the same tryptic peptide as S179 and mutations of S179 could hypothetically alter the ability of the Q-TRAP instrument to detect these peptides.
Figure 3.14. Optimisation of Src kinase assay: increasing phosphorylation stoichiometry for mass spectrometric analysis.

Src phosphorylation of syndecan-4 cytoplasmic domain was optimised by increasing concentration of Src and kinase reaction times. Phosphorylation was monitored by IB using a phosphotyrosine antibody (A & B) and radio-labelling (C & D). Lane 2 represents normal assay conditions: 1.8 ng/µl Src (1xSrc) and 15 min incubation. Increasing Src concentration to 5.4 ng/µl (3xSrc) dramatically enhanced detection of syndecan-4 phosphorylation after 1 hr incubation at 30°C (lanes 5).
Figure 3.15. Optimisation of kinase assay: use of phospho-specific gel stain to visualise syndecan-4 phosphorylation levels prior to mass spectrometric analysis.

GST-Syn4WT and GST-Syn4S179A constructs were incubated with 5.4 ng/µl Src at 30°C for 1 hr. Levels of phosphorylation were detected by Phospho-tag 540 gel stain prior to coomassie staining (A). Comparison between GST-Syn4WT and GST-Syn4S179A using Phospho-tag confirmed previous IB and radio-labelling results: Src-mediated phosphorylation of GST-Syn4S179A was significantly enhanced compared to GST-Syn4WT (B) (n=4). (**p<0.01).
Figure 3.16. Q-TRAP mass spectrometer detection of phospho-Y197 in syndecan-4 cytoplasmic domain.

Extracted ion chromatogram (XIC) signal for GST-Syn4WT (upper panel) and GST-Syn4S179A (lower panel) peptides are shown in A. Signals corresponding to phosphorylated peptide $e$ (KAPTNEFpYA) were detected in both samples at retention time of ~24.3 min. The precursor $m/z$ for peptide $e$ was subsequently targeted, in positive ion mode, for validation by MS/MS (B).
Figure 3.17. Q-TRAP mass spectrometer detection of phospho-Y180 in syndecan-4 cytoplasmic domain.

Extracted ion chromatogram (XIC) signal for GST-Syn4WT (upper panel) and GST-Syn4S179A mutant (lower panel) peptides are shown in A. No signal was detected in GST-Syn4WT sample whereas a definite peak corresponding to phosphorylated peptide b (KDEGApYDLGK) was observed for GST-Syn4S179A at retention time of ~18.6 min. The precursor m/z for peptide b in GST-Syn4S179A sample was subsequently targeted, in positive ion mode, for validation by MS/MS (B).
3.5.3 Increasing sensitivity of detection and quantitative analysis of phosphopeptides: LTQ Velos ion-trap mass spectrometer

Further experiments focused on increasing sensitivity for detection of phosphopeptides such as Y180 in syndecan-4 WT samples, and establishing a valid quantification method for analysing and comparing Src-mediated tyrosine phosphorylation between syndecan-4 WT and mutants of the PKCδ-phosphorylation site.

To improve mass spectrometry identification of low abundance phospho-peptides such as phospho-Y180, the amount of sample was increased three-fold and subsequently run on an LTQ Velos mass spectrometer, which combines faster scan rates and multiple fragmentation techniques thereby enabling sensitive detection of peptides and post-translational modifications. The ratios of detected phosphorylated to unphosphorylated peptides were calculated and compared to the basal unphosphorylated S179 condition (GST-Syn4WT) and following perturbation of the PKCδ-phosphorylation site (GST-Syn4S179D and GST-Syn4S179A).

Using LTQ Velos, phosphorylated peptide c, encompassing Y180 and Y188 residues, was isolated from GST-Syn4WT, GST-Syn4S179D and GST-Syn4S179A samples (Fig. 3.18B). MS/MS data corresponding to peptide c revealed phosphorylation to be specifically on Y180 and not Y188 residue (Fig. 3.19). Moreover, mutations of the PKCδ-phosphorylation site (GST-Syn4S179D and GST-Syn4S179A) dramatically increased the ratio of phosphorylated to unphosphorylated peptide c compared to GST-Syn4WT (3.4- and 3.95-fold increase, respectively) (Fig. 3.20A and B). Importantly, these data are in agreement with previous results (Figs. 3.11 and 3.12) demonstrating an increase in Src-mediated Y180 phosphorylation in GST-Syn4S179A and GST-Syn4S179D samples. Despite mass spectrometry confirming previous data, phospho-Y180 was also isolated in a second tryptic peptide (peptide b) in the GST-Syn4S179A sample alone (Fig. 3.18B). It is unclear whether detection of phosphorylated peptide b in GST-Syn4S179A samples is due to enhanced Y180 phosphorylation over other samples or a consequence of mutation that improves mass spectrometer detection of GST-Syn4S179A peptides.

Unlike phospho-Y180, phosphorylation of Y197 was present in all tryptic variants (peptides d and e) and detected in all samples (Fig. 3.18B). Initial analyses, comparing phosphorylated and non-phosphorylated forms of peptides d and e suggested a slight decrease in phosphorylation of Y197 in GST-Syn4S179D and GST-Syn4S179A mutants.
However, examination of the specific phosphorylated peptides that were detected revealed a different tryptic profile from the previously characterised digestion of syndecan-4. In unphosphorylated forms, peptides $d$ and $e$ existed in a 1:20 ratio, i.e. for the sequence surrounding the Y197 residue the most abundant digestion product was peptide $e$, which contains an additional C-terminal lysine residue. Analysis of the phosphorylated pools of peptides $d$ and $e$ in the same sample revealed a two-fold reduction in $d$ to $e$ ratio ($\text{phospho-}d:\text{phospho-}e = 1:10$), suggesting an altered tryptic activity when syndecan-4 is phosphorylated on Y197 (Fig. 3.20D). In addition to altering trypsin digestion, the basic environment of the lysine in peptide $e$ may also mask the negative charge of the added phosphate group and diverge from expected $m/z$ characteristics, interfering with MS identification of the phosphorylated peptide. For these reasons it was concluded that direct comparison of phosphorylated to unphosphorylated peptides was inappropriate.

The tryptic peptides containing Y197 (peptides $d$ and $e$) do not contain S179 and therefore were not affected by any mutations and so these peptides were used in the search for a valid quantitation method to be applied in the analysis of phosphorylation events.

### 3.5.4 Normalisation of MS results using GST peptides and Progenesis software

GST was chosen as an alternative candidate for normalisation of MS results due to its presence in all syndecan-4 constructs and because it remains unmodified by Src kinase. To assess levels of syndecan-4 peptides, a software package called Progenesis was used to normalise all samples to their respective GST peptides. Following normalisation, direct comparisons were made between syndecan-4 WT and S179 mutants. Using this technique, Src-mediated levels of Y197 phosphorylation were decreased in GST-Syn4S179D compared to WT with no significant difference seen in GST-Syn4S179A samples (Fig. 3.21). However, the pattern of unphosphorylated peptides detected after GST normalisation was similar to the pattern of phosphorylated peptides and therefore a clear conclusion could not be drawn (Fig. 3.22). For example, while lower levels of phosphorylated peptide $e$ were detected from S179D to WT sample, lower levels of unphosphorylated peptide $e$ were also detected in S179D compared to WT.
Figure 3.18. LTQ Velos mass spectrometer: detection of phosphorylated peptides.

WT syndecan-4 (GST-Syn4WT) and PKCδ-phosphorylation site mutants (GST-Syn4S179D, GST-Syn4S179A) (A) were incubated with 5.4 ng/µl for 1 hr at 30°C and analysed on an LTQ Velos mass spectrometer. Phosphorylation of Y180 was detected in peptide c for GST-Syn4WT and GST-Syn4S179D and in peptides b and c for GST-Syn4S179A. Phospho-Y197 was detected in all samples (table B).
Figure 3.19. MS/MS analysis of peptide c.

Phosphorylation of peptide c is specific to Y180 and not Y188 phosphorylation as demonstrated in GST-Syn4WT (A), GST-Syn4S179A (B) and GST-Syn4S179D (C) samples.
### Figure 3.20. LTQ-Velos mass spectrometer: ratio of phosphorylated to unphosphorylated peptides.

To compare phosphorylation levels of GST-Syn4WT, GST-Syn4S179D and GST-Syn4S179A, detection of phosphorylated peptides was normalised to unphosphorylated peptides (A). Phosphorylation of Y180 was increased (B) and phosphorylation of Y197 decreased in GST-Syn4S179D and GST-Syn4S179A samples (C), compared to GST-Syn4WT. However, the efficiency of tryptic digestion appeared to be altered upon addition of phosphate group (D).
Figure 3.21. Progenesis software analysis of phosphorylated Y197 peptides normalised to GST background.

The characteristic $m/z$ and retention times of peptides were mapped by Progenesis software using GST-Syn4WT sample as a reference (A). Green box indicates phosphorylated Y197 in peptide $e$ at $m/z$ 560.74 with retention time of $\sim$21.38 min (A). Isotopic variants of phosphorylated peptide $e$ from 3 repeat runs are shown in B. Normalised abundance of phosphorylated peptide $e$ relative to GST background was compared between WT and PKC$\delta$-site mutants (C). Decreased levels of Y197 phosphorylation were observed in the presence of phosphomimetic PKC$\delta$ site (GST-Syn4S179D) with no difference seen in GST-Syn4S179A (C & D). (Error bars are representative of three standard errors from the mean).
Figure 3.22. Progenesis software analysis of unphosphorylated Y197 peptides normalised to GST background.

The characteristic m/z and retention times of peptides were mapped by Progenesis software using GST-Syn4WT sample as reference point (A). Green box indicates unphosphorylated Y197 in peptide e at m/z 520.76 with retention time of ~21.23 min (A). Isotopic variants of unphosphorylated peptide e from three repeat runs are shown in B. Normalised abundance of unphosphorylated peptide e relative to GST background was compared between WT and PKCδ-site mutants (C). A modest decrease in unphosphorylated peptide e containing Y197 was observed in presence of phosphomimetic PKCδ site (GST-Syn4S179D), whereas, GST-Syn4S179A samples exhibited a modest increase in levels of unphosphorylated peptide e containing Y197 (C & D). (Error bars are representative of three standard errors from the mean).
The suitability of GST peptides for sample normalisation was investigated by measuring average levels of detection of three GST peptides in three repeat runs. Fold changes of GST peptides, unphosphorylated peptides \(d\) and \(e\) and phosphorylated pools of the same peptides were then calculated in GST-Syn4S179D and GST-Syn4S179A samples, relative to GST-Syn4WT. Levels of phosphorylated Y197 peptides \((d\) and \(e)\) were suppressed in S179D relative to WT, while the levels of non-phosphorylated Y197 peptides \((d\) and \(e)\) and GST peptides were increased with S179D samples (Fig. 3.23), indicating that Src phosphorylation of Y197 was indeed inhibited by phosphomimetic PKC\(\delta\) site, as suggested by the Progenesis software (Fig. 3.21). By contrast, levels of both phosphorylated and nonphosphorylated Y197 peptides were reduced in GST-Syn4S179A samples (Fig. 3.23).

Despite these data being consistent with previous findings (Figs. 3.19 and 3.21), the presence of extra GST peptides compared to unphosphorylated peptides \(d\) and \(e\) within the same samples raised concerns for normalising against GST. It was expected that syndecan-4 and GST exist in a 1:1 ratio and therefore any fold change from WT sample would be the same for unphosphorylated Y197 peptides and GST peptides. Due to variation in detection of unphosphorylated and GST peptides it was difficult to draw solid conclusions.

In summary, the use of mass spectrometry to map the Src phosphorylation sites directly confirmed the enhanced phosphorylation observed in the presence of PKC\(\delta\) mutants GST-Syn4S179D/A. However, attempts to quantify the results highlighted several problems associated with mass spectrometric analysis of phosphorylation events that rely on mutations. Future experiments could involve the use of synthetic syndecan-4 cytoplasmic domain peptides, with or without additional phosphate groups on the different tyrosine residues to optimise mass spectrometry identification of phosphorylated peptides. Indeed, as demonstrated previously (Fig. 3.19) syndecan-4 peptides that include a phosphate group on S179 exhibit enhanced levels of Src-dependent syndecan-4 phosphorylation and would be ideal for further investigations.

The Progenesis software will be used for quantification of results as it has several advantages such as allowing the simultaneous analysis of all isotopic variants of peptides and the ability to search for further protein modifications that may have occurred as a result of sample preparation and may interfere with mass spectrometric analysis.
Fold change compared to GST-Syn4WT

Figure 3.23. Suitability of GST peptides for normalisation of data.

Detection of GST peptides and phosphorylated and unphosphorylated Y197 peptides \( d \) and \( e \) from three replicate runs was calculated and expressed as a fold change from GST-Syn4WT sample (A & B). Increased levels of unphosphorylated \( d \) and \( e \) and GST peptides were detected in GST-Syn4S179D sample, whereas phosphorylated \( d \) and \( e \) were reduced (A) and unchanged (B), respectively, compared to GST-Syn4WT sample, indicating overall decrease in Y197 phosphorylation. Pattern of fold change in GST-Syn4S179A samples was very similar for unphosphorylated, phosphorylated and GST peptides, making interpretation of results difficult.
3.6 Conclusions and discussion Part I

The aim of this chapter was to determine whether Src tyrosine kinase played a role in post-translational modification of syndecan-4 by directly phosphorylating the cytoplasmic domain of the syndecan-4 receptor. The results, confirmed by several techniques, demonstrate that Src specifically and directly phosphorylates syndecan-4 at two distinct sites; Y180 and Y197. Furthermore, modulation of the PKCδ-phosphorylation site, S179, significantly enhances phosphorylation of syndecan-4 by Src (Fig. 3.24). Together, the data suggested that phosphorylation of S179 specifically promotes Src-mediated phosphorylation of Y180. Much of the data presented in this chapter also suggest that modification of the S179 residue can suppress phosphorylation of Y197 by Src. This would suggest that PKCδ-mediated phosphorylation can prime syndecan-4 for Src-mediated phosphorylation of Y180 while suppressing the ability of Src to phosphorylate Y197. However, attempts to prove this categorically by quantitative mass spectrometric analysis, while still supporting this hypothesis, were not conclusive.

Determining whether PKCδ-phosphorylation of syndecan-4 inhibits Y197 phosphorylation in addition to the observed increase in Y180 phosphorylation will be important for understanding Src-dependent syndecan-4 signalling. Y197 is in the PDZ-binding motif of syndecan-4 and therefore its phosphorylation state potentially could regulate binding of PDZ proteins to syndecan-4. Indeed, it has been suggested that syntenin, a PDZ-domain containing protein, binds to syndecan-1 upon dephosphorylation of the equivalent conserved tyrosine residue in syndecan-1 C2 domain (Y309). However, the kinases responsible for phosphorylation of this residue have not been identified (Sulka et al., 2009). Syntenin-syndecan interaction has been identified as a key mechanism regulating syndecan recycling back to the membrane. Thus, identifying pathways modulating syntenin-syndecan binding will be critical in understating how syndecan recycling is controlled.

NMR studies have suggested a conformational change in both the C1 and C2 (PDZ-binding) domains of syndecan-4 in the presence of phosphorylated S179. It is therefore likely that these conformational changes are responsible for the S179-dependent switch in specificities of Src-mediated phosphorylation from Y197 to Y180. Mass spectrometry experiments could be used to quantify the relative shift in Src specificity and structural studies could reveal how phosphorylation by PKCδ modulates the ability of Src to
phosphorylate syndecan-4, e.g. whether conformational changes directly reveal Y180 to Src or mask Y197 phosphorylation site, thereby allowing Y180 to become the preferential Src substrate. Improving mass spectrometry identification of peptides and subsequent quantification will be key to answering these questions.

Although this work has demonstrated a role for Src-dependent phosphorylation of syndecan-4, it is important not to dismiss the potential phosphorylation of syndecan-4 by other Src family kinase members (e.g. Yes and Fyn). The identification of phosphatases responsible for the reverse phospho-modification of S179, Y180 and Y197 will also be fundamental to an understanding of the dynamic regulation of syndecan-4 signalling events.

PKCδ-dependent syndecan-4 phosphorylation offers a mechanism for differential tyrosine phosphorylation of syndecan-4, resulting in a shift in the balance of phosphorylated Y180 over Y197 (Fig. 3.24). Thus, it would appear that syndecan-4 functions as a nexus to integrate PKCδ and Src signals, in which the phosphorylation of S179 dictates the effect of Src activity of syndecan-4. It is possible that, in migrating cells, the spatiotemporal control of PKCδ activity functions to coordinate differential Src-dependent syndecan-4 signalling.

Following on from the biochemical analyses reported in this chapter, the roles of Src-mediated syndecan-4 phosphorylation and specifically how differential phosphorylation of Y180 and Y197 affects syndecan-4 signalling and cell behaviour will be investigated next.
Figure 3.24. Model: PKCδ-dependent syndecan-4 phosphorylation functions to coordinate differential tyrosine phosphorylation of syndecan-4 by Src.

Src tyrosine kinase directly phosphorylates syndecan-4 cytoplasmic domain on two tyrosine residues, Y180 and Y197 (A). PKCδ-dependent phosphorylation of S179, primes syndecan-4 for Src-mediated phosphorylation of Y180 and not Y197 (B), thereby restricting Src-dependent syndecan-4 signalling.
4. Src-mediated phosphorylation of syndecan-4: Regulation of GTPases and integrin receptor recycling

**Aims and background:**

Efficient cell migration requires the formation and dissociation of cell-ECM contacts in a spatially- and temporally-controlled manner. FAs are macromolecular assemblies that provide a mechanical linkage to the ECM and regulate signalling downstream of integrin receptor binding and clustering (Wozniak et al., 2004). The physical linkage from the ECM provided through integrins and the actin cytoskeleton regulates tension-associated cell behaviour and morphology, and the unique signalling properties of integrins allow for bidirectional exchange of information across the plasma membrane (Hynes et al., 2002).

The principal integrin receptors involved in Fn engagement include α5β1 and αVβ3 (Humphries et al., 2006). Engagement of different integrin heterodimers, such as α5β1 and αVβ3, elicits very different signalling events that regulate cell fate, morphology and migration. For example, engagement of αVβ3 stabilises FAs, suppresses RhoA activation and promotes directionally-persistent cell migration. In contrast, α5β1 promotes FA turnover and random cell migration through the activation of RhoA (Danen et al., 2002; Danen et al., 2005). Furthermore α5β1 regulates matrix assembly at sites of Fn fibrillogenesis (fibrillar adhesions) (Clark et al., 2005; Mao and Schwarzbauer, 2005). Therefore the relative availability of α5β1 and αVβ3 for ECM engagement dictates different modes of cell motility and regulating this balance is fundamental for efficient cell migration.

Integrin recycling is a process involving endocytosis of integrin receptors followed by redelivery back to the plasma membrane (Balasubramanian et al., 2007). Rather than being degraded, a significant proportion of the internalised pool of integrins is redistributed to the cell surface to reengage matrix and form new adhesions. Much is known about the endocytosis of integrin heterodimers, but the mechanisms underlying redelivery and targeting of α5β1 and αVβ3 back to the membrane are not fully understood. Arf6, a small Ras family GTPase, is a multifunctional regulator of receptor trafficking and membrane remodelling. Arf6 has been implicated in the recycling of β1 integrins from Rab11-positive compartments but the specific integrin heterodimer remains unidentified (Powelka et al.,
Arf6 is also involved in recycling of lipid rafts, a process key to adhesion-dependent GTPase regulation (D'Souza-Schorey and Chavrier, 2006).

Syndecans are characterised as co-receptors for a diverse range of transmembrane receptors including integrins. Recent work demonstrated a role for Arf6-mediated recycling of syndecans from perinuclear recycling compartments back to the membrane. Importantly, the perturbation of syndecan recycling following mutations of syntenin, a syndecan PDZ-binding domain partner, or expression of dominant negative Arf6 resulted in accumulation of syndecans, FGF receptor and β1 integrin in the PNRC (Zimmermann et al., 2005). This raises the intriguing possibility that recycling of β1 integrins may be coupled to that of syndecans.

Recently activation of Arf6 has been demonstrated upon cell spreading on Fn (Balasubramanian et al., 2007; Humphries et al., 2009). Furthermore, specific engagement of syndecan-4 with a Fn fragment containing the major heparin-binding domain (H/0) increased Arf6 activity, demonstrating a direct role for syndecan-4 receptor in modulating Arf6 activation. In addition, cells expressing a phosphomimetic Y180 syndecan-4 receptor (Syn4Y180E) exhibit low basal Arf6 activity and suppressed α5β1 recycling. By contrast, both a phospho-null mutant (Syn4Y180L) and a fast cycling Arf6 mutant (Arf6T157A) promoted recycling of α5β1 and inhibited membrane redelivery of αVβ3 (Mark Morgan, unpublished data). Thus, the phosphorylation status of syndecan-4 Y180 regulates Arf6 activity to coordinate differential heterodimer-specific recycling of α5β1 and αVβ3 integrins.

In the previous chapter it was demonstrated that Y180 is phosphorylated by Src - an adhesion-associated tyrosine kinase - and that Src-dependent phosphorylation of Y180 is regulated by PKCδ-mediated phosphorylation of S179 in the syndecan-4 cytoplasmic domain. In this chapter the aim was to determine whether PKCδ–mediated phosphorylation of syndecan-4 controls Arf6 activity by regulating Src-dependent phosphorylation of syndecan-4 at key tyrosine residues. As Arf6 activity is implicated in receptor trafficking, integrin recycling will also be investigated.
4.1 PKCδ knockdown: Arf6 activation and integrin recycling

To understand the role of PKCδ-phosphorylation of syndecan-4 in Arf6 activation and integrin recycling, PKCδ expression was suppressed in cells expressing wild-type syndecan-4 (Syn4WT). The efficiency of 4 siRNA oligos directed against mouse PKCδ was tested. Following 2 rounds of transfection, oligos 6 and 7 exhibited the highest levels of knockdown of PKCδ and oligo 6 was chosen for subsequent experiments (Fig. 4.1A). PKCδ expression was monitored after the second transfection to determine a profile of knockdown efficiency over time. PKCδ expression was reduced by 82%, relative to control siRNA-transfected cells, 2 days post-transfection. Levels of PKCδ increased gradually over time and had fully recovered within 5 days (Fig. 4.1B). All experiments with PKCδ-knockdown cells were therefore carried out 2-3 days post-transfection.

4.1.1 Regulation of Arf6 activity

Arf6 activity was assessed through the isolation of GTP-bound Arf6 from cell lysates by effector pull-down (using GST-GGA3). PKCδ and control siRNA-transfected cells were plated on Fn for 4 hrs, in the presence of 2% FCS, to analyse steady-state Arf6 activity. No significant change in steady-state Arf6 activity was observed as a result of PKCδ knockdown (Fig. 4.2A).

The ability of PKCδ-deficient cells to induce Arf6 activity in response to ECM stimuli was examined next. Arf6 activity was analysed in PKCδ- and control-siRNA transfected cells in suspension and following 1 hr spreading on Fn. Although no change was seen in steady-state levels of Arf6 activity, reduced expression of PKCδ suppressed the induction of Arf6 activity that was observed with control cells in response to ECM engagement (Fig. 4.2B).

Arf6 GTPase is a key regulator of receptor recycling from PNRCs to the membrane. Integrin β1 is among receptors known to be modulated by Arf6-dependent recycling pathways. Recent work in this laboratory has shown that cells exhibiting defects in Arf6 activation have altered α5β1 and αVβ3 recycling (Syn4Y180E/L) (Mark Morgan, unpublished data). As modulation of Arf6 activity was perturbed in PKCδ-knockdown cells, the expression of these specific integrins on the cell surface was examined.
Figure 4.1. siRNA-mediated PKCδ knockdown.
PKCδ expression was suppressed in MEFs expressing endogenous syndecan-4 using 4 different siRNA oligos directed against mouse PKCδ (A). Efficiency of knockdown was assessed following single and double rounds of transfection (A) and over 5 days post-transfection (B). Oligo 6 was chosen in subsequent experiments to suppress PKCδ expression.
N.B. Dashed line denotes exclusion of additional lanes in the same blot.
Figure 4.2. Suppression of PKCδ expression prevents Arf6 activation in response to ECM engagement.

Control- (Ctrl) and PKCδ-siRNA-treated MEFs, expressing endogenous syndecan-4 were spread on 10 µg/ml Fn. GTP-bound Arf6 was isolated in effector pull-down (PD) with GGA3 and Arf6 activity calculated relative to total Arf6 protein levels. No change in steady-state Arf6 activity was detected as a result of PKCδ knockdown following 4 hr spreading on Fn in the presence of 2% FCS (A). Ctrl and PKCδ-siRNA-treated MEFs, were kept in suspension and spread on Fn for 1 hr (B). Suppression of PKCδ resulted in a failure to induce Arf6 activity in response to Fn engagement (B) (n=4). (*p<0.05)
4.1.2 Cell-surface expression and recycling of specific integrin heterodimers

Cell-surface expression of Fn receptors α5β1 and αVβ3, following PKCδ knockdown, was assessed by flow cytometry using specific antibodies directed against α5, β1, αV and β3 integrin subunits and fold change from isotype specific IgG was calculated. Antibodies against α4 integrin were used as a control as α4β1 is an integrin believed to function independent of syndecan signalling.

Initial experiments revealed a high level of α5 integrin on the cell surface of PKCδ-deficient cells compared to control-siRNA treated cells (Fig. 4.3A); however, these data were not consistent. On subsequent repeats, α5 levels remained unchanged (Fig. 4.3B), and an increase in αV surface levels was observed in one experiment (Fig. 4.3C).

Integrin recycling dynamics in PKCδ-deficient cells were investigated by monitoring redelivery of internalised surface-labelled integrins back to the membrane. ELISA pull-down of labelled integrins using specific antibodies revealed the same inconsistencies observed with flow cytometry (Fig. 4.4). The variability in integrin expression and recycling profiles between experiments may result from different levels of PKCδ knockdown or be influenced by the alternative phosphorylation targets of PKCδ. PKCδ is a ubiquitously expressed serine/threonine kinase and syndecan-4 is not its only target. Using siRNA-mediated knockdown of PKCδ to study PKCδ-mediated syndecan-4 specific signalling may therefore not be appropriate.
Figure 4.3. Suppression of PKCδ expression: Cell-surface expression of Fn receptors, α5β1 and αVβ3.

Cell-surface expression of Fn-binding integrins, α5β1, αVβ3 and α4β1 was assessed using flow cytometry. Levels of α4, β1 and β3 remained unchanged in three independent experiments for Ctrl and PKCδ-siRNA-treated MEFs, expressing endogenous syndecan-4. Surface levels of α5 and αV were inconsistent between experiments. Increased α5 expression was observed in assay 1 (A), high αV observed in assay 2 (B) and no change was observed in assay 3 as a result of PKCδ suppression (C).
Figure 4.4. Suppression of PKCδ expression: Recycling profile of Fn receptors, α5β1 and αVβ3 integrins.

Serum-stimulated redelivery of integrins back to the membrane was monitored at 7, 15, 30 and 45 min at 37°C and calculated as a percentage of the total internalised pool of labelled integrins. Suppression of PKCδ initially caused an increase in αV recycling (A). However, subsequent experiments showed no difference in integrin recycling profiles between Ctrl- and PKCδ-siRNA treated cells (B).
4.2 Expression of PKCδ site phosphorylation mutants in cells

In order to delineate the syndecan-4-specific function of PKCδ from the numerous other pathways regulated by this kinase, it was necessary to interfere directly with the PKCδ-mediated phosphorylation of the syndecan-4 cytoplasmic domain.

Syndecan-4 null MEFs (Syn4-/-) were stably transfected with either full-length HA-tagged wild-type human syndecan-4 receptor (Syn4WT) or HA-tagged syndecan-4 receptors containing mutations of the PKCδ-phosphorylation site. Cell lines were generated that expressed syndecan-4 cytoplasmic domain mutants that cannot be phosphorylated by PKCδ (Syn4S179A) or to mimic constitutive PKCδ-mediated phosphorylation of syndecan-4 (Syn4S179D). Following transfection, syndecan-4-expressing cells were selected using puromycin and expression of the syndecan-4 receptor was assessed by quantitating cell-surface levels using flow cytometry with an anti-human specific syndecan-4 antibody. Following three rounds of cell sorting (FACS), Syn4WT- and Syn4S179A-transfected cells had comparable levels of syndecan-4 receptor present at the cell surface. However, Syn4S179D cells had only a small amount of syndecan-4 at the cell surface, as detected by flow cytometry (Fig. 4.5A). This low level of surface expression suggested that either Syn4S179D receptor was not expressed or that the phosphomimetic mutation was perhaps causing protein misfolding and retention of the receptor within the cell. The total expression of syndecan-4 was therefore analysed using reverse transcription PCR and specific syndecan-4 primers. The data demonstrated that all cell lines contained syndecan-4 cDNA (Fig. 4.5B) and that Syn4S179D was indeed expressed within cells.

Furthermore, cell-surface biotinylation, followed by cell lysis and antibody-mediated capture using an anti-HA antibody, demonstrated that substantial levels of Syn4S179D were expressed at the cell surface and that Syn4S179D expressed similar levels of syndecan-4 as Syn4WT and Syn4S179A cells (Fig. 4.5C). The low levels of syndecan-4 detection by flow cytometry may either be the result of syndecan-4 extracellular domain shedding or the rapid internalisation of the receptor following cell detachment from ECM. Syndecan-4 internalisation will be explored later. As Syn4S179D was expressed by cells and present at the cell surface, the Syn4S179D cell line was suitable for future studies.
4.3 PKCδ-mediated phosphorylation of syndecan-4 regulates integrin recycling

4.3.1 Regulation of Arf6 activity

Steady-state and inducible Arf6 activity was assessed in Syn4S179A- and Syn4S179D-expressing cells. Steady-state Arf6GTP levels were substantially reduced in the absence of S179 phosphorylation (Syn4S179A), and remained unchanged in Syn4S179D-expressing cells (Fig. 4.6A). Arf6 activity in Syn4WT cells increased 1.9-fold upon adhesion to Fn; however, both Syn4S179A- and Syn4S179D-expressing cells were unable to induce Arf6 activity in response to matrix engagement, demonstrating that PKCδ modulation of syndecan-4 is important for Arf6 activation (Fig. 4.6B).

In the previous chapter, it was established that phosphorylation of the PKCδ site that modulate phospho-competence of S179, enhanced Src-mediated phosphorylation of Y180. Phosphomimetic Syn4Y180E-expressing cells suppress the activation of Arf6 upon ligand binding (Mark Morgan, unpublished data). Together, these data suggest that PKCδ-mediated regulation of Arf6 activity may be a consequence of modulation of Y180 phosphorylation by Src.

4.3.2 Cell-surface expression and recycling of specific integrin heterodimers

As modulation of Arf6 activity was perturbed in PKCδ-phosphorylation mutants of syndecan-4, and Arf6 regulates heterodimer-specific integrin trafficking, expression of integrin subunits on the cell surface was investigated by flow cytometry. Increased levels of αV were detected in Syn4S179A cells with no significant difference in surface expression of other integrins relative to Syn4WT cells (Fig. 4.7). Cells mimicking constitutive syndecan-4 phosphorylation by PKCδ (Syn4S179D) displayed increased surface expression of α5, β1, αV and β3 integrin subunits but not α4, by a factor of 2.4, 2.05, 3.33 and 1.61, respectively, suggesting either increased expression of integrins or a defect in integrin delivery to the membrane.
Figure 4.5. Expression of syndecan-4 receptor mutants in syndecan-4-null cells.

HA-tagged wild-type human syndecan-4 (Syn4WT) and mutants of the PKCδ-phosphorylation site (Syn4S179A and Syn4S179D) were generated and overexpressed in syndecan-4-null (Syn4-/-) MEFs. Expression of syndecan-4 was monitored by flow cytometry (A), RT-PCR (B) and ELISA capture of surface labelled syndecan-4 using an anti-HA antibody (12CA5) (C). Syn4S179D cells exhibited low levels of syndecan-4 at cell surface as assessed by flow cytometry (A). RT-PCR primers against syndecan-4 cytoplasmic domain demonstrated equal expression of human syndecan-4 in cells transfected with the receptor (B). ELISA revealed similar expression of all syndecan-4 mutants at the cell membrane.
**Figure 4.6. PKCδ-mediated phosphorylation of syndecan-4 regulates Arf6 activation.**

MEFs were spread on 10 µg/ml Fn. GTP bound Arf6 was isolated in PD with GST-GGA3 and Arf6 activity calculated relative to total Arf6 protein levels. Steady-state Arf6 activity was suppressed in cells expressing Syn4S179A receptor following 4 hr spreading on Fn in the presence of 2% FCS (A & B). Induction of Arf6 activity in response to ECM was abolished in both Syn4S179A and Syn4S179D expressing cells (C & D) (n=4). (*p<0.05).

N.B. Dashed line denotes exclusion of additional lanes in the same blot.
Figure 4.7. PKCδ-dependent phosphorylation of syndecan-4 regulates surface expression of Fn receptors, integrin α5β1 and αVβ3.

Cell-surface expression of Fn-binding integrins, α5β1, αVβ3 and α4β1 was assessed using flow cytometry. Levels of α4 remained unchanged. The expression of Syn4S179A promoted αV expression at the cell surface. Expression of the converse syndecan-4 mutant, Syn4S179D, increased surface levels of all integrin subunits (n=5).
To determine whether the changes in surface expression of integrin receptors were a consequence of altered integrin recycling dynamics, the constitutive trafficking of internalised surface-labelled $\alpha_5\beta_1$ and $\alpha V\beta_3$ integrins was monitored. In all instances, delivery of the integrin receptor back to the membrane was very rapid with half of the internalised pool returned by 10 min. Compared to Syn4WT cells, Syn4S179A cells had increased $\alpha V\beta_3$ recycling with no effect seen on $\alpha_5\beta_1$ delivery rates (Fig. 4.8A). Importantly, the expression of a syndecan-4 receptor mimicking constitutive Y180 phosphorylation by Src (Syn4Y180E) (Mark Morgan, unpublished data) or direct suppression of Arf6 activity using siRNA-mediated knockdown, recapitulated the increase in $\alpha V\beta_3$ recycling with no effect on $\alpha_5$ delivery (Fig. 4.8B). Furthermore, expression of Syn4S179D, which suppresses Arf6 activation, demonstrated similar integrin recycling dynamics as Arf6 knockdown or Syn4S179A expression (Fig. 4.8C). Together, these data demonstrate that syndecan-4-dependent Arf6 activity regulates integrin delivery back to the membrane.

An alternative way to study integrin trafficking is by serum stimulation of the recycling pathways. Under serum-stimulated conditions, Syn4S179A-expressing cells exhibited a similar pattern of integrin recycling, relative to Syn4WT cells, as with constitutive (serum-free) conditions: increased $\alpha V\beta_3$ delivery to the membrane and no effect on $\alpha_5\beta_1$ recycling (Fig. 4.9A). However, suppression of Arf6 levels similar to expression of Syn4Y180E (Mark Morgan, unpublished data), had no effect on $\alpha V\beta_3$ recycling but delivery of $\alpha_5\beta_1$ to the membrane was dramatically reduced (Fig. 4.9B).

These results indicated that Syn4S179A cells were unresponsive to serum modulation of syndecan-4-dependent receptor recycling pathways. Similar to Syn4S179A, expression of Syn4S179D induced increased recycling of $\alpha V\beta_3$; however, under these conditions, Syn4S179D cells also exhibited enhanced levels of $\alpha_5\beta_1$ trafficking to the membrane (Fig. 4.9C). These data correlated well with flow cytometry results obtained earlier (Fig. 4.7), where the increased expression of integrins on the cell surface could be explained by increased efficiency in recycling of internalised integrin heterodimers.

These data demonstrate an important role for PKC$\delta$-mediated phosphorylation of syndecan-4 in integrin recycling. The differences observed under serum-stimulated conditions possibly represent additional signals, the response to which differentially regulates $\alpha_5$ recycling in a PKC$\delta$-dependent manner.
Cells expressing Syn4S179A and Syn4S179D are unable to activate Arf6 in response to ECM engagement, yet under serum stimulation produce integrin recycling profiles that differ from the suppression of Arf6 expression or the introduction of Syn4Y180E in cells. The previous chapter demonstrated the importance of PKCδ-mediated phosphorylation of syndecan-4 in regulating Src phosphorylation of Y180. These results indicate a complex role for PKCδ-dependent phosphorylation of syndecan-4 in integrin recycling that may be independent of Y180 phosphorylation by Src and Arf6 activation. However, PKCδ-phosphorylation site mutants of syndecan-4, although promoting Src-mediated Y180 phosphorylation, are susceptible to factors that regulate and localise Src activity. By contrast, Syn4Y180E cells represent a constitutively phosphorylated Src target and any signalling downstream of Y180 phosphorylation will be independent of Src kinase activity.

Further experiments are required to clarify these findings. The introduction of active Src kinase/constitutively active Arf6 in cells expressing PKCδ-phosphorylation site mutants of syndecan-4 will be key in determining whether an alternative syndecan-4-dependent integrin recycling pathway does indeed exist.
Figure 4.8. PKCδ-dependent phosphorylation of syndecan-4 regulates recycling of Fn receptors, integrin α5β1 and αVβ3.

Redelivery of integrins back to the membrane was monitored under constitutive (no serum) conditions at 37°C for 7, 15, 30 and 45 min and calculated as a percentage of the total internalised pool of labelled integrins. Syn4S179A cells, exhibited enhanced redelivery of αVβ3 with no change in α5β1 recycling (A). Suppression of Arf6 expression (B) - by siRNA mediated knockdown – or the expression of Syn4S179D (C), which cannot induce Arf6 activation, recapitulated the enhanced αVβ3 integrin recycling with no change in α5 redelivery as observed with Syn4S179A cells (n=2-3).
Figure 4.9. PKCδ-dependent phosphorylation of syndecan-4 regulates recycling of Fn receptors, integrin α5β1 and αVβ3.

Redelivery of integrins back to the membrane was monitored under serum-stimulated conditions at 37°C for 7, 15, 30 and 45 min and calculated as a percentage of the total internalised pool of labelled integrins. Under serum stimulation Syn4S179A cells, exhibited enhanced redelivery of αVβ3 with no change in α5β1 recycling (A). By contrast, suppression of Arf6 expression - by siRNA mediated knockdown – dramatically reduced α5β1 recycling with no effect on αVβ3 redelivery (B). Expression of Syn4S179D (C) enhanced αVβ3 integrin with additional increase in α5β1 recycling (n=3).
4.4 Phosphorylation of Y197 by Src regulates protein interaction with syndecan-4 PDZ-binding motif

As discussed in the previous chapter, Src-mediated phosphorylation of syndecan-4 occurs at two distinct sites and is dependent on PKCδ modulation of the syndecan-4 receptor. Y180 phosphorylation was enhanced in GST-tagged syndecan-4 constructs containing mutations of the PKCδ-phosphorylation site (S179). Mass spectrometric analysis of Src-phosphorylated GST-syndecan-4 constructs suggested a possible decrease in Y197 phosphorylation in GST-Syn4S179D mutants, suggesting that PKCδ phosphorylation of syndecan-4 may suppress Y197 phosphorylation, in addition to promoting Y180 phosphorylation.

The impact of PKCδ-mediated Y180 phosphorylation in syndecan-4-dependent Arf6 activity and integrin recycling has been examined. The second Src phosphorylation site, Y197, lies within the PDZ-binding motif in the C-terminal domain of syndecan-4 and as such its phosphorylation by Src may influence protein binding to the syndecan-4 receptor. Syntenin, a PDZ-domain containing protein, interacts with syndecan-4 via the PDZ-binding motif of syndecan-4 and has been shown to drive the exit of the receptor from recycling vesicles and deliver syndecan-4 back to the membrane. Therefore the ability of syntenin to bind to various phosphorylation mutants of syndecan-4 was examined. GST-tagged syndecan-4 cytoplasmic domains were incubated with Syn4WT MEF lysate and binding proteins isolated by GST pull-down. Levels of syndecan-4 cytoplasmic domain-associated syntenin were detected by immunoblotting and compared between syndecan-4 mutants. Phosphomimetic mutations of Y197 (GST-Syn4Y197E), which emulate Src-mediated Y197 phosphorylation, had a striking effect on syntenin binding, reducing levels to approximately 10% of wild-type levels (Fig. 4.10). These results indicate that optimal syntenin binding to syndecan-4 PDZ-binding motif is firstly dependent on an intact Y197 residue and that phosphorylation of Y197 may be an important mechanism regulating syndecan-4-syntenin interaction.

Intriguingly, GST-Syn4S179D, a mutant predicted to reduce Src-dependent Y197 phosphorylation, and GST-Syn4Y180E constructs promoted the binding of syntenin compared to wild-type syndecan-4 (GST-Syn4WT) by a factor of 1.7 and 1.56 respectively, whereas binding of syntenin to GST-Syn4S179A remained unaffected. The phospho-null Syn4Y180L mutant reduced binding of syntenin by 64%. These results
indicate a role for PKCδ phosphorylation of syndecan-4 in regulating protein binding to PDZ motif of syndecan-4 (Fig. 4.10). However, whether this is a consequence of direct modulation of Y197 phosphorylation by Src or a result of increased phosphorylation of Y180 residue remains to be determined.
Figure 4.10. PKCδ-dependent phosphorylation of syndecan-4 promotes syntenin binding to GST-tagged syndecan-4 cytoplasmic domain.

Syndecan-4 binding partners were isolated in pull-down with GST-tagged syndecan-4 cytoplasmic domains. Syntenin binding to GST-Syn4Y197E was suppressed (A & C) and enhanced to GST-Syn4S179D and GST-Syn4Y180E constructs (B & C) (n=3). Dashed line denotes exclusion of additional lanes in the same blot. (*p<0.05, **p<0.01, ***p<0.001).
4.5 Syntenin-syndecan-4 interaction: regulation of Arf6 activity and integrin recycling

To investigate the function of syntenin-syndecan-4 interactions, a cell line was utilised in which the C-terminal amino acid residue in the PDZ-binding motif is deleted (Syn4ΔPDZ), thereby preventing interaction between syndecan-4 and PDZ-binding proteins.

4.5.1 Regulation of Arf6 activity

Arf6 activity in Syn4ΔPDZ- and Syn4WT-expressing cells was determined by effector pull-down assay. No change in steady-state activity was observed (Fig. 4.11A and B); however, unlike Syn4S179A and Syn4S179D cells, expression of Syn4ΔPDZ resulted in enhanced Arf6 activity in the absence of ECM engagement and Syn4ΔPDZ cells were unable to induce activation of Arf6 beyond these constitutively high levels (Fig. 4.11C and D).

4.5.2 Cell-surface expression and recycling of specific integrin heterodimers

Given that cells expressing Syn4ΔPDZ had elevated Arf6 activity, cell-surface expression and integrin recycling were investigated. Surprisingly, similar to Syn4S179D cells which exhibited suppressed Arf6 activity, αVβ3 surface levels were elevated in Syn4ΔPDZ cells and corresponded to an apparently small increase in redelivery of αVβ3 back to the membrane. By contrast, surface levels of α5 remained unchanged although β1 expression and the recycling of α5 appeared to be increased (Figs. 4.12 & 4.13).

4.5.3 Syndecan-4-dependent endocytosis of integrin heterodimers

Preliminary experiments monitoring constitutive receptor internalisation revealed a defect in endocytosis of all integrin receptors in Syn4ΔPDZ cells (Fig. 4.14). This decrease in integrin endocytosis may be a contributing factor to the increased surface expression of integrins observed by flow cytometry. Furthermore, as the internalised pool of labelled integrin is so dramatically reduced, it may denigrate the recycling data, that is any interpretation of recycling rates is difficult. However, these data do highlight the importance of syndecan-4-PDZ-binding protein interactions in regulating integrin endocytosis.
Figure 4.11. Arf6 is constitutively activated in cells expressing syndecan-4 mutant lacking PDZ-binding motif.

MEFs were spread on 10 µg/ml Fn. GTP bound Arf6 was isolated in PD with GST-GGA3 and Arf6 activity calculated relative to total Arf6 protein levels. No change in steady-state Arf6 activity was observed in cells expressing Syn4ΔPDZ following 4 hr spreading on Fn in the presence of 2% FCS (A & B). However, Syn4ΔPDZ cells exhibited elevated Arf6 activity in suspension, similar to ECM-induced Arf6 levels observed with Syn4WT cells spread on Fn for 1hr. Unlike Syn4WT cells, Syn4ΔPDZ expressing cells were unable to induce Arf6 activity further upon Fn engagement (C & D) (n=4). (*p<0.05).
Figure 4.12. Disrupting the PDZ-binding motif of syndecan-4 alters surface expression of Fn receptors, α5β1 and αVβ3 integrins.

Cell-surface expression of Fn-binding integrins, α5β1, αVβ3 and α4β1 was assessed using flow cytometry. Similar to Syn4S179D cells, the expression of Syn4DPDZ increased surface levels of αV and β3 integrins. Expression of α5 remained at Syn4WT levels. However, β1 cell-surface expression appeared to be increased (n=5).
Figure 4.13. Disrupting the PDZ-binding motif of syndecan-4 enhances recycling of Fn receptors, integrin α5β1 and αVβ3.

Redelivery of integrins back to the membrane was monitored under serum-stimulated conditions at 37°C for 7, 15, 30 and 45 min and calculated as a percentage of the total internalised pool of labelled integrins. Expression of Syn4ΔPDZ receptor, enhanced redelivery of αV, β3 and α5 to the membrane (n=2).
The internalisation of integrin receptors was further investigated in cells expressing Syn4S179A and Syn4S179D syndecan-4 receptors. No effect was seen on αV or β3 internalisation; however, α5 endocytosis was inhibited in Syn4S179D cells similarly to Syn4ΔPDZ expressing cells, and to a lesser extent in Syn4S179A cells. These results imply that PKCδ-mediated phosphorylation of syndecan-4 may be an important modulator of α5β1 internalisation as well as recycling (Fig. 4.14).

The internalisation of syndecan-4 was also substantially reduced in Syn4ΔPDZ expressing cells suggesting a direct link between mechanisms regulating syndecan-4 internalisation and the syndecan-4-mediated integrin endocytosis (Fig. 4.15).
Figure 4.14. Disrupting the PDZ-binding motif of syndecan-4 inhibits integrin receptor internalisation.

Internalisation of surface labelled integrin receptors was triggered in the absence of serum at 37°C, for 30 min and the total internalised pool was assessed. Dramatic defects in endocytosis of all integrins was observed with Syn4ΔPDZ mutants (A), whereas, expression of PKCa-phosphorylation site mutants of syndecan-4 reduced α5 internalisation rate only (n=3) (B & C).
Figure 4.15. Disrupting the PDZ-binding motif of syndecan-4 inhibits syndecan-4 internalisation.

Internalisation of surface labelled syndecan-4 receptor was triggered in the absence of serum at 37°C, for 30 min and the total internalised pool was assessed. The rate of syndecan-4 endocytosis was substantially reduced in Syn4ΔPDZ expressing cells (A), whereas, expression of PKCδ-phosphorylation site mutants had no significant effect (B & C) (n=3).
4.6 Conclusions and discussion part II

The data in this chapter demonstrate the Arf6-dependence of both α5β1 and αVβ3 integrin recycling. Suppression of Arf6 activity by either siRNA-mediated knockdown or expression of Syn4Y180E, that emulates constitutive Src-mediated phosphorylation of syndecan-4 at Y180, enhancing αVβ3 redelivery back to the membrane under constitutive (serum-free) conditions. The expression of PKCδ-phosphorylation site mutants of syndecan-4, Syn4S179A and Syn4S179D, that increase phosphorylation of Y180 (determined by GST constructs) inhibits Arf6 activation in response to ECM engagement and similarly enhances αVβ3 recycling with no effect on α5β1 membrane delivery under constitutive conditions. These results indicate that PKCδ-mediated phosphorylation of syndecan-4 may be an upstream control point for Arf6-dependent integrin recycling.

However, in Arf6-knockdown and Syn4Y180E cells, integrin recycling was susceptible to serum stimulation and resulted in suppressed α5β1 integrin recycling with no change in αVβ3. Conversely, Syn4S179A expressing cells were unable to modulate integrin recycling in response to serum. Similarly, under serum stimulation, Syn4S179D cells retained the enhanced αVβ3 recycling observed in constitutive recycling; however, serum stimulation enhanced α5β1 redelivery back to the membrane. The phosphorylation of syndecan-4 by PKCδ may therefore regulate α5 recycling independently of Arf6 activity downstream of growth factor stimulation. The mechanism for this regulation however remains unclear.

One potential explanation for the differences observed in integrin recycling in Syn4S179A, Syn4S179D and Syn4Y180E cells, is the modulation of Y180 by Src. Syn4S179A and Syn4S179D are susceptible to factors that modulate Src activity and localisation, whereas Syn4Y180E represents a constitutively Src-phosphorylated target. Furthermore, Src-dependent phosphorylation of an alternative site within syndecan-4 cytoplasmic domain (Y197) was demonstrated in the previous chapter. The phosphorylation of this residue (GST-Syn4Y197E) dramatically reduced binding of syntenin to the syndecan-4 cytoplasmic domain. Syntenin binding was also enhanced in mutants predicted to have reduced Y197 phosphorylation (GST-Syn4S179D). Syntenin plays a key role in syndecan-4 exocytosis; thus, Src-mediated phosphorylation of Y197 may be an important mechanism that restricts syndecan-4 signalling by regulating syndecan-4 membrane delivery. Moreover, cells expressing a syndecan-4 mutant, Syn4ΔPDZ, which is unable to bind
PDZ-domain containing proteins such as syntenin, exhibits defects in syndecan-4 internalisation. Intriguingly, integrin endocytosis is also dramatically reduced, suggesting a common mechanism underlying the internalisation of syndecan-4 and integrin heterodimers. The accumulation of syndecan-4 and β1 integrins in the same recycling compartments - following mutations of syntenin - further demonstrate this possibility. The elevated Arf6 activity observed in Syn4ΔPDZ in the absence of matrix engagement may also indicate that interactions at the PDZ-binding motif of syndecan-4 are crucial for regulating Arf6 activity in response to ECM signals.

Together, these data indicate that integration of adhesion signals is important in regulating syndecan-4-dependent Arf6 activity to influence receptor recycling and that PKCδ-mediated phosphorylation of syndecan-4 may be a key locus for integration of adhesion- and growth factor-dependent signals (Fig. 4.16). The balance between of α5β1 and αVβ3 membrane expression coordinates ECM signals, to regulate force transmission, cytoskeletal reorganisation and membrane protrusion and directs cell migration in response to extracellular cues (Bershadsky et al., 2003; Danen et al., 2005; Roca-Cusachs et al., 2009).
Figure 4.16. Model: The spatiotemporal control of PKCδ activity functions to coordinate differential tyrosine phosphorylation of syndecan-4 in order to restrict Src-specific intracellular signals. Src tyrosine kinase directly phosphorylates syndecan-4 cytoplasmic domain on Y180 and Y197 to regulate Arf6 activity (A). PKCδ-mediated syndecan-4 phosphorylation promotes Src-dependent Y180 phosphorylation and suppresses Src-dependent Y197 phosphorylation (B & C). Enhanced phospho-Y180 inhibits Arf6 activity (B). Reduced phospho-Y197 promotes syntenin binding to syndecan-4 cytoplasmic domain (C).
5. PKCδ-mediated phosphorylation of syndecan-4: Regulation of Rho GTPases and cell migration

Aims and background:

Rho GTPase family members are key modulators of cell migration that provide a signalling network linking receptor engagement to organisation of the actin cytoskeleton (Nobes and Hall, 1995a). Formation of nascent adhesion contacts and regulation of cytoskeletal remodelling are mediated primarily by 3 members of the Rho GTPase family, RhoA, Rac1 and Cdc42. Formation of focal complexes and membrane protrusions is controlled by Rac1, whereas RhoA regulates formation of FAs, promotes actin stress fibre assembly and tail retraction. For efficient cell migration, the opposing roles of Rac1 and RhoA must be tightly regulated in a spatial and temporal manner and this, in part, is coordinated by engagement of adhesion receptors such as integrins and syndecans.

Work in this laboratory has demonstrated the role of syndecan-4 in localising Rac1 activity to the leading edge of the cell, leading to persistent cell migration. Furthermore the engagement of both α5β1 integrin and syndecan-4 is required for transient activation of Rac1 in response to the ECM (Bass et al., 2007b). By contrast, RhoA activation is suppressed upon at early stages of cell spreading on Fn or as a consequence of syndecan-4 stimulation. The early suppression of RhoA activity is followed by a subsequent reactivation of RhoA. The RhoA activity profile during spreading on Fn is regulated by syndecan-4-dependent redistribution of p190RhoGAP (a negative regulator of RhoA function) to the cell membrane. Importantly, PKCα activation is fundamental in syndecan-4-dependent control of Rac1 and RhoA function (Bass et al., 2007b; Dovas et al., 2006). PKCα binds to the variable domain of syndecan-4 and this interaction is unique to syndecan-4 and absent in other syndecan family members. Expression of a mutant syndecan-4 defective in PKCα binding (Syn4Y188L), or suppression of PKCα expression, renders MEFs incapable of activating Rac1 or redistributing p190RhoGAP and thus perturbs cell migration (Bass et al., 2007a; Bass et al., 2008).

It has been suggested that oligomerisation of the syndecan-4 receptor and the formation of a ternary syndecan-4-PIP2-PKCα complex leads to the superactivation of PKCα (Murakami et al., 2002). Interestingly, the use of recombinant syndecan-4 mutants has suggested that PKCδ-mediated phosphorylation of syndecan-4 may be involved in
regulating PKCα activity. In these studies, phosphorylation of the equivalent PKCδ site in rat syndecan-4 (S183) suppressed PKCα activity in a PIP2-dependent manner (Murakami et al., 2002). However, the role of syndecan-4 in regulating PKCα activity in cells and biological consequences of this regulation especially in response to syndecan-4-ECM interaction has not been established.

Stimulation of PKCα has been shown to increase dynamin-dependent endocytosis of β1 integrins leading to enhanced cell migration in carcinoma cells (Ng et al., 1999). In the previous chapter a potential role for PKCδ-mediated phosphorylation of syndecan-4 in integrin recycling as a consequence of dysregulated Arf6 activity was established. The data suggested that the control of integrin surface expression by syndecan-4 and thus cell migration may involve signalling downstream of both syndecan-4-dependent PKCα activation and Arf6 activity. Several studies have implicated Arf6 activity in Rac1-mediated processes (Radhakrishna et al., 1999; Santy et al., 2005; Wong and Isberg, 2003) suggesting that PKCδ phosphorylation of syndecan-4 may be a nexus for a multitude of signalling pathways regulating cell-surface dynamics, actin organisation and cell migration.

In this chapter the aim was to first determine the activation profile of PKCα following stimulation of syndecan-4 by ECM ligand, second, to examine the consequences of PKCδ-mediated phosphorylation of syndecan-4 on PKCα and Rho GTPase activation, and third, to investigate the effect of perturbing PKCδ-mediated phosphorylation of syndecan-4 on FA formation and cell migration.

### 5.1 Syndecan-4-dependent PKCα activity

PKCα activity was investigated by measuring phosphorylation of a PKC substrate, myelin basic protein (MBP), in the presence of [γ-33P ATP] as a phosphate donor. First, recombinant active PKCα was utilised to optimise the phospholipid environment that is required for maintaining PKCα kinase activation. Active PKCα (10 ng/μl) was incubated with MBP (40 μg/ml), and a mixture of lipids that included either 1 mg/ml phosphatidylserine (PS) and 1 ng/ml TPA - or 0.1mg/ml PS and 0.01mg/ml DAG (lipid activator mix) for the recombinant active PKCα activity assay. Phosphorylation of MBP was visualised by radio-labelling and used to measure the activity of the recombinant PKCα. Phosphorylation of MBP was enhanced in the presence of 1x lipid activator (Fig. Results III 165
5.1 A), rather than 1 mg/ml PS and 1 ng/ml TPA. Therefore, 1x lipid activator was used in subsequent experiments to analyse PKCα activity.

5.1.1 Kinase activity in the presence of total cell lysates

To test kinase activity in cells, MEFs expressing Syn4WT or Syn4S179A were spread on Fn for 2hrs and lysates incubated with MBP as described above. Phosphorylation of MBP was detected in presence of cell lysates. TPA treatment of Syn4WT cells prior to lysis increased MBP phosphorylation, whereas the use of a PKC pharmacological inhibitor (BIM) resulted in inhibition of MBP phosphorylation, consistent with a role for PKC activity (Fig. 5.1A and B). Surprisingly, MBP phosphorylation levels appeared to be reduced in Syn4S179A-expressing MEFs, indicating an involvement for S179 in PKC activity modulation (Fig. 5.1A and B). However, the role of other protein kinases in MBP phosphorylation cannot be ruled out in these experiments. To determine the contributions of PKCα activity to MBP phosphorylation requires further refinement of these assays.

5.1.2 Immunoprecipitation of PKCα

To examine activity of PKCα in cells directly, it was necessary to immunoprecipitate PKCα from cell lysates, prior to incubation with MBP. Optimal conditions for PKCα immunoprecipitation were determined by either pre-coating protein G beads with antibody or prior incubation of lysate with antibody. Immunoprecipitation was carried out for 2 hrs or overnight at 4°C (Fig. 5.2A & B). Maximal levels of PKCα immunoprecipitation were obtained when beads were pre-coated with 1 µg of anti-PKC antibody (MC5) for 30 min at 4°C, followed by 2 hrs immunoprecipitation (Fig. 5.2B, condition 2a). Accordingly, this condition was used for all subsequent PKCα activity assays.

5.1.3 PKCα is transiently activated in response to syndecan-4 engagement and is suppressed in Syn4S179A expressing cells

PKCα activity was monitored in MEFs spread on 50K (a Fn fragment containing the α5β1 integrin-binding site), i.e. in the absence of syndecan-4 ligation, or on Fn for 2 hrs. Syn4S179A-expressing cells exhibited a substantial reduction in PKCα activity, to approximately 45% and 38% of wild-type Syn4 cells, on 50K and Fn, respectively (Fig. 5.3). This is consistent with initial observations of reduced total kinase activity in...
Syn4S179A cell lysates (Fig. 5.1) and indicates a specific role for syndecan-4 in PKCα activation.
Figure 5.1. Inhibiting PKCδ-mediated phosphorylation of syndecan-4 suppresses serine/threonine kinase activity.

Recombinant PKCα activity in the presence of different phospholipid constituents was analysed by monitoring PKC-dependent phosphorylation of MBP substrate (A). Enhanced phosphorylation of MBP was observed in the presence of lipid activator mix as detected by radio-labelling (A). Analysis of serine/threonine kinase activity in cell lysates revealed decreased MBP phosphorylation in Syn4S179A-expressing cells (B). No kinase activity was detected in the absence of lysate (B).
**Figure 5.2. Immunoprecipitation of PKCα for direct analysis of kinase activity.**

PKCα was immunoprecipitated from MEFs expressing Syn4WT under different IP conditions (outlined in A). Preincubation of protein G beads with anti-PKC antibody (MC5) (30 min, 4°C) followed by 2 hr IP was chosen as optimal IP conditions for isolation of PKCα from cell lysates (B). Blue arrows indicate PKCα in IP samples (B) and remaining PKCα in lysate following IP (C) (MW 78KDa).
Figure 5.3. Disrupting PKCδ-mediated phosphorylation of syndecan-4 suppresses basal PKCα activity.

MEFs were spread on 10 μg/ml 50K or Fn. PKCα was immunoprecipitated, incubated with MBP and phosphorylation levels detected by radio-labelling. Syn4S179A-expressing cells exhibited suppressed basal PKCα activity on 50K and Fn ligands, compared to syndecan-4 WT-expressing cells (Syn4) (A, B and quantified in C). (n=1-4). (*p<0.05, **p<0.01).
Syndecan-4 has been implicated in activation of PKCα in vitro (Horowitz and Simons, 1998a); however, the specific effect of syndecan-4 engagement on PKCα activity has not been established. This is surprising considering that syndecan-4-mediated regulation of PKCα activity has been studied widely in FA formation, GTPase activity and cell migration (Bass et al., 2007b; Dovas et al., 2006).

To determine the effect of syndecan-4 engagement on PKCα activity, wild-type Syn4 or Syn4S179A-expressing cells, plated on 50K, were stimulated with H/0, a soluble Fn fragment encompassing binding sites for syndecan-4. The profile of PKCα activity was established at 0, 10, 30, 60 and 90 mins post-stimulation. Syndecan-4 engagement induced a transient wave of PKCα activation in wild-type Syn4-expressing cells with a distinct peak of activity observed at 30 min representing approximately a 2.4-fold increase from basal (0 min) activity (Fig. 5.4A, C & D). By contrast, expression of Syn4S179A suppressed the rate and magnitude of PKCα activation in response to H/0 (Fig. 5.4B, C & E). Thus, the already low basal levels of PKCα activity in Syn4S179A cells were only increased 1.6-fold (Fig. 5.4B & C).

These data show for the first time that syndecan-4 engagement specifically induces PKCα activation and that S179 (PKCδ-phosphorylation site) is important for the regulation of syndecan-4-specific PKCα activity.

5.2 Abrogating PKCδ phosphorylation of syndecan-4 suppresses Rac1 but not RhoA activation

Engagement of syndecan-4 by ECM is necessary for activation of the Rho GTPases Rac1 and RhoA. Syn4-/- cells have constitutively high Rac1 activity and an inability to regulate Rac1 in response to Fn engagement. Expression of wild-type syndecan-4 leads to a transient wave of Rac1 activity that is dependent on syndecan-4-PKCα binding (Bass et al., 2007b).

As Syn4S179A cells exhibited suppressed PKCα activation their ability to modulate Rac1 activation was investigated. To establish the contributions made by syndecan-4 to activation of Rac1, MEFs were initially spread on 50K for 2 hrs in the absence of syndecan-4 ligation and basal Rac1 activity was assessed using an effector (PAK1) pull-down assay. Suppression of PKCδ expression by siRNA knockdown (as described in Fig.
4.1B) decreased basal Rac1 activity to 63% of control siRNA-treated cells (Fig. 5.5A). Furthermore, expression of Syn4S179A, recapitulated the suppressed basal Rac1 GTP levels observed with PKCδ knockdown (Fig. 5.5B), consistent with a requirement for PKCα activity for Rac1 activation. By contrast, Rac1 activity was enhanced by expression of Syn4S179D (1.8-fold increase above levels observed in Syn4WT expressing cells) (Fig. 5.5C). These data indicate that PKCδ-mediated phosphorylation of syndecan-4 promotes Rac1 activity.

To examine the effect of syndecan-4 ligation on activation of Rac1, MEFs expressing Syn4WT or Syn4S179A were spread on 50K for 2 hrs and stimulated with H/0. Rac1 GTP levels were monitored at 0, 10, 30, 60, 90 and 120 min post syndecan-4 stimulation. As previously described (Bass et al., 2007b), engagement of syndecan-4 resulted in transient activation of Rac1 with a peak at 10 min in Syn4WT cells (Fig. 5.5D & E). Similarly, Syn4S179A cells also exhibited a transient wave of Rac1 activation (Fig. 5D & F). However, due to low levels of basal Rac activity, engagement of Syn4S179A failed to restore Rac1 activation to Syn4WT levels (Fig. 5.5D).

Syndecan-4 regulation of RhoA activity has also been demonstrated to occur in a PKCα-dependent manner (Bass et al., 2008; Dovas et al., 2006). RhoA activity was therefore investigated in Syn4S179A cells that exhibit suppressed PKCα activity. Basal RhoA activity was monitored using a Rho GTP effector pull-down assay following 4 hrs spreading on Fn. In contrast to the dramatic effects seen with Rac1 activation, basal RhoA levels remained unaffected upon expression of Syn4S179A (Fig. 5.6)

These data may suggest that syndecan-4-mediated regulation of Rac1 activity is independent of its ability to modulate RhoA, and that PKCδ-mediated phosphorylation of syndecan-4 may not affect syndecan-4-dependent RhoA activity. However, to understand this fully, further experiments are required to examine the profile of RhoA activation following syndecan-4 stimulation.
Figure 5.4. Syndecan-4 engagement by ECM regulates PKCα activation and is dependent on the integrity of the PKCδ-phosphorylation site within syndecan-4 cytoplasmic domain.

MEFs were spread on 10 µg/ml 50K and PKCα activity monitored following H/0 stimulation of syndecan-4 at 0, 10, 30, 60 and 90 min. Syn4 cells induced a transient wave of PKCα activation (peak at 30 min) (A, C & D), that was suppressed in Syn4S179A-expressing cells (B, C & E).
Figure 5.5. PKCδ-mediated phosphorylation of syndecan-4 regulates Rac1 activity.

MEFs were spread on 10 µg/ml 50K. GTP-bound Rac1 was isolated in PD with GST-PAK and activity calculated relative to total Rac1 levels. Suppression of PKCδ expression (A) or the expression of Syn4S179A (B) reduced basal Rac1 activity. Conversely, expression of Syn4S179D enhanced basal Rac1 activity (C), compared to Syn4WT cells. H/0 stimulation of syndecan-4 induced a wave of Rac1 activity in Syn4WT cells (peak at 10 min) (D & E) that was suppressed in Syn4S179A-expressing cells. (*p<0.05, **p<0.01).
Figure 5.6. Disrupting PKCδ-mediated phosphorylation of syndecan-4 does not affect basal RhoA activity.

MEFs were spread on 10 µg/ml 50K. GTP-bound RhoA was isolated in ELISA assay and activity calculated relative to total RhoA protein. Basal RhoA activity was unaffected in cells expressing Syn4S179A. Syn4WT cells treated with C3 botulinum toxin (potent inhibitor of RhoA activity) was used as negative control.
5.3 Focal adhesion formation is enhanced in the absence of PKCδ-mediated syndecan-4 phosphorylation

Coordinated activation of Rac1 and RhoA is fundamental for FAs formation and regulation of cell migration. Therefore to determine the importance of PKCδ-dependent syndecan-4 regulation of PKCα and Rac1 activity, the ability of cells to form FAs and to migrate efficiently on CDMs was investigated next.

The requirement for dual engagement of α5β1 and syndecan-4 in the formation of FAs is well established. In the absence of syndecan-4 ligation, MEFs spread on 50K but fail to organise their actin cytoskeleton into stress fibres and form FAs. FA formation requires dynamic regulation of Rho GTPases Rac1 and RhoA. As demonstrated, cells unable to modulate PKCα activity also fail to activate Rac1 fully and PKCδ-dependent phosphorylation of syndecan-4 appears to be key to regulating these processes (Fig. 5.5). Therefore the role of PKCδ-mediated phosphorylation of syndecan-4 in FA formation was examined.

Expression of PKCδ was suppressed by siRNA-mediated knockdown (as described in Fig. 4.1B) and MEFs were plated on 50K for 2 hrs and then stimulated, for 60 min, in the absence or presence of H/0. Adhesion area was calculated prior to and post syndecan-4 stimulation. Control siRNA-treated wild-type Syn4 cells displayed very low levels of adhesion complex formation in the absence of H/0, as described previously (Bass et al., 2007a; Bass et al., 2007b). However, suppression of PKCδ expression resulted in the appearance of small vinculin-containing focal complexes even in cells spread on 50K alone. Moreover, stimulation of PKCδ knockdown cells with H/0 induced maturation of these complexes into larger FAs (Fig. 5.7). Quantification of adhesion area demonstrated that FA area was augmented in PKCδ-suppressed cells on both 50K and upon syndecan-4 engagement (Fig. 5.8). Significantly, PKCδ knockdown in Syn4/- cells did not promote FA formation. These data indicate that the effects of PKCδ knockdown were dependent on syndecan-4 signalling (Fig. 5.7 and quantified in Fig. 5.8). Furthermore, direct inhibition of PKCδ-mediated phosphorylation of syndecan-4 by expression of SynS179A resulted in enhanced adhesion complex formation on 50K and H/0 similar to the effect of PKCδ knockdown in Syn4WT cells (Fig. 5.9), providing further evidence for the role of PKCδ-mediated phosphorylation of syndecan-4 in regulation of FA formation.
Figure 5.7. Suppression of PKCδ expression enhances FA formation in a syndecan-4-dependent manner.

Ctrl siRNA-treated MEFs were spread on 50K and formed FAs only in the presence of WT-syndecan-4 receptor (Syn4WT) stimulated with H/0. Suppression of PKCδ expression in Syn4WT cells induced formation of small adhesions in the absence of syndecan-4 stimulation (yellow arrows). These adhesions were further enhanced upon syndecan-4 stimulation with H/0 (white arrows represent FA formation in response to H/0).
Figure 5.8. Suppression of PKCc expression enhances FA formation in a syndecan-4-dependent manner.

Adhesion area was quantified for 20 cells per condition using Image J. Suppressed PKCc expression in Sy4WT cells resulted in increased adhesion area on 50K and upon H/0 stimulation, as compared to Ctrl-treated cells. Ctrl and PKCc siRNA-treated Syn4-/- cells failed to form FAs on 50K or following H/0 stimulation. (*p<0.05, ***p<0.001, ****p<0.0001).
Figure 5.9. Disrupting PKCδ-mediated phosphorylation of syndecan-4 enhances FA formation.

Ctrl siRNA-treated MEFs were spread on 50K and formed FAs upon H/0 stimulation. Suppression of PKCδ expression in Syn4 cells, or the expression of Syn4S179A, induced formation of small adhesions in the absence of syndecan-4 stimulation (yellow arrows). These adhesions were further enhanced upon syndecan-4 stimulation with H/0 (white arrows represent FA formation in response to H/0).
5.4 PKCδ-mediated phosphorylation of syndecan-4 regulates directional cell migration

Efficient cell migration requires tight regulation of Rac1 and RhoA activity leading to dynamic and controlled adhesion turnover. A role for PKCδ-mediated phosphorylation of syndecan-4 in Rac1 activity and FA formation was identified here (see section 5.2 and 5.3). Thus, it is proposed that PKCδ-mediated syndecan-4 phosphorylation is a key mechanism for regulating cell migration.

As described previously, Syn4-/- and Syn4 WT-expressing cells migrating across 2D Fn substrate (Fn coated from solution) show no significant differences in migration speed (µm/min) and persistence (linear displacement (µm) divided by total distance travelled (µm)). Indeed, Syn4-/- and Syn4 WT cells migrate randomly and extend multiple protrusions (Bass et al., 2007b and Fig. 5.10). However, when cells are allowed to migrate across a fibrillar cell-derived matrix (CDM) (Cukierman et al., 2001), Syn4 WT cells adopt an elongated morphology, extending one or two dominant lamellae and migrate in a directionally-persistent manner along Fn fibrils. Importantly, this switch from random to directionally-persistent migration is dependent on an intact syndecan-4 receptor (Bass et al., 2007b and Fig. 5.11) and an ability to localise Rac1 activity to leading lamella (Bass et al., 2007b; Pankov et al., 2005).

To assess the role of PKCδ-mediated syndecan-4 phosphorylation in cell migration, Syn4S179A-expressing cells were allowed to migrate across 2D Fn and CDMs. Similar to Syn4 WT cells, Syn4S179A-expressing cells migrated randomly across 2D Fn; however, cells expressing Syn4S179A exhibited a highly dynamic membrane and quantification of the number of protrusions revealed that 80% of the cells examined had seven or more protrusions over the migration time course, compared to 5% and 15% in Syn4 WT and Syn4-/- cells, respectively (Fig. 5.10C). Furthermore, expression of Syn4S179A failed to restore persistent migration on a fibrillar matrix. However, unlike Syn4-/- cells, Syn4S179A-expressing cells displayed a dramatic decrease in migration speed on CDMs (Fig. 5.11). Together, these results suggest that dynamic phosphorylation of syndecan-4 by PKCδ is vital for efficient cell migration.
Figure 5.10. Cell migration on 2D Fn substrate.

MEFs were plated on Fn-coated dishes (10 µg/ml) and filmed for 20 hrs. Cells were manually tracked and average persistence (A) and speed (µm/min) of migration calculated (B). Syn4WT, Syn4-/− and Syn4S179A cells routinely showed no substantial differences in speed and persistence of migration (A & B). However, a manual count of average number of membrane protrusions demonstrated that Syn4S179A expressing cells had a highly dynamic membrane (C).

The above is a representative experiment (n=2). For each cell type approximately 90 cells were tracked.
Figure 5.11. Cell migration on 3D fibrillar matrix.

MEFs were plated on CDMs and filmed for 20 hrs. Cells were manually tracked across Fn fibrills (A) and average persistence (B) and speed of migration (µm/min) (C) calculated. Syn4WT-expressing cells migrated in a directionally-persistent manner (B and cell tracks are depicted in D), whereas Syn4-/- cells and Syn4S179A-expressing cells failed to migrate persistently across CDMs (B and cell tracks are depicted in D).

The above is a representative experiment (n=2). For each cell type approximately 90 cells were tracked. (**p<0.01, ****p<0.0001).
5.5 Conclusions and discussion part III

It has been thought for some time that syndecan-4 function modulates PKCα activity and that this regulation controls FA formation and cell migration. However, the dynamics of syndecan-4-mediated PKCα activity modulation have never been studied in cells. Here it was demonstrated that syndecan-4 engagement specifically induces a transient wave of PKCα activation. Furthermore, the wave of PKCα activity observed in wild-type Syn4 MEFs was dependent on an intact PKCδ-phosphorylation site, S179, in the syndecan-4 cytoplasmic domain. Cells expressing a phospho-null mutant of S179 (Syn4S179A) exhibited suppressed PKCα activity in the absence of syndecan-4 ligation. Moreover, stimulation of Syn4S179A with H/0 failed to restore PKCα activity to wild-type levels.

The suppression of PKCα activation in cells expressing Syn4S179A was unexpected, as previous studies have suggested that syndecan-4-dependent PKCα activity would be negatively regulated by PKCδ-mediated phosphorylation of syndecan-4. In these studies, recombinant PKCα activity was monitored first in in vitro kinase assays in the presence of PIP2 and syndecan-4 cytoplasmic domain peptides, with and without phosphorylation at S179. In subsequent studies, recombinant PKCα activity was assessed in the presence of syndecan-4 immunoprecipitated from endothelial cells overexpressing PKCδ mutants. Here overexpression of wild-type PKCδ suppressed PKCα activity, whereas dominant negative PKCδ led to increased recombinant PKCα activity (Murakami et al., 2002). These studies, although demonstrating a role for PKCδ-mediated regulation of syndecan-4-PKCα activity, did not investigate the specific role of matrix engagement in modulating syndecan-4-specific PKCα activation. Furthermore, endogenous PKCα activity in response to PKCδ overexpression was never established. As PKCα is an important kinase, required for formation of FAs downstream of syndecan-4 receptor clustering (Bass et al., 2007b, Dovas et al., 2006) it is important to understand how interaction of cell-surface receptors with ECM directly regulates its kinase activity within cells.

PKCα binding mutants of syndecan-4 exhibit suppressed Rac1 activity and are incapable of modulating Rac1 in response to ECM (Bass et al., 2007b), indicating that Rac1 activation is downstream of syndecan-4-dependent PKCα activation. Here it was shown that syndecan-4-dependent PKCα activity in cells is abrogated by the prevention of PKCδ-dependent phosphorylation of syndecan-4. Expression of Syn4S179A or siRNA-mediated knockdown of PKCδ resulted in suppressed Rac1 activity that correlated well with the
inability of Syn4S179A cells to modulate PKCα activation in response to ligand engagement. Together, these data suggest that the wave of PKCα activation is a prerequisite for transient activation of Rac1 observed in response to syndecan-4 engagement and is regulated by PKCδ-mediated phosphorylation of syndecan-4 (Fig. 5.12).

Rac1 activity promotes formation of focal complexes and membrane protrusions (Burridge and Wennerberg, 2004). Surprisingly, Syn4S179A cells that have suppressed Rac1 activation exhibit enhanced adhesion formation. The reasons for these contrasting effects on Rac1 activation and adhesion formation are unclear. It is possible that RhoA signalling or localisation are altered in the absence of antagonistic Rac1 signalling and thus drive FA formation, but this requires further investigation.

PKCα activation, Rac1 activity and the regulation of adhesion contacts are fundamentally involved in regulation of cell migration and the maintenance of directional persistence. Thus, the expression of a syndecan-4 receptor which is resistant to PKCδ-dependent phosphorylation suppresses signals that are required for directional persistence. Therefore, the inability of Syn4S179A-expressing cells to migrate persistently may represent defects in PKCα and Rac1 activation in response to ECM engagement. However, as demonstrated in the previous chapters, PKCδ-mediated phosphorylation of syndecan-4 influences differential syndecan-4 phosphorylation by Src, Arf6 activity (a GTPase involved in membrane remodelling), heterodimer-specific integrin recycling and syndecan-4-syntenin binding. Therefore, it is likely that a combination of these factors contribute to cell migration. Moreover, it is likely that there is a high level of cross-regulation between each of these processes. The challenge now will be to understand precisely how all of these processes are integrated in a spatially and temporally controlled manner.
Figure 5.12. PKC\(\delta\)-mediated phosphorylation of syndecan-4 regulates PKC\(\alpha\) and Rac1 activation. Dynamic phosphorylation of syndecan-4 by PKC\(\delta\) is required for regulation of syndecan-4-specific PKC\(\alpha\) activity. Inhibition of PKC\(\delta\)-mediated phosphorylation of syndecan-4 leads to suppressed PKC\(\alpha\) and Rac1 activation. Conversely, PKC\(\delta\)-mediated syndecan-4 phosphorylation results in enhanced Rac1 activation.
6. Discussion

Transmembrane receptors, such as integrins and syndecans, provide the principal interface between cells and their extracellular environment. The intracellular domains of these adhesion receptors provide a physical link between the contractile actin cytoskeleton and ECM, and trigger long range signalling events within the cell to control cell survival, proliferation and migration.

The synergistic relationship between integrins and syndecans has been appreciated for several years. Formation of FAs, regulation of Rho GTPase activity and directional cell migration are dependent on dual engagement of Fn receptors, α5β1 integrin and syndecan-4 (Bass et al., 2008; Bass et al., 2007b; Morgan et al., 2007). Recent work has also indicated a role for syndecan-4 clustering, by a laminin peptide, in β1-integrin activation and keratinocyte cell migration (Araki et al., 2009). These data, combined with the ubiquitous expression pattern of syndecan-4 indicate a central role for the syndecan-4 receptor as a regulator of integrin signalling in response to different ECM molecules. However, despite these advances, the molecular mechanisms that coordinate the regulation of cell behaviour by integrins and syndecans are largely unknown.

The aim of this study was to determine the molecular mechanisms regulating syndecan-4 phosphorylation and to characterise the functional consequences of this regulation. It was found that modulation of the phosphorylation state of syndecan-4 by two protein kinases, PKCδ (a serine/threonine kinase) and Src (a non-receptor tyrosine kinase), is fundamental to the regulation of GTPase activity, integrin recycling, FA formation and cell migration.

6.1 Src phosphorylates syndecan-4

The syndecan family of cell-surface receptors lack enzymatic activity and therefore rely on recruitment of adaptor/scaffolding proteins to their intracellular domains to propagate signals initiated at the cell surface. The PDZ-binding motif of syndecans, located in the membrane distal C2 domain, is ideal for performing this function. Syndecan-binding partners identified so far include PDZ-domain-containing proteins such as syntenin, GIPC1 and CASK and actin-interacting proteins such as cortactin.

In addition, syndecans include three conserved tyrosine residues, one conserved serine and one conserved threonine residue; each one a potential phosphorylation site. Ott et al (1998)
demonstrated endogenous tyrosine phosphorylation of syndecans; however, until now the tyrosine kinases responsible for syndecan-4 phosphorylation have not yet been identified (although phosphorylation was inhibited by the broad spectrum tyrosine kinase inhibitors genistein, herbimycin A and staurosporin, which can be indicative of SFK-mediated phosphorylation) (Ott and Rapraeger, 1998). Here, using GST-fusion and custom-made syndecan-4 peptides, Src tyrosine kinase was demonstrated to directly phosphorylate syndecan-4 cytoplasmic domain (Figs. 3.2, 3.6 and 3.7). Furthermore, mutagenesis experiments in combination with mass spectrometric analysis revealed two potential Src phosphorylation sites within syndecan-4 cytoplasmic domain, Y180 and Y197. It is important to note that these phosphorylation sites are conserved within all syndecans and therefore Src potentially could phosphorylate and regulate other syndecan family members. Indeed, Src has been isolated with syndecan-3 along with cortactin, Fyn (another SFK member) and tubulin (Kinnunen et al., 1998).

In addition to tyrosines Y180 and Y197, syndecan-4 contains another tyrosine in its variable domain. However, mutagenesis and mass spectrometry, suggested that the Y188 residue was not a target of Src-dependent phosphorylation. It is not known currently whether, or how, Y188 is phosphorylated; however, this residue has a fundamental role in syndecan-4-dependent PKCα activation (Lim et al., 2003). As Y188 is in the non-conserved variable domain of syndecan-4, unlike Y180 and Y197, it is likely that its regulation and function will be syndecan-4-specific.

The presence of multiple conserved phosphorylation sites within syndecans may suggest that regulation of syndecan-4 function could occur by differential phosphorylation by one or more kinases, or by coordination of a phosphorylation cascade. Indeed, in this study the phosphorylation status of S179, the well-characterised PKCδ-phosphorylation site (Murakami et al., 2002), differentially dictated which tyrosine residues (Y180 or Y197) would be phosphorylated by Src (Figs. 3.11 and 3.12) (the significance of this finding will be discussed further in 6.2).

Phosphotyrosines are potential binding sites for SH2-domain-containing proteins and therefore, Src-mediated syndecan phosphorylation may recruit other, as yet unidentified, syndecan-binding partners. Furthermore, phosphorylation may modulate binding of known syndecan partners. For example, it has been suggested that syntenin-syndecan-1 interaction requires tyrosine dephosphorylation of syndecan-1 PDZ-binding motif (Sulka et al., 2009).
In this study it was demonstrated that the syntenin-syndecan-4 interaction is abolished with syndecan-4 constructs harbouring a phosphomimetic Y197 residue (Fig. 4.10). Src-mediated phosphorylation of Y197 may therefore promote dissociation of syntenin from syndecan-4 (perhaps allowing recruitment of other PDZ-domain-containing proteins to syndecan-4 PDZ-binding motif).

Both Src and syndecan-4 have been implicated in pathways that regulate cell migration. Syndecan-4 has evolved as a key receptor involved in tissue repair, and Src is fundamentally associated with morphogenesis and tumour progression (Echtermeyer et al., 2001; Playford and Schaller, 2004; Sandilands and Frame, 2008; Shindo et al., 2008). Src-mediated phosphorylation of syndecan-4 may therefore be critical in syndecan-4-dependent wound healing processes.

While numerous PDZ-domain-containing syndecan-binding proteins have been identified, little is known about interactions with the C1 domain that contains the Src-substrate Y180. However, the equivalent residue in syndecan-2 (Y189) has been shown to be phosphorylated by EphB2 (a receptor tyrosine kinase) in neurons. EphB2-mediated phosphorylation regulates syndecan-2 clustering, formation of cellular protrusions and neural sprouting (Ethell et al., 2001); further highlighting the important role of this phosphorylation event in regulating migratory processes that rely on dynamic cell-ECM interaction. Interestingly, tyrosine phosphorylation of an unknown residue in syndecan-1 has been suggested to regulate ectodomain shedding of the receptor, so this may be an alternative syndecan phosphorylation-dependent regulatory mechanism for coordinating adhesion to ECM and cell migration (Reiland et al., 1996)

### 6.2 PKCδ controls Src-mediated phosphorylation of syndecan-4

As Src can phosphorylate syndecan-4 on two separate tyrosine residues, it was necessary to determine how these phosphorylation events are coordinated and whether they differentially regulate syndecan-4 signalling. The data in Chapters 3 and 4 suggest that PKCδ-mediated phosphorylation of syndecan-4 functions as a key regulator of Src-dependent syndecan-4 phosphorylation.

Syndecan-4 cytoplasmic constructs emulating phosphorylation of the PKCδ-site (GST-Syn4S179D) or phosphonull PKCδ-site (GST-Syn4S179A) enhanced Src-mediated
phosphorylation of syndecan-4 specifically at Y180. Intriguingly, Y197 phosphorylation appeared to be reduced in GST-Syn4S179D constructs, indicating that perhaps PKCδ-mediated phosphorylation of syndecan-4 acts to disrupt the specificity of Src-mediated syndecan-4 phosphorylation from Y197 to Y180 (Figs. 3.11, 3.12, 3.20 and 3.23). In agreement with reduced tyrosine phosphorylation at Y197, syntenin binding to GST-Syn4S179D mutants was enhanced indicating a role for PKCδ-mediated phosphorylation of syndecan-4 in regulating syntenin binding to syndecan-4 cytoplasmic domain. Therefore, PKCδ-mediated control of differential Src-mediated syndecan-4 phosphorylation may play a role in regulating syndecan-4-syntenin-dependent events.

Determination of the structure of the syndecan-4 cytoplasmic domain by NMR has revealed a potential mechanism whereby PKCδ-mediated phosphorylation of syndecan-4 could alter phosphorylation of syndecan-4 by Src. Phosphorylation of S179 in C1 domain of syndecan-4 induces a conformational change in both the C1 and C2 (PDZ-binding) domains (Koo et al., 2006). It is likely that this conformational change differentially regulates the exposure of residues Y180 and Y197 as targets for Src kinase activity, thus altering the stoichiometry of their phosphorylation.

Thus, serine phosphorylation by PKCδ offers a mechanism to differentially control tyrosine phosphorylation of syndecan-4 by Src. Intriguingly, due to the conserved nature of all of these residues, this newly identified role for PKCδ may also influence tyrosine phosphorylation of other syndecan family members.

6.3 Syndecan-4 regulates Arf6 activity and integrin recycling

Arf6 is a central regulator of intracellular trafficking and actin reorganisation (D'Souza-Schorey et al., 1997). Adhesion to Fn induces a wave of Arf6 activity (Balasubramanian et al., 2007; Humphries et al., 2009). Importantly, syndecan-4 ligation is sufficient to promote Arf6 activation, and this is dependent on the phosphorylation state of Y180 residue in syndecan-4 cytoplasmic domain (Mark Morgan, unpublished data). Moreover, the phosphorylation status of syndecan-4 Y180 regulates differential recycling of α5β1 and αVβ3 integrins. Thus, cells expressing phosphorylation resistant Y180L exhibit enhanced recycling of α5β1 and reduced membrane delivery of αVβ3, whereas cells expressing phosphomimetic Y180E display suppressed Arf6 activity and reduced α5β1 recycling (Mark Morgan, unpublished data). The data presented in Chapter 3 demonstrate that Y180
is a substrate for Src-dependent phosphorylation, and that Src-mediated phosphorylation of Y180 is dependent on the phosphorylation status of S179. Furthermore, it was shown (in Chapter 4) that disruption of the PKCδ-phosphorylation site (S179) perturbs adhesion-dependent Arf6 activity and integrin recycling. Together these data suggest that PKCδ-mediated phosphorylation of syndecan-4 regulates Arf6 activity by modulating Src-dependent phosphorylation of Y180. Arf6 has been implicated in a number of pathways that regulate cell migration including recycling of β1 integrin (Powelka et al., 2004; Dunphy et al., 2006) and syndecans (Zimmermann et al., 2005).

In this study a role for syndecan-4-dependent Arf6 activity was identified in modulating heterodimer-specific redelivery of integrins back to the membrane. Suppression of Arf6 activity, by either siRNA-mediated knockdown or expression of syndecan-4 mutants, highlighted a syndecan-4- and Arf6-dependent reciprocal relationship between delivery of α5β1 and αVβ3 integrin heterodimers. Upon Arf6 suppression, recycling of αVβ3 was enhanced whereas α5β1 integrin recycling was reduced (Figs. 4.8 and 4.9). As Src-mediated phosphorylation of syndecan-4 promotes membrane delivery of αVβ3, in an Arf6-dependent manner, it is possible that this pathway could be reinforced by a positive feedback mechanism: whereby the activation of Src mediated by β3 integrins (Arias-Salgado et al., 2003; Shattil, 2005) leads to phosphorylation of syndecan-4, inhibition of Arf6 activity and enhanced αVβ3 recycling back to the membrane. Moreover, as syndecans can also be recycled in an Arf6-dependent manner (Zimmerman et al., 2005), and syndecan-4 ligation actually promotes Arf6 activation (Mark Morgan, unpublished data). It is possible that syndecan-4-ECM engagement potentiates syndecan-4 recycling back to the membrane and reinforces syndecan-4 signalling. Given the scope for such positive feedback mechanisms, it is important that Src-mediated phosphorylation of syndecan-4 is tightly controlled. The role of PKCδ in regulating the tyrosine-specificity of Src-mediated syndecan-4 phosphorylation (i.e. whether Y180 or Y197 is phosphorylated) suggests that PKCδ activity may function as a “gate-keeper” to restrict inappropriate propagation of syndecan-4-dependent signals. Therefore, it will now be important to determine what extracellular signals stimulate PKCδ- and Src-mediated phosphorylation of syndecan-4 and precisely how these converging signals are integrated in a spatially and temporally restricted manner in a migrating cell.

Interestingly, under serum-stimulated conditions, cells expressing syndecan-4 with a phosphomimetic PKCδ site (Syn4S179D) displayed enhanced α5β1 integrin redelivery to
the membrane in addition to increased αVβ3 integrin recycling, despite having low Arf6 activity. These results suggest that under certain conditions, PKCδ-mediated phosphorylation of syndecan-4 may modulate α5β1 integrin recycling independently of Arf6 activity.

Syndecan-4 plays a critical role in wound healing, a process that relies fundamentally on the regulation of cell migration (Echtermeyer et al., 2001). The ability of syndecan-4 to differentially regulate integrin recycling is an important discovery that will further our understanding of cell migration during both physiological, repair and pathological processes. Most ECM molecules, such as Fn, contain binding sites for more than one integrin heterodimer and different combinations of ECM-integrin receptor pairs can elicit very different cellular responses. Adhesion to Fn via α5β1 integrin promotes a contractile, fibroblastic morphology and highly dynamic adhesion turnover. RhoA activity modulated by α5β1 leads to random cell motility. Cells using αVβ3 integrin display static adhesions, an inability to modulate RhoA activity and migrate in a directionally-persistent manner (Danen et al., 2002; Worth et al., 2010). Integrin recycling is a key mechanism for regulating cell-surface integrin expression and therefore dynamic interaction with the ECM and cell migration (Caswell et al., 2009; Caswell et al., 2008). The ability of syndecan-4 to differentially regulate integrin trafficking could be vital for regulation of cell migration and wound healing. Coordination of integrin recycling has also been implicated in cancer progression, such that enhancement of integrin recycling pathways can promote invasive behaviour (Caswell et al., 2007; Caswell et al., 2008). Indeed, mutations in tumour suppressor protein p53 have recently been shown to increase α5β1 integrin and EGF receptor recycling to support random cell motility and metastatic behaviour (Muller et al., 2009).

The differential recycling of integrins regulated by syndecan-4 may be indicative of reciprocal integrin recycling pathways being activated and modulated downstream of syndecan-4. Whilst α5β1 and αVβ3 can be trafficked through the same pathway (Roberts et al., 2004), there are accumulating data suggesting that α5β1 and αVβ3 preferentially follow reciprocal and antagonistic recycling pathways. PDGF stimulation of fibroblasts has been shown to uncouple integrin αVβ3 integrin recycling from α5β1 by promoting recycling of αVβ3 integrins from Rab4-positive early endosomes, allowing faster delivery (“short-loop” recycling) of αVβ3, over α5β1, to the membrane (Roberts et al., 2001). Also, RCP, an effector of Rab11 and a member of the FIP family, binds directly to both αVβ3 and α5β1
integrins and is believed to mediate a reciprocal relationship between α5β1 and αVβ3 integrin recycling. Inhibition of αVβ3 engagement or signalling drives recruitment of RCP to the cytoplasmic domain of α5β1 integrin and promotes assembly of Rab11-positive endosome complexes containing RCP, α5β1 integrin and EGF receptor 1 (Caswell et al., 2008).

Although a direct connection between RCP and Arf6 activity has not been established, other FIP family members (FIP3 and FIP4) have been shown to bind Arf family members, most notably Arf6 (Fielding et al., 2005; Shin et al., 1999). It is believed that Arf6 recruits FIP3 and FIP4 to cleavage furrows in dividing cells and regulates cytokinesis. As syndecan-4 regulates Arf6 activity and coordinates differential heterodimer-specific recycling of α5β1 and αVβ3, it is possible that one or more FIP family members may play a role in syndecan-4-dependent integrin recycling. Since the discovery of the FIP family, numerous studies have demonstrated FIPs to be critical in regulating cellular functions (Horgan et al., 2005) including cell division and cell migration.

It is notable that the regulation of growth factor function (e.g. PDGF and EGF) is intrinsic to regulation of the reciprocal α5β1 and αVβ3 recycling pathways. As syndecans have been shown in this study to differentially regulate heterodimer-specific recycling of α5β1 and αVβ3, and as syndecans have the capacity to act as both ECM receptors and growth factor co-receptors (Bass and Humphries, 2002a), it is an intriguing possibility that syndecans might function to integrate matrix and growth factor stimuli to spatially and temporally coordinate integrin recycling. This possibility will offer an exciting avenue for further investigation.

In this study, Src was shown to phosphorylate Y197 in the PDZ-binding domain of syndecan-4, and GST pull-down assays suggested that this inhibits interaction with syndecan-binding partners such as syntenin (Fig. 4.10). Interestingly, perturbation of the syndecan-4 PDZ-binding site in cells, led to a substantial defect in internalisation (endocytosis) of both α5β1 and αVβ3 integrins (Fig. 4.14). Furthermore, internalisation of syndecan-4 was also abrogated in these cells (Fig. 4.15), indicating that perhaps integrin endocytosis is coupled to syndecan-4 internalisation and requires protein interaction with syndecan-4 PDZ-binding motif. Intriguingly, Syn4ΔPDZ-expressing cells exhibited constitutively elevated levels of Arf6 activity (Fig. 4.11); this is in contrast to perturbation of the PKCδ-phosphorylation site which suppressed adhesion-dependent Arf6 activity (Fig. 192).
4.6). Arf6 activation may therefore involve not only Y180 phosphorylation but also modulation of protein binding to the syndecan-4 PDZ-binding motif; this could be regulated by PKCδ-mediated syndecan-4 phosphorylation leading to differential phosphorylation of syndecan-4 by Src. It will now be important to determine how molecules that interact with the syndecan-4 PDZ-binding motif regulate receptor internalisation, and to elucidate how this impinges on Arf6 activity.

Interestingly GIPC1 and syntenin, which are both syndecan PDZ-binding proteins, have been shown to regulate receptor trafficking. GIPC1 is a single PDZ-domain containing protein that interacts with a number of receptors including Nrp-1 (Cai and Reed, 1999) and syndecan-4 (Gao et al., 2000) PDZ-binding motifs. VEGF receptor endocytosis is delayed in GIPC1−/− cells (Lanahan et al., 2010). GIPC1 interaction with Nrp-1 regulates α5β1 integrin endocytosis and supports endothelial cell adhesion to Fn (Valdembri et al., 2009). GIPC1 binding to syndecan-4 PDZ-binding motif has been suggested to regulate cell migration (Gao et al., 2000; Tkachenko et al., 2006). Interestingly, a role for GIPC1 has already been identified in regulating the activation of RhoG in a complex with syndecan-4 and RhoGDI1. So it will be interesting to determine whether syndecan-4-mediated GIPC1-dependent RhoG activity regulates receptor trafficking. Another syndecan-interacting PDZ protein, syntenin, also has been implicated in coordinating receptor trafficking. PDZ-mediated syntenin-binding regulates internalisation of the Delta-1 receptor (Estrach et al., 2007) and suppresses AP2-dependent endocytosis of the tetraspanin CD63 (Latysheva et al., 2006). Syntenin also co-localises with Rab5 and Rab11 in endocytic vesicles (Fialka et al., 1999). Moreover, association of syntenin with the C-terminus of syndecans and PIP2 has been shown to regulate Arf6-dependent syndecan recycling (Zimmermann et al., 2005).

In order to understand fully how syndecan-4 regulates adhesive function it will be necessary to determine specifically how the effects of PKCδ and Src on syndecan-4 are integrated with Arf6 activity and binding of PDZ proteins to syndecan-4 during integrin and syndecan endocytosis and recycling.

6.3.1 Arf6 activity and cell migration

Arf6 activity is fundamental to the regulation of cell migration, but this is not limited to redistribution of integrin and syndecan receptors to the cell surface, it also involves
regulation of cell-cell adhesion, response to growth factors and cross-talk with other GTPases (D'Souza-Schorey and Chavrier, 2006). Thus, in epithelial cells, hepatocyte growth factor stimulation of Arf6 promotes cell migration by instigating the internalisation of E-cadherin to early endosomes and the dissociation of adherens junctions (Palacios and D'Souza-Schorey, 2003; Palacios et al., 2001). During tumourogenesis loss of cell-cell adhesion is necessary for cells to undergo epithelial to mesenchymal transition (EMT) (cell scattering) and adopt a motile phenotype (Berx et al., 1995; Birchmeier et al., 1996; Handschuh et al., 1999; Vermeulen et al., 1995).

Formation of dominant lamellipodia in migrating cells requires localisation of Rac1 activity to the leading edge. Spatial restriction of Rac1 signalling during cell migration is achieved partly by Rab5 mediated endocytosis of Rac1 (Palamidessi et al., 2008). Furthermore, clustering of β1 integrins leads to Rac1 localisation in lipid microdomains (Del Pozo et al., 2002). Others have highlighted an intimate connection between Arf6 and Rac1 activity; however, the precise relationship is poorly understood (Boshans et al., 2000; D'Souza-Schorey et al., 1997; Nishiya et al., 2005; Radhakrishna et al., 1999; Zhang and Xiong, 1999). Expression of an Arf GEF protein, Arf nucleotide-binding site opener (ARNO), induces activation of Rac1 and formation of large lamellipodia in migrating epithelial cells. A dominant-negative form of a Rac GEF, Dock180/Elmo, inhibits Rac1-mediated ARNO activation, suggesting that this Rac GEF may couple ARNO and Arf6 signalling to Rac activation (Santy et al., 2005). Further studies have identified kalirin, a Rho family GEF, as a binding partner for Arf6. Kalirin binding to Arf6 facilitates its recruitment to the membrane promoting localised Rac1 activation (Koo et al., 2007). The precise coordination of Arf6 and Rac1 activities can induce epithelial cell migration by promoting dissociation of cell-cell adhesions, loss of cellular polarity and acquisition of fibroblast-like morphology in a process termed EMT. EMT is a characteristic feature of physiological and pathological events including development, tumour cell invasion and wound healing (Kalluri, 2009), so deducing the mechanism whereby syndecan-4 coordinates activities of both Arf6 and Rac1 may further our understanding of these processes.
6.4 Regulation of PKCα and Rho GTPase activation

In addition to the role of syndecan-4 in regulating Src-, PKCδ- and Arf6-dependent receptor trafficking (as demonstrated in this study), syndecan-4 also has a well-characterised role in regulating PKCα and Rac1 activity. The syndecan-4 variable domain encompasses a binding site for PKCα and the phospholipid PIP2 (Oh et al., 1998). Syndecan-4-dependent PKCα activity has been implicated in FA formation and regulation of Rac1 and RhoA activity downstream of receptor engagement (Bass et al., 2008; Bass et al., 2007b; Dovas et al., 2006). Spatiotemporal coordination of Rac1 and RhoA activity regulates cell spreading and cell migration. Therefore, the ability of syndecan-4 to bind and modulate PKCα activity is a significant step in mediating syndecan-4 function. Despite this, PKCα activation in response to syndecan-4 engagement has never been shown. Therefore, the role of syndecan-4 ligand-engagement in regulating PKCα activity was examined. The data in Chapter 5 demonstrated a transient activation of PKCα upon syndecan-4 stimulation. Moreover, PKCα activation was dependent on the integrity of the PKCδ-phosphorylation site (S179). Abrogating PKCδ-mediated phosphorylation of syndecan-4, by expressing Syn4S179A in cells, suppressed PKCα activation in response to matrix engagement. The inability of syndecan-4 PKCδ-phosphorylation site mutants to induce PKCα activity resulted in suppressed Rac1 activation (Fig. 5.5), demonstrating a crucial role for syndecan-4-ECM-dependent activation of PKCα in coordinating GTPase activity.

It will now be important to identify downstream effectors of PKCα to understand how Rac1 is activated following syndecan-4 ligation. Data suggests that PKC may phosphorylate Tiam1 in response to PDGF stimulation, which in turn leads to Rac1 activation (Fleming et al., 1998). Tiam1 contains type II PDZ domains and therefore could potentially bind other PDZ-domain-containing proteins (Fanning and Anderson, 1999) such as syntenin or other PDZ-binding motifs (e.g. syndecan). Moreover, syndecan-2 expression has been shown to increase membrane localisation of Tiam1 and activate Rac1, whereas syndecan-2-mediated cell migration is inhibited in Tiam1 deficient cells (Choi et al., 2009). However, whether Tiam1 interacts with syndecans directly or indirectly requires further validation. The link between PIP2 and Tiam1 (Buchanan et al., 2000), and recent work demonstrating association of Tiam1-PDZ domain with syndecan-1 cytoplasmic domain (Shepherd et al., 2010), may be indicative of a direct role of syndecans in activation of this GEF.
 Syndecan-4-dependent PKCα activity has also been implicated in the regulation of other Rho family members such as RhoA (Dovas et al., 2006; Bass et al., 2008) and more recently RhoG (Elfenbein et al., 2009). It is believed that PKCα activity is required for syndecan-4-dependent RhoA activation, FA formation and stress fibre assembly. Activation of PKC by stimulation with phorbol ester or by syndecan-4 clustering leads to redistribution of p190RhoGAP to membrane ruffles (Brouns et al., 2000; Bass et al., 2008) and ultimately inhibition of RhoA. The results in this thesis show RhoA activity levels were not perturbed in Syn4S179A cells that have reduced PKCα activity (Fig. 5.6). Furthermore, Syn4S179A cells have larger FAs, indicating that perhaps RhoA activity is not abrogated in these cells (Fig. 5.9). However, further experiments are required to analyse RhoA activation in response to syndecan-4 engagement and clustering. RhoA activation upon ECM engagement is a biphasic event, involving firstly an initial suppression of activity followed by a transient wave of activation. The inactivation and activation of RhoA would require the coordinated activities of both GAPs and GEFs, respectively. Syndecan-4-dependent PKCα activity has been demonstrated as a crucial component in p190RhoGAP redistribution to the membrane, thereby leading to RhoA inactivation (Bass et al., 2008). The role of PKCα in RhoA reactivation and how this may be influenced by PKCδ activity remains unclear and requires further study.

RhoG, another member of the Rho family of GTPases, has been proposed to activate Rac1 upstream of Rac GEF complex Dock180/Elmo (Katoh and Negishi, 2003) leading to morphological changes such as membrane ruffling. Recently, RhoG has been shown to regulate Rac-dependent and -independent pathways by which it contributes to cell migration (Meller et al., 2008). It has been also suggested that syndecan-4-mediated PKCα activity leads to the dissociation of RhoG from RhoGDI1 via formation of a previously unidentified syndecan-4-GIPC1-RhoGDI1 complex. Thus, syndecan-4 regulates a multitude of Rho GTPase family members by modulating PKCα activity.

6.5 A role for syndecan-4 dephosphorylation

In this study the role of kinases in the modulation of syndecan-4 function was examined. However, spatial and temporal coordination of phosphorylation-dependent signalling is controlled by dynamic cycles of phosphorylation and dephosphorylation. Therefore, to fully understand syndecan-4 signalling it will also be important to identify the
phosphatase(s) responsible for dual control of syndecan-4 phosphorylation. Inhibition of PTPs using H$_2$O$_2$ or pervanadate increases tyrosine phosphorylation of syndecans (Ott and Rapraeger, 1998), indicating that transient phosphorylation of syndecans is under the control of both tyrosine kinases and phosphatases. Intriguingly, the syndecan-4 binding protein syntenin has been shown to bind the rat homologue of PTPη (Iuliano et al., 2001). The role of the PTPη-syntenin interaction is not completely understood, as PTPη does not reduce tyrosine phosphorylation of syntenin itself. However, syntenin contains two PDZ domains and therefore can bind two PDZ domains simultaneously. Therefore, it is possible that syntenin-PTPη interaction facilitates recruitment of other PTPη substrates through its second PDZ domain; raising the intriguing possibility that syntenin, a protein that binds syndecans in a phosphorylation-dependent manner (Fig. 4.10; Sulka et al., 2009), might regulate delivery of phosphatases to syndecan-4.

The ubiquitously expressed PTP, PTP-PEST (proline (P), glutamic acid (E), serine (S) and threonine (T) rich phosphatase), associates with a number of FA proteins including p130Cas (Garton and Tonks, 1999) and paxillin (Shen et al., 2000). Overexpression of PTP-PEST in fibroblasts (Garton and Tonks, 1999) or conversely its gene deletion (Angers-Loustau et al., 1999; Sastry et al., 2006) results in inhibited cell migration. Other substrates for PTP-PEST are upstream regulators of Rac1 and RhoA, such as p190RhoGAP and VAV2 (a Rho GTPase GEF). It has been suggested that PTP-PEST couples cell protrusion and cell retraction by modulating activities of these substrates (Sastry et al., 2006). The intimate role of PTP-PEST in a number of signalling cascades that regulate cell adhesion and migration and its ubiquitous distribution, make PTP-PEST an alternative molecule that may regulate dephosphorylation of syndecan-4.

6.6 Signalling complexity

The evidence presented in this thesis clearly demonstrates a vital role for syndecan-4 in regulating integrin signalling that goes far beyond synergistic control of Rho GTPases, Rac1 and RhoA. Indeed, syndecan-4 regulates the activity of another GTPase family member, Arf6, to influence the dynamic expression of integrin heterodimers, α5β1 and αVβ3, at the cell surface.

A novel Src- and PKCδ-dependent mechanism regulating syndecan-4 phosphorylation, in order to coordinate GTPase activity, syntenin-binding and receptor trafficking, has been
defined. Specifically, Src phosphorylates syndecan-4 at two alternative tyrosine phosphorylation sites and this Src-mediated phosphorylation of syndecan-4 regulates Arf6 activation. Thus, in a migrating cell, phosphorylation and dephosphorylation of syndecan-4 by Src must be under tight spatiotemporal regulation - in order to coordinate membrane delivery of specific integrin heterodimers with activation of GTPases. The dual role of PKCδ-mediated phosphorylation of syndecan-4 to regulate both PKCα activity and Src-mediated phosphorylation of syndecan-4 offers a potential control point for integrating syndecan-4-dependent Rho GTPase activity and localised integrin delivery. Furthermore, syndecan-4 as a receptor for ECM and co-receptor for growth factor receptors has the potential to be modulated by, and integrate, many different extracellular signals to tightly regulate adhesion dynamics and cell migration.

It is now important to determine the extracellular signals that regulate activation of PKCδ and Src kinase activity to constrain the syndecan-4-dependent pathways regulating Arf6 activation and integrin recycling. However, there are numerous indications from the published literature of both adhesion- and growth factor-dependent regulation of PKCδ and SFKs (Li et al., 1994b); and conversely, PKCδ- and SFK-mediated regulation of adhesion and growth factor function (Abram and Courtneidge, 2000; Bass and Humphries, 2002a; Chae et al., 2010; Parsons and Parsons, 1997; Playford and Schaller, 2004; Romanova et al., 2010; Uchiyama et al., 2009; Yacoub et al., 2006). So it is likely that the precise nature of this regulation will be rather complicated. Moreover, PKCδ and SFKs have the capacity to directly modulate the function of each other. SFKs and PKCδ can form a complex (Shanmugam et al., 1998; Zang et al., 1997) and Src-mediated phosphorylation of PKCδ can either suppress or promote PKCδ activity and destabilise the interaction (Joseloff et al., 2002; Li et al., 1994a; Zang et al., 1997). By contrast, PKCδ can serine-phosphorylate Src and promote Src activity (Song et al., 1998). It is also interesting to note that PKCδ may indirectly regulate Src kinase activity via phosphorylation of PTPα (Brandt et al., 2003).

Consequently, it is likely that in vivo, the regulation and integration of the processes described in this study will be very complex. They can potentially be subject to both positive and negative feedback mechanisms and will depend fundamentally on the spatiotemporal restriction of kinase and GTPase activity being coordinated by extracellular signals. To address such levels of complexity, it will be necessary to use sophisticated proteomic approaches, to define syndecan-4 regulatory networks, combined with systems biology to determine how those networks are dynamically coordinated.
6.7 Final conclusions

Cell migration is central to many physiological and pathological events and adhesion to ECM is indispensable for regulation of migratory processes. Integrin and syndecan families of cell-surface receptors are the primary points of interaction between cells and their extracellular environment. It is clear that synergistic signalling downstream of integrins and syndecan-4 is vital for modulation of dynamic cell-ECM adhesion and cell migration. The mechanism whereby syndecan-4 contributes to adhesion turnover and directional cell migration is an area under intense investigation.

The results in this study have provided the first evidence of a direct interaction between Src and syndecan-4. Furthermore, PKCδ-mediated phosphorylation of syndecan-4 is an upstream regulator of this interaction; differentially regulating phosphorylation of two syndecan-4 tyrosine residues (Y180 and Y197) to coordinate Arf6 activity and syntenin-binding. Thus, a syndecan-4 phosphorylation cascade regulates GTPase activity, FA formation and integrin recycling. The convergence of these signals to allow the spatiotemporal regulation of GTPase activity and heterodimer-specific integrin delivery to the membrane is a fundamental mechanism by which syndecan-4 regulates directional cell migration.
References


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and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* 377, 539-44.


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Motif Is Required for Multiple Angiogenic Events During Early Embryonic Development. 


References


### Appendix

1. Oligonucleotides used for mutagenesis, PCR and sequencing

1. A. Mutagenesis primers for the generation of GST-syndecan-4 mutants in PGEX4T1 vector

<table>
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<th>Anti-sense</th>
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GST-Syn4Y180/188L GST-Syn4Y180L Used GST-Syn4 Y188L mutagenesis primers

GST-Syn4Y180/197L GST-Syn4Y180L Used GST-Syn4Y197L mutagenesis primers

GST-Syn4Y188/197L GST-Syn4Y188L Used GST-Syn4Y197L mutagenesis primers

GST-Syn4S179D GST-Syn4WT 5-GAAGGATGAAGGCGacTATGACCTGGGCAAGAAAC-3

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GST-SD-Y197L GST-Syn4Y197L Used GST-Syn4S179D mutagenesis primers

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GST-SA-Y188L GST-Syn4Y188L Used GST-Syn4S179A mutagenesis primers
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1. B. primers for sequencing and PCR

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<td></td>
<td>Anti-sense 5-AGGCACCGAGGGATGGAC-3</td>
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<tr>
<td></td>
<td></td>
<td>Anti-sense 5-CGGAGCTGCATGTGTCAGAGG-3</td>
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2. Band excision and in-gel trypsin digestion protocol (provided by the Mass Spectrometry Facility, University of Manchester)

Digestion of Silver and Coomassie Stained Proteins Using 96 Well Plates

Perform as much of digestion and extraction as possible under a fume hood.

- Assemble the plate by fitting the covered perforated well plate on top of the storage well plate.
- Excise the bands of interest from the gel using either a clean razor blade or with the tip of a glass pipette, and place pieces in a well in the perforated well plate. Label the samples by writing on the cover above the well.
- Shrink by washing in 50 µl acetonitrile for 5 min.
- Remove acetonitrile by centrifuging the plate for 1 min at 1500 rpm using the 96 well plate rotor. The liquid will be centrifuged from the perforated plates into the storage wells which can then be emptied after centrifugation.
- Dry the pieces in a vacuum centrifuge ~ 15 min using the 96 well plate rotor.
- Add a volume (enough to cover the gel) of 10 mM DTT in 25 mM NH₄HCO₃ and reduce the proteins at 56°C for 1 hr. (0.00154 g DTT/1ml NH₄HCO₃)
- Cool to RT, then centrifuge out the DTT, 1 min 1500 rpm, before adding the same volume of 55 mM iodoacetamide in 25 mM NH₄CO₃ to the gel pieces and incubating for 45 min at RT in the dark. (0.0080 g iodoacetamide/1ml NH₄HCO₃)
- Remove iodoacetamide solution by centrifuging (1 min 1500 rpm) and wash the gel pieces with 50-100 µl 25 mM NH₄HCO₃ for 10 min
- Wash with acetonitrile, enough to cover pieces (~50 µl) (5 min) then centrifuge,
- Wash with 25 mM NH₄HCO₃ (5 min) then centrifuge,
- Wash again in acetonitrile (5 min), centrifuge out the liquid phase and completely dry the gel pieces in a vacuum centrifuge (~15 min)
- Add 5 µl 12.5ng/µl trypsin solution, and 45 µl 25 mM NH₄CO₃ to the samples and place in an ice cold bath for 45 min for gel pieces to absorb the buffer. If after this time the gel pieces have no liquid on top add enough 25 mM NH₄CO₃ buffer to make sure pieces are covered.
- Place well plate in a container with enough water to cover the container bottom then incubate overnight at 37°C

**Extraction**

- Extract the peptides with ~50 µl 20 mM NH₄HCO₃ for 20 min then centrifuge plate using a fresh storage plate. Do not empty the storage plate.
- Extract a further two times (20 min) with 5% formic acid in 50% acetonitrile, using the same storage plate during each centrifugation to pool all the supernatant into the same well tube per sample.
- Concentrate the pooled supernatants to ~20 µl in the vacuum centrifuge. (if sample is found to have dried completely add 20 µl of buffer (0.1% formic acid) and leave to re-dissolve peptides.)
- Store Peptides at < -20 °C until mass spec.
- If using the Maldi-Tof, peptides need to be desalted using a zip tip (C18).

*Make stock trypsin (Promega - sequencing grade – 20 µg powder) up in 160 µl of 50 mM NH₄HCO₃, to give a stock solution of 125 ng/µl, keep at < -20°C.*