Tuning Cell Motility – Roles of Nestin and Vimentin in Cancer Cell Invasion

Claire Louise Hyder

Department of Biosciences, Åbo Akademi University
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University of Turku and Åbo Akademi University,
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“Whatever it is you're seeking won't come in the form you're expecting.”

-Haruki Murakami

To those who have loved and supported me on this endeavour, you know who you are.
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ABSTRACT

The cytoskeleton is a key feature of both prokaryotic and eukaryotic cells. It is comprised of three protein families, one of which is the intermediate filaments (IFs). Of these, the IFs are the largest and most diverse. The IFs are expressed throughout life, and are involved in the regulation of cell differentiation, homeostasis, ageing and pathogenesis. The IFs not only provide structural integrity to the cell, they are also involved in a range of cellular functions from organelle trafficking and cell migration to signalling transduction. The IFs are highly dynamic proteins, able to respond and adapt their network rapidly in response to intra- and extra- cellular cues. Consequently they interact with a whole host of cellular signalling proteins, regulating function, and activity, and cellular localisation.

While the function of some of the better-known IFs such as the keratins is well studied, the understanding of the function of two IFs, nestin and vimentin, is poor. Nestin is well known as a marker of differentiation and is expressed in some cancers. In cancer, nestin is primarily described as is a promoter of cell motility, however, how it fulfils this role remains undefined. Vimentin too is expressed in cancer, and is known to promote cell motility and is used as a marker for epithelial to mesenchymal transition (EMT). It is only in the last decade that studies have addressed the role that vimentin plays in cell motility and EMT. This work provides novel insight into how the IFs, nestin and vimentin regulate cell motility and invasion. In particular we show that nestin regulates the cellular localisation and organisation of two key facilitators of cell migration, focal adhesion kinase and integrins. We identify nestin as a regulator of extracellular matrix degradation and integrin-mediated cell invasion. Two further studies address the specific regulation of vimentin by phosphorylation. A detailed characterisation study identified key phosphorylation sites on vimentin, which are critical for proper organisation of the vimentin network. Furthermore, we show that the bioactive sphingolipids are vimentin network regulators. Specifically, the sphingolipids induced RhoA kinase-dependent (ROCK) phosphorylation at vimentin S71, which lead to filament reorganisation and inhibition of cell migration. Together these studies shed new light into the regulation of nestin and vimentin during cell motility.
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications and manuscripts, which are referred to in the text by their Roman numerals. In addition some unpublished results are included.


II  Isoniemi, K.O, Hyder, C.L., Eriksson, J.E. Structure and migration of vimentin phosphorylation. Manuscript


*Equal contribution

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### Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>A</td>
<td>Alanine</td>
</tr>
<tr>
<td>AchR</td>
<td>Acetylcholine receptor</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
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<tr>
<td>ADLD</td>
<td>Adult-onset autosomal dominant leukodystrophy</td>
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<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
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<tr>
<td>ATP</td>
<td>Adenine triphosphate</td>
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<tr>
<td>AxD</td>
<td>Alexander disease</td>
</tr>
<tr>
<td>Cdc</td>
<td>Cell division control protein 42 homolog</td>
</tr>
<tr>
<td>Cdc42GAP</td>
<td>Cdc42 gtpase-activating protein</td>
</tr>
<tr>
<td>Cdk</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CLEM</td>
<td>Correlative light and electron microscopy</td>
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<tr>
<td>CMT</td>
<td>Charcot-Marie-Tooth disease</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>D</td>
<td>Aspartate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DRG</td>
<td>Dorsal root ganglion</td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran sodium sulphate</td>
</tr>
<tr>
<td>EB</td>
<td>Epidermylosis bullosa</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix components</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-related kinase</td>
</tr>
<tr>
<td>ERM</td>
<td>Ezrin radixin moesin</td>
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<tr>
<td>FA</td>
<td>Focal adhesions</td>
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<td>FAK</td>
<td>Focal adhesion kinase</td>
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<tr>
<td>FB</td>
<td>Fibrillar adhesions</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>FRAP</td>
<td>Fluorescent recovery after photobleaching</td>
</tr>
<tr>
<td>FX</td>
<td>Focal complex</td>
</tr>
<tr>
<td>GAN</td>
<td>Giant axonal neuropathy</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acid protein</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HGPS</td>
<td>Hutchinson-Gilford progeria syndrome</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish-peroxidase</td>
</tr>
<tr>
<td>Htt</td>
<td>Huntingtin protein</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>IDE</td>
<td>Insulin degrading enzyme</td>
</tr>
<tr>
<td>IF</td>
<td>Intermediate filament</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin growth factor</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILK</td>
<td>Integrin-linked kinase</td>
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<tr>
<td>IP3</td>
<td>Inositol trisphosphate</td>
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<tr>
<td>IP3R1</td>
<td>Inositol 1,4,5-trisphosphate receptor type 1</td>
</tr>
<tr>
<td>IRBIT</td>
<td>IP(3)R binding protein released with inositol 1,4,5-trisphosphate</td>
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<tr>
<td>JNK</td>
<td>C-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KO</td>
<td>Knock out</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen-activated protein</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal to epithelial transition</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MT</td>
<td>Microtubules</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa enhancer binding protein</td>
</tr>
<tr>
<td>NF-L</td>
<td>Neurofilament light</td>
</tr>
<tr>
<td>NF-H</td>
<td>Neurofilament heavy</td>
</tr>
<tr>
<td>NF-M</td>
<td>Neurofilament medium</td>
</tr>
<tr>
<td>NIFID</td>
<td>Neuronal intermediate filament inclusion disease</td>
</tr>
<tr>
<td>NMJ</td>
<td>Neuro-muscular junction</td>
</tr>
<tr>
<td>NRG</td>
<td>Neuregulin</td>
</tr>
<tr>
<td>P</td>
<td>Proline</td>
</tr>
<tr>
<td>p130Cas</td>
<td>p130 Crk-associated substrate</td>
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<tr>
<td>P1f</td>
<td>Plectin 1f</td>
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<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) polymerase 1</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>pFAK</td>
<td>Phosphorylated FAK</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<tr>
<td>PKG</td>
<td>Protein kinase G</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>Plk</td>
<td>Polo-like kinase</td>
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<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PP</td>
<td>Protein phosphatase</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
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<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>Rac</td>
<td>Ras-related C3 botulinum toxin substrate</td>
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<tr>
<td>RACK</td>
<td>Receptor for activated C kinase</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homolog gene family, member A</td>
</tr>
<tr>
<td>RNAi</td>
<td>Ribonucleic acid interference</td>
</tr>
<tr>
<td>ROCK</td>
<td>RhoA kinase</td>
</tr>
<tr>
<td>S</td>
<td>Serine</td>
</tr>
<tr>
<td>S1P</td>
<td>Sphingosine 1-phosphate</td>
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<td>S1PR</td>
<td>Sphingosine 1-phosphate receptor</td>
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<td>SERT</td>
<td>Serotonin transporter</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>SK</td>
<td>Sphingosine kinase</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphisms</td>
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<td>SPC</td>
<td>Sphingosylphosphorylcholine</td>
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<td>STED</td>
<td>Stimulated emission depletion microscopy</td>
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<td>T</td>
<td>Threonine</td>
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<td>TACE</td>
<td>Tumor necrosis factor-α converting enzyme</td>
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<td>TGase</td>
<td>Transglutaminase</td>
</tr>
<tr>
<td>TGF</td>
<td>Tumour growth factor</td>
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<tr>
<td>TIRFM</td>
<td>Total internal reflection fluorescence microscopy</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>ULF</td>
<td>Unit length filament</td>
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<tr>
<td>VASP</td>
<td>Vasodilator-stimulated phosphoprotein</td>
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<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cells</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskott-Aldrich syndrome protein</td>
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<tr>
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<td>Wild-type</td>
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<td>Tyrosine</td>
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<td>ZO-1</td>
<td>Zonal occludins protein 1</td>
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<td>β3-AR</td>
<td>B3-adrenergic receptor</td>
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1 INTRODUCTION

The cytoskeleton is an incredible protein network within the cell. It consists of three distinct systems of protein polymers, all of which are engaged in essential functions for maintaining proper cell function, such as structural support, protein and organelle transport, cell shape, cell motility, mechanosensing, intra- and extracellular signalling. The least known of the cytoskeletal systems, the intermediate filaments (IF), also comprises the largest gene family coding for nearly 70 different proteins, each of which has distinct functions within the body. Within the IF family there are two proteins of interest in this thesis, nestin and vimentin. Nestin is a rather unique entity in that it is expressed briefly, in very specific cell types, such as muscle, kidney and the central nervous system, at very specific times during a cell’s life – during development and regeneration. This very specific timing and location of expression suggests that nestin has a very specific function in allowing cells to transition through these processes. Nestin is also found to be expressed in a wide array of cancer types, yet until now its function in cancer has remained a mystery. Vimentin is the antithesis of nestin, it is expressed in many tissues often at later stages of development where it will continue to be expressed into adulthood. In pathological situations vimentin is known to contribute to specific neurodegeneration types, cataract formation and it is most well known as a characteristic of very aggressive and invasive cancers.

When the term “war on cancer” was coined in 1971, cancer was thought to be a single disease that could strike in different parts of the body. We now know that not only is this completely wrong, but that even the specific description of a cancer type actually refers to a wide range of heterogeneous diseases classified under the cancer umbrella. Even as recently as 2003, a challenge was given to eliminate the “suffering and death from cancer, and to do so by 2015”. It is now 2014 and we are nowhere near reaching that goal.

The leading cause of death in cancers is not from the primary tumour, but from the secondary tumours that arise because of metastasis. Metastasis occurs when cells in the primary tumour acquire the ability to invade and migrate, a term known as malignant transformation. The intrinsic changes a cell undergoes during this transition is commonly known as epithelial to
mesenchymal transition (EMT). During this transition, tumour cells are able to break out from the primary tumour and invade into the surrounding tissue. Some cells will invade into the network of blood and lymph vessels that a tumour attracts to feed itself (intravasation). From there they can travel to many distal sites in the body, such as the lungs, liver and bones. In order to begin the reverse process of colonising the tissue to which they have travelled, the cells need to exit the blood or lymph vessels (extravasation) into the stroma. The metastasized tumour cells will then undergo reverse EMT, also known as mesenchymal to epithelial transition (MET), whereby they lose their motile abilities and reacquire some of the characteristics of the primary tumour. Understanding the fundamental processes involved in cell motility is of importance when trying to understand metastatic processes. Since acquiring metastatic abilities is a transitory process, there are many molecules that will be expressed either at the beginning, throughout the transition and at the end.

Both nestin and vimentin are associated with cell motility. Vimentin is known as a regulator of EMT and nestin is most often expressed in intermediary stages of malignant transformation. In the case of nestin, very little is known about how it influences cell migration and invasion. Vimentin, on the other hand, is well known to influence cell migration, but only recently are precise details about how it does this are coming to light. Using cancer cells as a model, this thesis explores how both nestin and vimentin are able to influence cell migration and invasion.
2 REVIEW OF THE LITERATURE

2.1 OVERVIEW OF THE INTERMEDIATE FILAMENTS

2.1.1 Intermediate filaments: The most diverse protein family of the cytoskeleton

In 1903, a Russian biologist, Nikolai Koltsov proposed that the shape of a cell is determined by fibrous or filamentous network that resembles the human skeleton. He called this protein network the “cytoskeleton”. We now know that this group consists of 3 protein families, the microtubules (MT) composed of tubulin, the intermediate filaments (IF) and the microfilaments, composed of actin. With more than 70 genes encoding IF proteins they are the most diverse members of the cytoskeleton. The mammalian IF family consists of six types of IF protein as determined by DNA and amino acid sequence homology: keratins, found in epithelia are type 1 and 2; desmin, glial fibrillary acid protein (GFAP), peripherin and vimentin belong to type 3; type 4 consists of α-internexin, the neurofilaments -light, -medium and – heavy (NF-L, NF-M and NF-H), nestin, nestin homolog synemin and syncoilin; the nuclear lamins are the only IFs in type 5 and the eye lens intermediate filaments, phakinin and filensin make up the type 6 IFs (Szeverenyi et al., 2008). Each of the IF proteins has a specific developmental and tissue-specific expression pattern. Some IF types can form polymers with themselves, known as homopolymers. Other IF types cannot polymerise with themselves. Instead they require another IF type to co-polymerise with, and form heteropolymers. While the IFs are classed as one large family, each of the subfamilies and individual proteins have distinct properties which impart a great range of flexibility when it comes to specialised cell type specific mechanical and signalling functions.

IFs were first described as a distinct cytoskeletal filament group in the cell in 1968, when the authors used electron microscopy to examine skeletal muscle of chick embryos (Ishikawa et al., 1968). Rapidly following that several papers were published describing distinct classes of tissue specific IF family members (Lazarides, 1980). Proteins within the IF family are highly conserved throughout evolution as demonstrated by the cross-reactivity of vimentin antibodies in several diverse species, vertebrate and invertebrate as
well as by alignment of protein sequences across species (Bartnik and Werber, 1989; Franke et al., 1978; Franke et al., 1979; Guérette et al., 2007; Hyder et al., 2008; Szeverenyi et al., 2008).

IFs were first thought to be important for mechanical integrity, implying that early researchers would have believed that their only function was to provide structure and that understanding of their cellular function has come recently. This is not strictly true, even in the earlier days of IF research they were found to be important for maintaining organelle position and integrity (Lazarides, 1980). Now it is widely accepted that not only do they provide mechanical integrity, they are also important hubs in the regulation of cell signalling and in a plethora of other cellular functions ranging from cell stress to mechanosensing to inflammatory responses (Eriksson et al., 2009; Hyder et al., 2008; Ivaska et al., 2007; Kim and Coulombe, 2007; Pallari and Eriksson, 2006).

2.1.2 Intermediate filament structure and assembly characteristics

Compared with the other members of the eukaryotic cytoskeleton, actin and tubulin, the IFs are more elastic and able to resist shear stress without breaking (Janmey et al., 1991; Wagner et al., 2007). Early studies suggested that one of the primary functions of the IFs was maintenance of the basic structural integrity of the cell (Janmey et al., 1991). This elasticity and resistance to shear stress can be attributed to the hierarchical structure and specific structural changes during assembly of the IF filaments themselves (Qin et al., 2009).

Microtubules are tubular polymers formed by the polymerisation of $\alpha$ and $\beta$-tubulin. $\alpha$- and $\beta$-tubulin dimerise, where the $\alpha$ subunit is known as the minus end and the $\beta$ subunit is known as the plus end. The dimers join end to end to form protofilaments which assemble laterally to form microtubules (MT). MTs can be elongated by the addition of more protofilaments to the plus end of the MT in a zipper-like fashion and this requires tubulin bound GTP. As the MT grows, the GTP is hydrolysed to GDP. Eventually the hydrolysis of GTP to GDP catches up with the end of the filament. GDP bound tubulin has a lower binding affinity and this leads to MT disassembly and shrinkage of the MT. Actin filaments are made up of two helical intertwined polymers made up of actin monomers, known as G-actin. Like MT, actin filaments are polarised, G-actin has what are known as a pointed
end (minus) and a barbed end (plus end). Unlike MT, actin filaments assemble from the plus end and disassemble from the minus end and both grow and reposition themselves in the cell via a process known as treadmilling, whereby one end (plus) grows while the other one shrinks (minus). Unlike MT, both ends of actin filaments are capable of extending albeit with differing properties. This polarity is necessary for the unidirectional transport of motor proteins (Herrmann et al., 2009). IFs on the other hand lack polarity and do not require ATP or GTP to assemble.

**Figure 1: Intermediate filament assembly.** A) Intermediate filament proteins consist of an N-terminal head domain (N) and a C-terminal tail domain (C) which dimerise in parallel. B) Two IF dimers assemble in an anti-parallel fashion to form tetramers. C) Two tetramers assemble to form unit-length filaments (ULF). D) Filaments assemble and elongate through end-to-end annealing.

IF proteins consist of an N-terminal “head” domain, a central rod and a C-terminal “tail” domain (Figure 1). Unlike other IF proteins, nestin and synemin have a very short N-terminus and a long C-terminus, from which
they are primarily regulated. However, they do assemble in the same way as other IFs. IFs are assembled hierarchically. Dimers are formed through interaction of two rod domains to form a coiled coil. The dimers assemble in an anti-parallel fashion to form tetramers which then assemble into unit-length filaments (ULFs) composed of eight tetramers. Kinetic and mathematical modelling, immunofluorescence, and total internal reflection fluorescence microscopy (TIRFM) studies have demonstrated that filaments form and elongate by the assembly and addition of ULFs by end-to-end annealing (Colakoğlu and Brown, 2009; Czeizler et al., 2012; Kirmse et al., 2007; Winheim et al., 2011). IF polymerisation is a dynamic process and there is a steady state exchange of IF subunits between a soluble pool and IF filaments (Vikstrom et al., 1992) whereby subunit exchange occurs along the length of the filament (Colakoğlu and Brown, 2009). Use of such in silico techniques combined with advanced microscopy techniques are really helping us to understand in more detail the kinetics and process of IF assembly and will hopefully allow us in the future to probe effects of mutational or chemical modulation of the IF network on IF assembly dynamics.

In vitro studies of neurofilaments (NF) indicate that the rod domain is a determinant of the filament architecture, and the termini regulate assembly. In particular the N-terminus promotes lateral assembly while the C-terminus regulates the actual assembly process and prevents unconstrained lateral filament growth (Heins et al., 1993). Similar work with the vimentin C-terminus supports this data and showed that conserved motifs in coil 2B in the vimentin rod domain were also important for filament length and network architecture (Figure 2) (McCormick et al., 1993). Sometime ago a study showed that specific domains, coil 1A, 1B and 2B on the rod, in nestin determines its integration into vimentin intermediate filaments. These domains also determine nestin’s subcellular localisation. Remarkably this study’s findings have not been followed up and deserve more attention (Marvin et al., 1998). The long C-terminal tail of nestin and synemin is proposed to act as a cross-bridge/linker between filament bundles (Steinert et al., 1999).

The properties of IF filaments are not only conferred by their inherent structure and hierarchical assembly, the elastic properties of IFs are also regulated by ions. Support for the idea that the vimentin C-terminus is important for filament architecture come from a study showing that divalent
cations, such as Ca$^{2+}$ and Mg$^{2+}$, interacting with the last 11 amino acids on the vimentin tail promote stiffening of the filament network by crosslinking IF filaments (Lin et al., 2010).

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### 2.1.3 Intermediate filament homo- and hetero- polymerisation characteristics

IFs can either form filaments as homo- or hetero- polymers. Some IF types can only form heteropolymers whereas others can be both homo- and hetero- polymeric. Type 1 and 2 keratins form inter-type heteropolymers. Nestin can only form heteropolymers, with vimentin and α-internexin (Steinert et al., 1999) and while nestin interacts with vimentin and desmin in skeletal muscle and SW13 cells it is not entirely clear if they polymerise directly (Marvin et al., 1998; Sejersen and Lendahl, 1993; Sjöberg et al., 1994b). A newly identified nestin isoform interacts with NF and peripherin in dorsal root ganglia neurons (Su et al., 2013). NF also form heteropolymers, however, this is typically within IF subtype. NF-L cannot

![Figure 2: Schematic diagram illustrating the regions of vimentin that regulate different assembly characteristics.](image)
form polymers by itself, instead it requires NF-M or NF-H at a threshold ratio of 10% or more to polymerise (Lee et al., 1993). Classically vimentin forms homopolymers, however, in disease states such as the induction of reactive astrocytes, vimentin can co-polymerise with GFAP (Satoh et al., 2004). In reactive astrocytes, synemin is only able to incorporate into GFAP filament networks if vimentin is present, implying that vimentin acts as a co-polymerisation partner for filament incorporation. In cells with GFAP alone, synemin binds to GFAP but is unable to be integrated into the network (Jing et al., 2007). This ability to incorporate into IF networks is conferred by two specific motifs in the rod 2A and 2B domains as well as the requirement for a functional N-terminus similar to the vimentin head domain, which is lacking in synemin (Khanamiryan et al., 2008). As such, synemin may exploit vimentin’s functional characteristics in order to integrate into the GFAP network.

The necessity for some IFs to form heteropolymers gives rise to additional flexibility in IF function. It is well documented in the NF field that the ratio of NF-L, NF-M and NF-H subunits incorporated into NF filaments is important for determining NF characteristics such as expression levels and PTMs. Changes in NF subunit ratios can alter cellular characteristics, such as axonal calibre (Tu et al., 1995; Wong et al., 1995; Xu et al., 1996). The ratio of nestin to vimentin in cells has a direct effect on the assembly of the vimentin network during mitosis and potentially other cell processes, of which migration could be a good example considering that cell motility requires a dynamic IF cytoskeleton (Chou et al., 2003; Steinert et al., 1999).

2.1.4 Microtubule and actin networks are important for spatially regulated intermediate filament assembly

The question of how IF subunits are available at the locations in the cell where they are required can be answered in part by looking to the interactions between IFs and MT. MT have been implicated in the transport of IF proteins to their site of polymerisation. It is well known that MT depolymerisation also induces IF perinuclear reorganisation (Herrmann and Aebi, 2000). Interfering with MT cytoskeletal dynamics interferes with the translocation of short vimentin filaments known as “squiggles” as well as steady-state subunit exchange between soluble and polymerised vimentin (Yoon et al., 1998). MT dependent IF transport is bidirectional along microtubule tracks and is dependent on kinesin, dynein and dynactin.
(Liovic et al., 2003). It is possible that plectin as a cytoskeletal cross-linker may regulate MT-dependent vimentin assembly (Spurny et al., 2008). MT may also be involved in de novo network formation as well as IF network extension and organisation since there is evidence that MT participate in the dynamic co-translation of peripherin mRNA and formation of non-filamentous IF particles (Chang et al., 2006). These interactions maybe important for vimentin translocation and network organisation and during both cell spreading, mitosis and neurite outgrowth (Helfand et al., 2002; Prahlad et al., 1998; Shim et al., 2008). In motile cells, IF assembly is initiated in regions of increased actin turnover such as lamellipodia and focal adhesions (FA) whereby IF precursors are transported along actin stress fibres prior to integration with the existing IF network (Kölsch et al., 2009; Tsuruta and Jones, 2003; Windoffer et al., 2006). This close association of these cytoskeletal networks must be kept in consideration when observing disease states or treatments affecting the MT and actin cytoskeletons.

2.1.5 Intermediate filaments are expressed in a temporal and tissue specific manner

IF’s are expressed in a very tissue-specific temporal manner. Nestin is transiently expressed during development of the central nervous system (CNS), peripheral nervous system (PNS), muscle and kidney. As differentiation of tissue proceeds nestin is downregulated and replaced by other tissue specific IFs such as vimentin (mesenchymal tissues), desmin (in muscles), GFAP or NF (CNS). Similarly, in the CNS, α-internexin is expressed in early development alongside peripherin. As differentiation proceeds α-internexin levels decrease and NF-L, NF-M and NF-H will replace it. The co-expression of α-internexin and NF is maintained at very specific stochiometry in homeostasis. The difference between α-internexin and nestin expression is that while nestin is rarely found in the adult during homeostasis, in some tissues α-internexin will remain expressed into adulthood and is sometimes the sole IF expressed by a cell type. Keratins too exhibit succession of expression in the skin during keratinocyte maturation (Fuchs and Coulombe, 1992).

Vimentin is expressed in cells of mesenchymal origin, including endothelial cells and vascular smooth muscle cells, and the CNS, among others. Typically, it is expressed throughout embryonic development and differentiation. Depending on the cell type it may be replaced by a tissue
specific IF upon terminal differentiation. These changes in IF expression are regulated, in part, by different growth factors and signalling pathways activated throughout differentiation. The same growth factor may differentially regulate IFs in the same cell type as is the case with TNFα which can increase GFAP and nestin transcription in cells, but only regulates vimentin turnover (Zhou and Skalli, 2000). Interestingly, IF expression patterns in the same cell type are differentially regulated by extracellular matrix (ECM) components (Sultana et al., 1998), suggesting that the cellular environment is also important for IF expression (Table 1).

2.1.6 Intermediate filaments contribute to a diverse range of pathologies

The identification of IF functions in the cell is often a result of their disease-association. Some of the first identifications of intermediate filaments in disease was in cancer (Ben-Ze’ev et al., 1986; Miettinen et al., 1982). Now the IF protein family is associated with a wide array of often quite debilitating diseases which affect almost all parts of the human body (Table 1).

Common characteristics of many IF-related diseases arise from abnormalities with filament assembly, filament organisation or aberrant regulation (PTMs and degradation), all of which can have downstream effects on protein-protein interactions and cell signalling. In some cases, the IF in question is upregulated, other times, as with Hutchinson-Gilford Progeria Syndrome (HGPS) and the keratin skin blistering diseases, the problem arises from function-altering mutations. Many disorders associated with type 4 IFs are characterised by abnormal protein aggregation arising from misregulation of PTMs and elevated protein expression.

Understanding the role the IFs play in disease has facilitated the understanding of these proteins’ function during homeostasis. What is clear from the literature is that those IFs with overt roles in pathology are currently the best understood and researched. Those with more subtle roles in pathology such as vimentin and nestin still require much more study in order to understand their cellular function.
Table 1: IF subtypes, tissue expression and associated diseases

<table>
<thead>
<tr>
<th>Type</th>
<th>IF</th>
<th>Tissue</th>
<th>Diseases</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>I &amp; II</td>
<td>Keratins</td>
<td>Skin, Stratified epithelia, e.g. nails, hair</td>
<td>Pancreatitis, Liver disease, skin and hair-related tissue fragility disorders e.g. Epidermylosis Bullosa (EB)</td>
<td>(Haines and Lane, 2012)</td>
</tr>
<tr>
<td>III</td>
<td>Desmin</td>
<td>Striated and smooth muscle</td>
<td>Myopathies, Cardiomyopathy</td>
<td>(Clemen et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>Glial fibrillary acidic protein (GFAP)</td>
<td>Astrocytes, Central nervous system (CNS)</td>
<td>Alexander Disease (AxD), Neurodegenerative diseases inc. Alzheimer’s (AD), Parkinson’s (PD), Amyotrophic lateral sclerosis (ALS)</td>
<td>(Middeldorp and Hol, 2011)</td>
</tr>
<tr>
<td></td>
<td>Peripherin</td>
<td>CNS, peripheral nervous system (PNS)</td>
<td>ALS, motor neuron disease, neurodegeneration</td>
<td>(Liem and Messing, 2009)</td>
</tr>
<tr>
<td></td>
<td>Vimentin</td>
<td>Tissues of mesenchymal origin</td>
<td>Cataract formation, giant axonal neuropathy (GAN), EMT and metastatic cancer marker</td>
<td>(Bornheim et al., 2008; Ivaska, 2011; Mahammad et al., 2013; Müller et al., 2009a)</td>
</tr>
<tr>
<td>IV</td>
<td>α-internexin</td>
<td>CNS</td>
<td>Neuronal intermediate filament inclusion disease (NIFID)</td>
<td>(Liem and Messing, 2009)</td>
</tr>
<tr>
<td></td>
<td>Neurofilaments (NF)</td>
<td>CNS, PNS</td>
<td>Sensory and motor neuropathy diseases e.g. Charcot-Marie-Tooth disease (CMT), ALS, PD, AD, diabetes and multiple sclerosis</td>
<td>(Lépinoux-Chambaud and Eyer, 2013; Liem and Messing, 2009; Liu et al., 2004; Teunissen and Khalil, 2012)</td>
</tr>
<tr>
<td></td>
<td>Nestin</td>
<td>CNS, PNS, heart, kidney, muscle</td>
<td>Cancer and AD</td>
<td>(Lépinoux-Chambaud and Eyer, 2013)</td>
</tr>
<tr>
<td></td>
<td>Synemin</td>
<td>Kidney, muscle, skin, CNS, liver, heart</td>
<td>AxD, glioma, myopathy, liver fibrosis</td>
<td>(Lépinoux-Chambaud and Eyer, 2013; Pekny et al., 2013)</td>
</tr>
<tr>
<td>V</td>
<td>Lamins</td>
<td>Nuclear lamina</td>
<td>Lipo and muscle -dystrophies, CMT, cardiomyopathies, Adult-onset autosomal dominant leukodystrophy (ADLD) and premature ageing diseases e.g. HGPS</td>
<td>(Schreiber and Kennedy, 2013)</td>
</tr>
<tr>
<td>VI</td>
<td>Phakinin, Filensin</td>
<td>Eye</td>
<td>Cataracts</td>
<td>(Song et al., 2009)</td>
</tr>
</tbody>
</table>
2.2 PHYSIOLOGICAL FUNCTIONS OF NESTIN AND VIMENTIN

2.2.1 Nestin expression characteristics

Nestin is the primary IF protein expressed in the early stages of differentiation in muscle, testis, kidney, and the CNS among others (Bertelli et al., 2007; Chen et al., 2006; Fröjdman et al., 1997; Lendahl et al., 1990; Sejersen and Lendahl, 1993). It is also expressed in neovasculature (Teranishi et al., 2007). It has been proposed to be a marker of multi-lineage cells and is expressed after initiation of differentiation in embryonic stem cells (Wiese et al., 2004). As differentiation of the tissue proceeds, nestin is replaced by other tissue specific IFs such as GFAP, NF, vimentin or desmin. In the adult, nestin is expressed in the pancreas, pituitary gland and adrenal glands. It has an important role in maintaining the morphology and cytoarchitecture of terminally differentiated podocyte foot processes in the adult kidney (Bertelli et al., 2002; Chen et al., 2006; Krylyshkina et al., 2005; Street et al., 2004; Toti et al., 2005; Yoshida et al., 2013).

The tissue specific expression of nestin is determined by regulatory elements within its three introns. The first intron has an enhancer coding for muscle specific expression, it also controls nestin expression in endothelium (Aihara et al., 2004; Zimmerman et al., 1994). The second intron regulates nestin expression in pluripotent stem cells, the CNS, the heart and kidney (Jin et al., 2006; Sejersen and Lendahl, 1993; Wagner et al., 2006; Zimmerman et al., 1994). There are also Sp1 and Sp3 transcription factor binding sites in the nestin promoter region (Cheng et al., 2004). Persistent nestin expression in the adult kidney is due to lack of proteasomal degradation as opposed to transcriptional control (Sakairi et al., 2007).

Nestin protein expression is, perhaps unsurprisingly, induced and inhibited by a range of growth factors and ligands. It is clear that the majority of the research has been into growth factor induction of nestin protein during development. Some ligands have distinct, sometimes opposing (most likely tissue dependent), effects on nestin protein expression during differentiation, regeneration and disease. These ligands include, EGF (Esnı et al., 2004; Means et al., 2005; Oikawa et al., 2010), FGF (Krylyshkina et al., 2005; Wroblewski et al., 1997), Neurotrophin-3 (Kim et al., 2011a; Kuo et al., 2007), thrombin (Huang et al., 2008; Huang et al., 2009a; Wautier et al., 2007),
PDGF (Oikawa et al., 2010; Sultana et al., 1998) and Wnt (Cui et al., 2013; Nikolova et al., 2007). In some cases induction of nestin expression can be affected by a combination of ligands or signalling pathways. One example is glioblastoma, whereby K-Ras signalling is required for Notch induced nestin expression (Shih and Holland, 2006). In neural progenitor cells both FGF and IGF are required for upregulation of nestin expression in neural progenitor cells (Kalluri et al., 2007). Although a great degree of work has been done to identify factors that induce and regulate nestin expression, functional signalling studies are still lacking, particularly with regard to the timing of the induction and cessation of nestin expression.

Why is nestin even necessary in the cell since it is transiently expressed in association with other IFs which then often replace it? As will be highlighted below, nestin has some unique signalling functions that are not a function of its polymerisation partner. Additionally, related to Section 2.1.3, nestin:vimentin polymers are less stable and more able to reassemble and disassemble in dividing and proliferating cells (Chou et al., 2003). As such nestin imparts a higher degree of flexibility on its polymerisation partner. So, transient expression of nestin may reflect the physiology of cells that need to be more flexible. Nestin expression in rapidly proliferating and differentiating cells and in cancer cells may reflect a requirement for a less stable and more flexible cytoskeleton that is quicker to adapt to its environment (Flørenes et al., 1994; Sejersen and Lendahl, 1993).

### 2.2.2 Nestin in differentiation and angiogenesis

It is only in recent years that a knock-out (KO) mouse has been developed for nestin. Three different groups created a nestin KO mouse using different approaches. Curiously the first published attempt using targeted deletion of exon 1 found that the mouse was embryonic lethal (Park et al., 2010). Another approach targeted exon 1 and part of the 5’UTR (Mohseni et al., 2011) and the third approach used RNAi targeting (Yang et al., 2011). The latter two approaches found that the nestin KO mouse is viable and does not exhibit an overt phenotype. The viability difference appears to arise from the approach used to create the mouse. Nevertheless, despite the lack of an overt phenotype these mice have elicited some fundamental insights into nestin function that would not have been possible in a traditional in vitro setting. The studies by Mohseni et al., 2011 and Yang et al., 2011 demonstrate that although nestin is not necessary for CNS development, it is
important for peripheral motor function and development of the neuromuscular junction (NMJ). Nestin regulates acetylcholine receptor (AchR) clustering at the NMJ. AchR clustering occurs during development of the postsynaptic membrane and is important for regulating the localisation and stabilisation of the developing NMJ. If this is altered, as is the case with nestin KO mice, motor function is impaired. Nestin’s regulation of AchR clustering occurs from its ability to scaffold and regulate Cdk5 activity, which is required for AchR cluster dispersal at the NMJ (Mohseni et al., 2011; Yang et al., 2011).

Nestin is transiently expressed during myogenesis and is understood to regulate the pace at which myogenesis occurs by acting as an inhibitory scaffold for Cdk5, a myogenesis promoting kinase (Lazaro et al., 1997; Pallari et al., 2011). This is mediated partially by PKCζ which acts upstream to regulate Cdk5 activation. PKCζ phosphorylates the Cdk5 activator p35 which triggers calpain mediated cleavage of p35 into p25, a more stable Cdk5 activator leading to sustained Cdk5 activation (de Thonel et al., 2010). Increased Cdk5 activity stimulates nestin reorganisation following phosphorylation at threonine 316 (T316), releasing Cdk5 from the nestin scaffold allowing myogenesis to proceed (Sahlgren et al., 2003).

Nestin clearly has a role to play in angiogenesis during regeneration and pathogenesis. It is also expressed in proliferating and metabolically active endothelium, independent of developmental and neoplastic processes (Mokrý et al., 2004; Mokrý et al., 2008; Sökmensüer and Sökmensüer, 2007). The expression of nestin in angiogenesis may well be regulated by growth factors since mature endothelial cells cultured in the absence of growth factors had attenuated nestin expression (Suzuki et al., 2010). GFP is often coupled to a nestin regulatory element as a reporter to visualise angiogenesis during tumour progression (Amoh et al., 2005; Amoh et al., 2006).

Nestin is expressed in the adult angiogenic vasculature following myocardial infarct, particularly in arteriovenous malformations (Shimizu et al., 2006). In the pituitary gland nestin is expressed during capillary neovascularisation and is downregulated when pituitary infarcts transform to fibrotic tissue (Salehi et al., 2008). Comparable with development, nestin expression in neovascularisation is transient and as nestin is downregulated, vimentin expression is upregulated (Mokrý et al., 2008). While nestin
appears to be expressed in the proliferating endothelium in the adult, it is unclear whether this is a result of increased proliferation or whether it confers specific functions to the newly formed endothelial cells and vasculature (Brychtova et al., 2007). Under shear flow conditions in the endothelium, nestin expression is decreased, which may reflect a need for cells to alter their proteome in order to resist this mechanical stress (Sugawara et al., 2002).

2.2.3 Scaffolding and cytoprotective functions of nestin

Nestin and vimentin heteropolymeric filaments are required for cytoplasmic localisation of the inactive glucocorticoid receptor (GR), a ubiquitously expressed receptor for cortisol and glucocorticoids. The GR interacts with nestin:vimentin filaments. This anchoring of GR is necessary to prevent nuclear accumulation of GR and maintain proliferation (Reimer et al., 2009). This report is interesting since accumulation of GR in undifferentiated cells peaks prior to terminal differentiation, which is typically when nestin protein levels are decreasing, GR, nestin and vimentin co-expression and colocalisation is also a predictor of poor survival in patients with stage III and IV melanoma (Lai et al., 2012). In agreement with the former study Lai et al., 2012 suggest that nestin and vimentin anchoring of GR at the invasive front of the tumour may be important for maintaining the proliferative nature of the tumour, particularly since the colocalisation of all three molecules correlated with increased invasion and tumour size.

Nestin has a cytoprotective function in both neurons and in podocytes in the kidney and this is related to its interaction with and reciprocal regulation of Cdk5. Cdk5 regulates nestin filament organisation by phosphorylation at T316 (Sahlgren et al., 2003). Nestin sequesters Cdk5 and regulate its activity by modulating the Cdk5 activators p35/p25. During stress, such as oxidative stress or high glucose situations, nestin is degraded resulting in sensitization of the cells to Cdk5 pro-apoptotic activity (Liu et al., 2012b; Sahlgren et al., 2006). Under stress conditions, Cdk5 is upregulated and acts upstream of caspase-3 to mediate apoptosis in high glucose treated podocytes, nestin is able to attenuate this effect presumably by sequestering Cdk5 (Liu et al., 2013). In vascular smooth muscle cells oxidative stress leads to nestin upregulation which inhibits apoptosis by Cdk5 sequestration (Huang et al., 2009b).
As we can see here and from the myogenesis studies in Section 2.2.2, nestin is required as a scaffold in order to modulate kinase activity. In the case of Cdk5 it can regulate Cdk5 localisation. Cdk5 is only released once a threshold has been reached that removes the inhibitory scaffold either by phosphorylation-dependent reorganisation or through degradation. While the interactions with Cdk5 are the best characterised so far, other phosphorylation sites have been identified on nestin which will require further study (Namiki et al., 2012). As such the nestin Cdk5 interaction can be used as a paradigm by which to study new nestin interactions.

2.2.4 Nestin in regeneration

Not only is nestin expressed during development it is also transiently re-expressed in response to trauma and is typically associated with proliferation in this context. The fact that nestin expression rarely persists in fully repaired tissue lends itself to the notion that nestin plays a functional role in tissue repair. Nestin expression appears to be primarily in angiogenic structures and progenitor cells recruited to the regeneration site. During regeneration, nestin expression is induced by similar factors to those involved in differentiation (Frisen et al., 1995), suggesting that regeneration could be used as a model to study nestin protein expression and function and vice versa.

One of the first observations of the presence of nestin specifically in regenerating tissue comes from a study on patients with muscular dystrophy myositis. Nestin was found only in regenerating muscle fibres and was correlated with desmin upregulation. This co-regulation of nestin and desmin expression was suggested to be a consequence of similar transcriptional regulation mechanisms (Sjöberg et al., 1994a). A similar sequence of events was confirmed later in injured skeletal muscle whereby nestin and vimentin were upregulated in differentiating myoblasts localised at the injury site (Vaittinen et al., 2001). Nestin re-expression is induced rapidly after spinal or cerebral injury and nestin is highly expressed in glial scar tissue and reactive astrocytes and can persist for over a year (Duggal et al., 1997; Frisen et al., 1995). It is also upregulated in proliferating reactive ependymal cells in the forebrain following irradiation (Shi et al., 2002). The induction of nestin expression was hypothesised to be a dedifferentiation mechanism, as opposed to the recruitment of stem cells that is seen in the heart. In the heart following myocardial infarct nestin positive neural-like
stem cells and myofibroblasts are recruited to damaged area and was associated with angiogenesis and neural remodelling of the infarct region (reviewed in Calderone, 2012; Drapeau et al., 2005; Koenig et al., 2006; Lee et al., 2009). Nestin expression in the heart is controlled by the second intron of the nestin gene (El-Helou et al., 2013). In myofibroblasts, nestin regulates DNA synthesis and proliferation which accelerates the healing process following ischemia (Béguin et al., 2012).

In the kidney, nestin has a slightly different function. In proximal tubule cells, nestin is transiently upregulated in response to hypoxia and TGFβ and regulates the migration of immature renal cells to the site requiring regeneration (Sakairi et al., 2007; Wen et al., 2012). Nestin is also upregulated by serum and PDGF in damaged mesangial cells, which surround the glomerulus. Nestin was shown to regulate their proliferation, but not their migration (Daniel et al., 2008) highlighting the cell-type specificity of nestin function. Nestin is also re-expressed in the pancreas (Lardon et al., 2002), liver (Koenig et al., 2006), skin (Sellheyer and Krahl, 2010), retina (Luna et al., 2010) and teeth (About et al., 2000) following trauma, however, it’s function in these regenerating tissues is poorly characterised.

2.2.5 Nestin in pathology

For many years, nestin has been described as a marker of progenitor cells without any real understanding of its functional role in the cell. The fact that nestin is so widely found in progenitor cells, as well as its transient upregulation in muscle and nerve tissue following trauma, suggests that it may have an important role to play in cell function.

Since nestin is expressed in the CNS, it is natural that it is involved in CNS pathology. Nestin is overexpressed in AD and expression increases with advanced AD and this was attributed to increased proliferation (Perry et al., 2012). Additionally, nestin may have a role in diabetes and AD pathogenesis through its interactions with insulin degrading enzyme (IDE). Misregulation of insulin and amyloid-β levels contribute to type 2 diabetes and AD respectively (Qiu and Folstein, 2006). IDE works to maintain insulin and amyloid-β levels by enzymatic cleavage. Nestin inhibits the degradation of insulin and ubiquitin by binding IDE and regulating its activity, either through allosteric alteration of IDE conformation, or by regulating interaction of IDE and vimentin (Chou et al., 2009; Ralat et al., 2011). In this
way nestin may act to restrain insulin and amyloid-β breakdown thus contributing to Type 2 diabetes and AD pathology. Interestingly, nestin has also been identified in smooth muscle cells in coronary artery plaques and was correlated with increased angina severity (Suguta et al., 2007). Subsequently, two single nucleotide polymorphisms (SNPs) in the nestin gene were identified that correlate with early onset coronary heart disease (CHD) (Meng et al., 2008). However, it is unclear what nestin’s role is in CHD, whether it is pathological or whether nestin is expressed in response to injury caused by plaque formation.

<table>
<thead>
<tr>
<th>Cancer Type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature ovarian cystic teratomas</td>
<td>(Yoshikata et al., 2006)</td>
</tr>
<tr>
<td>Adenoma, Brain tumours</td>
<td>(Rotondo et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>(Chinnaiyan et al., 2008; Kim et al., 2011a; Rani et al., 2006)</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>(Lenz et al., 2011; Vizio et al., 2012)</td>
</tr>
<tr>
<td>Brain tumours</td>
<td>(Arai et al., 2012; Dahlstrand et al., 1992; Kitai et al., 2010; Tohyama et al., 1992; Wan et al., 2011; Yang et al., 2008; Zhang et al., 2008)</td>
</tr>
<tr>
<td>Melanoma, Non-small cell and other lung cancers</td>
<td>(Piras et al., 2010; Tanabe et al., 2010)</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>(Chen et al., 2010; Narita et al., 2014; Ryuge et al., 2011; Ryuge et al., 2012; Skarda et al., 2012; Takakuwa et al., 2013)</td>
</tr>
<tr>
<td>Oral squamous cell carcinoma</td>
<td>(Qin et al., 2012)</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>(Mascolo et al., 2012)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>(Zambo et al., 2012)</td>
</tr>
<tr>
<td>Ependyoma</td>
<td>(Liu et al., 2012a)</td>
</tr>
<tr>
<td></td>
<td>(Milde et al., 2012)</td>
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Table 2 Variability of nestin expression correlation with clinical outcomes in cancer
Nestin has been identified in a number of cancers including: osteosarcoma, prostate, breast testicular cancer, ovarian, skin cancers, gastrointestinal tract cancers, lung cancer, pancreatic cancer, anaplastic thyroid carcinoma, angiosarcoma, glioma and other CNS tumours to name a few (Ishiwata et al., 2011; O Krupkova et al., 2010; Ho and Liem, 1996). During development nestin is considered as a progenitor cell marker, it is also a marker for cells in early neoplastic stages and during angiogenesis. The mechanisms that regulate nestin expression during development and regeneration may also regulate nestin expression during transformation (Dahlstrand et al., 1992; Leach, 2005).

Several studies investigate the correlation of nestin expression in tumours with various clinical outcomes, such as prognosis, tumour grade, metastasis, recurrence and survival (Table 2). In some cases, nestin expression did correlate with worse clinical outcomes, such as worse tumour grade or metastasis. However, this was not always associated with decreased patient survival (Zambo et al., 2012). This variation could be due to study protocol differences, as well as a reflection of the potential complexity of nestin’s function in cancer. Much of the data is correlative and should be treated with care until there is better understanding of how nestin functions in cancer.

In vivo nestin expression may come from the tumour and metastasis itself, but in other cases it appears to be a response by the surrounding tissue to the “injury” caused by the tumour (Idoate et al., 2011). Nestin expressing progenitor cells can be recruited to the tumour margin and to the tumour itself, either by the host or by the tumour secreted growth factors (Glass et al., 2005). The nestin positive host cells recruited by tumours, such as gliomas, can both augment and inhibit functions such as tumour growth through angiogenesis and dissemination from the primary tumour (Duntsch et al., 2005; Glass et al., 2005; Najbauer et al., 2012; Tamagno and Schiffer, 2006). In other cases the nestin expression comes from local upregulation in the tissue proximal to the tumour (Kawamoto et al., 2009; Piras et al., 2010; Tanabe et al., 2010). One hypothesis suggests that immature cells accumulate at the invasive front of tumours and that these nestin positive cells may influence metastatic potential (Brychtova et al., 2007). This reflects nestin’s role in regeneration as opposed to a detrimental pathological role. It is critical in these cases to accurately differentiate between the cancer cells and
the tumour microenvironment. This begs the question of how much of what is seen of nestin expressing cells at the tumour margins actually is derived from the tumour itself, or whether it is a regeneration response from healthy tissue.

2.2.6 **Vimentin is an important scaffold for cell organelles and signalling molecules in homeostasis**

Vimentin is not critical for organism and cell survival as demonstrated by the viability and subtle phenotype of the vimentin knockout (KO) mouse (Bornheim et al., 2008; Colucci-Guyon et al., 1994; Eckes et al., 2000; Evans, 1998; Müller et al., 2009a; Nieminen et al., 2006; Wang et al., 1996). However, it does play an important part in maintaining tissue integrity and spatial localisation of cell organelles.

2.2.6.1 **Vimentin and microtubules regulate organelle targeting**

Vimentin is involved in all stages of the endosomal machinery from endocytosis and recycling to exocytosis. The vimentin filament network acts to scaffold late endosomes and recycling endosomes, and its primary function appears to be regulation of the localisation, composition and targeting of these organelles (Faigle et al., 2000; Styers et al., 2004). Interestingly, some of these interactions are bi-directional since the interaction (or lack of) can also alter vimentin filament organisation (Gao and Sztul, 2001; Gao et al., 2002; Styers et al., 2006). Intracellular organelles are organised and trafficked by MT and their respective motors (Caviston and Holzbaur, 2006; de Forges et al., 2012). In some cases MT have also been involved with vimentin-dependent trafficking. Since MT and vimentin interact and regulate each other vimentin’s role in organelle trafficking may be part of a co-ordinated process with MT (Styers et al., 2005).

Further evidence of a dual coordination between MT and vimentin in organelle positioning and regulation comes from a study showing that vimentin and the MT motor protein dynein regulate nuclear rotation and anchoring (Gerashchenko et al., 2009). Proper nuclear positioning is essential to maintain homeostasis in the cell, as without it, many functions of the cell are completely disrupted (Burke and Roux, 2009). Not only is the vimentin network required for nuclear positioning, it is also required for maintaining
nuclear morphology and proper chromatin organisation (Sarria et al., 1994; Shoeman et al., 2001).

2.2.6.2 Vimentin regulates mitochondria and golgi localisation

Mitochondria are motile organelles and vimentin interacts with and anchors mitochondria in a protein kinase C (PKC)-dependent manner, thereby restricting their motility. Although PKC is known to phosphorylate and cause vimentin reorganisation, this was not the reason for vimentin anchoring of the mitochondria. Instead it was hypothesised that PKC may regulate the tethering of mitochondria to vimentin (Nekrasova et al., 2007; Tang et al., 2008). Vimentin also regulates mitochondrial membrane potential and protects mitochondria from oxidative stress and the site on vimentin that regulates vimentin interaction with mitochondria is proline 57 on the N-terminus (Chernoivanenko et al., 2011; Matveeva et al., 2010; Nekrasova et al., 2011). Vimentin also interacts with and regulates the localisation of the golgi apparatus (Gao and Sztul, 2001; Gao et al., 2002) which is important for proper cell migration (Phua et al., 2009).

2.2.6.3 Vimentin and lipid homeostasis

Vimentin is involved in adipogenesis and lipid metabolism. It forms a cage around lipid droplets and scaffolds cytosolic phospholipase A2 and regulate its cellular localisation during arachidonic acid synthesis (Franke et al., 1987; Nakatani et al., 2000). Vimentin also interacts directly with hormone sensitive lipase in a PKA and insulin dependent manner and facilitates its translocation from the cytosol to lipid droplets (Shen et al., 2010). An intact vimentin network is also important for β3-adrenergic receptor (β3-AR) activation of ERK and lipolysis (Kumar et al., 2007; Shen et al., 2010). Not only is vimentin important for facilitating lipolysis, consistent with it’s role in endosomal trafficking, it participates in Rab9-dependent trafficking of LDL-cholesterol out of late endosomes to the ER (Walter et al., 2009). Further evidence of vimentin’s role in lipid homeostasis comes from the vimentin KO mice which exhibit decreased levels of various steroid hormones which was attributed to defects in the trafficking of lipids from lipid droplets to the mitochondria (Shen et al., 2012). It is clear from the aforementioned studies that vimentin plays a clear role in lipid trafficking which can impact directly on lipid biosynthesis and metabolism.
2.2.6.4 Vimentin maintains tissue integrity and function

The importance of vimentin in maintaining tissue integrity comes from several studies focused on the vimentin KO mouse. Vimentin KO mice have leaky endothelial vessels, disrupted homing of lymphocytes and impaired transcellular migration of lymphocytes (Nieminen et al., 2006). The role of vimentin in inflammation and the immune response is complex. Using the air pouch model the vimentin KO mouse appears to have normal acute inflammatory response to lipopolysaccharide (LPS) or interleukin (IL) -21 stimulation (Moisan et al., 2007). However, in an acute colitis model using dextran sodium sulphate (DSS) vimentin KO mice exhibited less gut inflammation (Mor-Vaknin et al., 2013). These results highlight the specificity of vimentin regulation of inflammation in different in vivo models.

Vimentin is also expressed in adult smooth muscle where it is important for muscle contraction and enables smooth muscle to respond properly to mechanical stress. In a study looking at vasodilation following renal ablation, vimentin was responsible for proper response to shear stress and endothelin mediated vasodilation in renal arteries (Terzi et al., 1997). It is proposed that vimentin has two key functions smooth muscle. Firstly, it is able to connect cells together via cell-cell junctions such as desmosomes, to facilitate intercellular mechanotransduction. Secondly, vimentin is reorganised upon stimulation of contraction, which may affect the actin cytoskeleton and facilitate cell signalling responses to mechanical stress (reviewed in Tang, 2008).

In the CNS, vimentin affects glial and purkinje cell morphology (Colucci-Guyon et al., 1999). Vimentin regulates the proper myelination of the PNS. Without vimentin, neuregulin 1 (NRG1) type III is elevated and this leads to altered downstream signalling through ERBB2 and Akt which contribute to the hypermyelination. Interestingly, the increased NRG1 type levels were dependent on vimentin interaction with TACE secretase which regulates NRG1 activation. It is unclear whether these findings are related to the impaired motor coordination seen in vimentin KO mice, however, since myelin is important for proper neural signal transduction, it is a possibility (Colucci-Guyon et al., 1999).
The emerging pattern from these studies is that although vimentin is not essential for organism and cell survival, it does play a very important role as a scaffold and organiser of signalling proteins and organelles. Perturbation of the proper localisation of many of these structures can have pathological consequences which will be expanded on in the following section.

2.2.7 Vimentin in pathogenesis

As demonstrated in Section 2.1.5, very many IFs are directly associated with specific pathology. However, the role of vimentin in disease is more subtle. This section will briefly go into the role of vimentin in some pathogenic situations (Figure 2). However, since some of these pathologies involve deregulation of vimentin phosphorylation they will be covered in detail in Section 2.4.2.

As stated previously, vimentin KO mice develop without any obvious phenotype. Recent approaches to vimentin mouse models have involved the introduction of single or multiple mutations into key regulatory sites on the protein. Others have challenged the KO mouse in a specific manner in order to identify more functions of the vimentin protein. Most of the energy looking at vimentin’s function has concentrated on the N-terminus which contains many PTM sites known to be important for vimentin organisation and scaffolding functions.

2.2.7.1 Vimentin contributes to cataract formation

A strategic mutation in the vimentin rod domain (Schietke et al., 2006) demonstrated the importance of this central domain in regulating the vimentin organisation and protein-stress response in the mouse which when unchecked can lead to the development of cataracts (Bornheim et al., 2008). This observation led to a follow-up study which found that a single amino acid change (G596A) in human vimentin exon 1 contributes to human cataract formation (Müller et al., 2009a). The mitotic vimentin phosphosites on the N-terminus also regulate eye and lens size in mice and are also associated with cataract formation in old age. The onset of cataract pathology in these mice differed from the aforementioned studies demonstrating that vimentin contributes to cataract formation in several different ways (Matsuyama et al., 2013).
2.2.7.2 Vimentin regulation of immunity

Vimentin has a key role in innate immunity, with a recent study suggesting that vimentin may hamper reactive oxidative species (ROS) production by macrophages and impede bacterial killing, and promote bacterial extravasation and inflammation in an experimental model of colitis which can lead to reduced survival (Mor-Vaknin et al., 2013). Citrullination of vimentin, the conversion of arginine to citrulline, is involved in Rheumatoid arthritis (RA) pathology. Citrullination of vimentin at the N-terminus unsurprisingly disrupts network organisation. Detection of auto-antibodies
against mutated citrullinated vimentin, in which glycine residues are mutated to arginine residues that are then citrullinated, is now being touted as a marker for RA and is being developed as a diagnostic test (Kuna, 2012).

2.2.7.3 Vimentin, epithelial-to-mesenchymal transition, wound healing and cancer

Vimentin expression is strongly associated with many, but not all, highly metastatic and aggressive cancers. It is classically known to confer a migratory and invasive phenotype but may also affect cell proliferation. It is classed as one of the hallmarks of EMT and its expression is regulated during EMT through TGFβ/TCF, NF-κB, Snail/Slug signalling (Gilles et al., 2003; Slabáková et al., 2011; Tsubaki et al., 2013; Vuoriluoto et al., 2010), as well as hypoxia (Liu et al., 2010) and various micro RNAs (Cai et al., 2013; Chen et al., 2013; Cheng et al., 2012; Deng et al., 2013; Shan et al., 2013; Sun et al., 2013; Yamasaki et al., 2012; Zhang et al., 2009). Poly [ADP-ribose] polymerase 1 (PARP-1) induced vimentin expression induces malignant transformation through integrin-linked kinase (ILK)/glycogen synthase kinase 3 (GSK3) signalling in melanoma (Rodríguez et al., 2013). Vimentin is described as a signalling hub for EMT due to the fact that the very presence of vimentin is enough to drive malignant transformation (Ivaska, 2011). How exactly vimentin is able to trigger this is unclear, whether it acts as a signalling scaffold, or whether the vimentin protein itself induces transcription. Although vimentin is not a classical transcription factor, there are lines of evidence that indicate it can be localised in the nucleus, interact with DNA and regulate transcription (Kumeta et al., 2012; Lian et al., 2009; Mergui et al., 2010; Tolstonog et al., 2005; Traub and Shoeman, 1994). Some insight comes from a recent paper that shows that Slug and K-Ras induced vimentin expression regulates the expression of the receptor tyrosine kinase, Axl, and this is necessary for vimentin-mediated EMT migration capabilities (Vuoriluoto et al., 2010). Vimentin and Slug expression are correlated in colorectal cancer and may be predictors of poor prognosis and metastatic disease (Toiyama et al., 2013)

The original studies with vimentin KO mice showed defects and delays in wound healing. A later study shown that induction of vimentin by the TGFβ SMAD pathway is necessary for lung wound healing following injury (Rogel et al., 2011). While delayed wound healing is not a pathogenesis in itself, delay of wound healing can lead to fibrosis and ulcer formation triggering
the molecular mechanisms involved in EMT. The molecular mechanisms behind wound healing and cancer are purported to be similar (Dvorak, 1986; Haddow, 1972; López-Novoa and Nieto, 2009; Schäfer and Werner, 2008). As such, deciphering the molecular mechanisms underlying vimentin’s involvement in wound healing may contribute to our understanding of the role that vimentin plays in cancer progression and EMT.

2.2.7.4 Vimentin, neurodegeneration and neuropathies

Although vimentin expression is low in the adult CNS and PNS it is involved in several neuropathies. Vimentin is re-expressed in astrocytes and neurons in AD brains (Yamada et al., 1992; Yen et al., 1983), in particular vimentin was expressed in the dendritic compartment of cells with AD pathology. Its re-expression in this compartment is understood to be in response to neuronal injury and loss of synaptic connections (Levin et al., 2009). In this sense it may be comparable to nestin expression during regeneration.

GAN is a sensory motor neuropathy characterised by mutations in gigaxonin, an E3 ligase adaptor protein, and NF disruption (Bomont et al., 2000). Gigaxonin interacts with vimentin and while WT gigaxonin is responsible for the proteasomal degradation of vimentin, mutant gigaxonin is unable to. In particular the central rod domain of vimentin is important for gigaxonin mediated vimentin degradation (Mahammad et al., 2013). Vimentin forms aggregates in GAN mutated cells. How GAN contributes to vimentin aggregate formation is unknown, although it is not a response to overexpression or hyperphosphorylation since vimentin protein and phosphorylation levels remain stable in GAN cells (Pena, 1982). As described before in Section 2.1.4, actin and MT networks are important for IF network organisation. MT network destabilisation contributes to vimentin aggregate formation in GAN. It was hypothesised that this may be due to disruptions in the balance of kinesin, dynein and vimentin, although this has yet to be studied (Bomont and Koenig, 2003; Cleveland et al., 2009; Helfand et al., 2002). Considering the contribution of IF aggregates in other diseases, it is likely that aggregated vimentin contributes to pathology in non-neuronal tissues.
2.3 INTERMEDIATE FILAMENTS IN CELL MIGRATION

2.3.1 Why do cells need to migrate?

Cell motility is important during embryogenesis, when cells are required to migrate to specific locations in the developing embryo in order to form various organs and limbs. In the developing brain neuronal cells need to migrate to the correct places in the brain. An inability to migrate properly can lead to many neuronal disorders. In the healthy adult most cells are immotile, motility tends to occur only during immunity, wound healing and tissue regeneration. Cell motility is also necessary during inflammation as immune cells are recruited to the affected tissue. Inflammatory disorders such as asthma and RA are a result of recruitment and migration of immune cells in response to environmental allergens and autoimmunity.

Cancer metastasis is arguably the most well known manifestation of disordered migration. Metastasis is the process by which cancer spreads from the primary tumour to adjacent and distal cites to form new tumours. Traditionally metastasis has been understood to occur at later stages of cancer, however, now it is believed that cancer cells can also disseminate even from pre-neoplastic lesions prior to the formation of a primary tumour (Rhim et al., 2012). Around 90% of deaths from solid tumours are caused by metastases, as such it is of vital importance to understand how metastasis occurs and the underlying processes guiding cell motility and invasion (Nguyen and Massagué, 2007). In order to disseminate, these cells need to undergo EMT whereby cell-cell adhesion junctions and cell polarity are lost and cells acquire a more motile and invasive phenotype to be able to invade through the basement membrane to the surrounding tissue. These cells need to enter and survive in the bloodstream before colonizing distant organs. Typically only a small fraction of cells in the primary tumour are able to undergo all the necessary changes in order for metastasis occur. Metastases usually occur in bone, lung, liver, brain and the peritoneal cavity. The studies presented in this thesis concentrate on cell motility, and therefore the ability of cells to move in their environment will be presented in more detail below.
2.3.2 Stages of cell migration and key signalling mediators

The steps of cell migration include: polarity, protrusion, adhesion, translocation and retraction. While there are a number of proteins that mediate motility the Rho, Rac and Cdc42 proteins sit at the hub of motility signalling. The upstream activator and the strength of the signalling will determine the response of these proteins to the signal and the consequent response of the cell. The balance between Rac and Rho levels determines the type of migration that a cell uses (Deakin et al., 2012)

Figure 4: Key structures and processes of cell motility
2.3.2.1 Lamellipodia and filopodia

Establishment of cell polarity comes from the formation of lamellipodia (Figure 4). Lamellipodia are typically characterised, as wide flat protrusions that resemble a fan. In some instances, cells will form lamellipodia that can form an arc spanning up to 180° of the cell body. Lamellipodia ruffle and move dynamically in order to sense the surrounding environment and prime the cell for directional movement. Actin is the main cytoskeletal element in lamellipodia and the rate of actin polymerisation drives the protrusion of the cell membrane and lamellipodia formation (Ryan et al., 2012). There is some controversy as to whether lamellipodia are required for cell motility (Steffen et al., 2013; Wu et al., 2012), possibly as a result of differences in cell models and experimental systems used in the studies.

Filopodia are long, protruding spikes formed at the leading edge of the cell (Figure 4). They are highly dynamic structures and extend past the lamellipodia to function as topographical, chemo- and mechano-sensors which enable the cell to co-ordinate its directed migration (Albuschies and Vogel, 2013; Heckman and Plummer, 2013). Filopodia and their associated proteins are associated with highly invasive behaviour of cancer cells (Machesky, 2008; Vignjevic et al., 2007). Filopodia are actin-rich and are regulated primarily by Cdc42 (Nobes and Hall, 1995). The morphology of actin in filopodia is more bundled and less branched than in lamellipodia which therefore requires different proteins to be involved in growth of the filaments.

Rac and Cdc42 act to coordinate filopodia and lamellipodia formation (Nobes and Hall, 1995) and it is understood that functional Rac signalling is required for Cdc42 and RhoG to participate in lamellipodia formation (Steffen et al., 2013). Rac and ERK/MAPK pathways activate the WAVE (a member of the Wiskott-Aldrich syndrome protein family (WASP)) complex at lamellipodia tips which in turn activates the Arp2/3 complex in order stimulate actin branching to generate actin filament networks (Mendoza et al., 2011; Steffen et al., 2013). Rac requires nascent FA formation in order to form lamellipodia, once FA start to form, Rac is activated and this activation spreads out in a wave as new FA are formed (Goldmann and Ingber, 2002; Xia et al., 2008), thus promoting actin nucleation and lamellipodia protrusion. FA proteins such as paxillin and vinculin are also required for proper positioning of lamellipodia for directed cell motility (Goldmann and
Ingber, 2002; Sero et al., 2011). Cdc42 is required to activate the Arp/3 actin nucleation complex and formins (mDia proteins) to the lamellipodia. The Rho GTPases regulate myosin and the formins in order to activate actomyosin contractility (Sixt, 2012).

The signalling pathways governing filopodia and lamellipodia are not independent of one another. Cdc42 has also been shown to induce filopodia formation by binding to IRSp53, WAVE2 and MENA (a member of the ENA/VASP family) to promote actin nucleation and filament growth. RhoA and its effector ROCK co-ordinate and switch signalling between Rac and Cdc42 to determine protrusion formation. Other proteins that are involved in actin dynamics in filopodia formation include: formins which are important for unbranched actin elongation and fascin, a key regulator of filopodial actin cross-linking, to name a few (El-Sibai et al., 2008; Mattila and Lappalainen, 2008).

### 2.3.2.2 Substrate adhesion

Focal adhesion (FA) is the name given to the intermediate complexes formed at the base of the cell which are key mediators of cell adhesion and transmission of signals from the ECM to the cell to stimulate pathways involved in cell survival, proliferation and migration. FA are the primary sites of adhesion and force transduction during cell motility (Figure 4). FA are formed through the recruitment of signalling effectors and scaffolding proteins to activated integrin tails at the interface of the plasma membrane and the ECM. FA are dynamic structures located in lamellipodia regions with a capacity for rapid turnover. At the last count it was found that FA can consist of well over 150 proteins also known as the integrin “adhesome” (Zaidel-Bar et al., 2007). Proteomics studies have identified many more proteins involved in FA composition, however further validation is required to identify their role in FA. The protein composition of adhesions fluctuates even within a single adhesion and is dependent on, for example, adhesion age, ECM composition and substrate tension, among others (Wolfenson et al., 2013; Zaidel-Bar et al., 2003).

Cell motility requires an optimal balance of cell adhesion, too little, the cell cannot generate enough traction to move, too much, and the cell is unable to translocate. During migration FA act to stabilise cell adhesion to the ECM to allow the contraction of actomyosin which enables the cell body to
translocate. FA are constantly assembled and turned over at the leading edge and disassembled at the rear of the cell (Nagano et al., 2012). Early stages of FA assembly is a Rac dependent process characterised by the sequential recruitment of specific proteins to the integrins such as, talin, paxillin, vinculin, α-actinin, FAK and vasodilator-stimulated phosphoprotein (VASP) (Zaidel-Bar et al., 2003; Zaidel-Bar et al., 2004; Zamir et al., 1999). At this point, the FA may either disassemble or continue to grow. This is characterised by a RhoA activation switch from Rac signaling and local mechanical force generated by actomyosin contractility. Spatial location of the FA relative to the trailing and leading edges is thought to be a trigger of FA maturation and to some extent disassembly (Berginski et al., 2011). How spatial localisation and mechanotransduction integrate with the signalling mechanisms regulating the dissolution of FA requires further investigation. It is currently unknown what the minimum signalling and FA composition requirements are for cell migration.

2.3.2.3 Translocation of the cell body

As the cell protrudes its membrane through the growth of actin filaments, the cell body needs to move forward. This is achieved through actin retrograde flow, which is occurs because of the opposing tension in the plasma membrane to the polymerisation of the actin during cell membrane protrusion. Actin retrograde flow pushes the actin network back to the cell body. Lamellipodia adhere to the substrate by the formation of FA and together with actomyosin contraction and the forces of the retrograde actin flow, the traction required for forward propulsion and thus translocation of the cell body is achieved (Sixt, 2012). The linkage between the actin cytoskeleton and FA is often described as a molecular clutch (Vicente-Manzanares et al., 2009).

2.3.3 Cell migration plasticity and invasion

Migration is stimulated by cues in the cell’s environment. The cues maybe directional and can be categorised as chemotactic, haptotactic, phototactic, mechanotactic, electrotactic or durotactic. Chemotactic cues may be small molecules or proteins such as growth factors; otherwise, they can be osmotic cues. Cells will respond to chemotactic stimuli depending on whether they are able to sense the chemotactic agent through ligand binding. Haptotactic migration is a response to a variation in concentration of a solid substrate.
Haptotactic cues are similar to chemotactic, but they are more subtle than a binary chemotactic cue. Phototaxis is the response of a cell to light cues. Mechanotactic migration results from changes tension of the physical surroundings, for example by wounding or changes in cell-cell adhesion. Electrotaxis is the response to an electric field and durotaxis is migration determined by ECM rigidity (Roussos et al., 2011).

The distinction between cell invasion and cell migration is rather murky. Often the terms are used interchangeably in the literature. Cell migration refers to the act of the cell translocation in a 2D or 3D environment. Cell invasion is more precise in that it refers to a cell moving through a 3D substrate, dependently or independently of proteolytic mechanisms (Hulkower and Herber, 2011). Cell motility can be divided into two types, single cell migration and collective cell migration and the type of migration a cell uses will depend on its environment and the cell type.

### 2.3.3.1 Single cell migration: amoeboid vs. mesenchymal

Amoeboid migration is characterised by rounded cells exhibiting a gliding or blebbing motion which is a result of poor adhesion and a poorly organised cytoskeleton. The actomyosin cytoskeleton is polarised and the traction required for movement is generated through actomyosin polymerisation and contraction, rather than by adhesion to the substrate. Mesenchymal migration is characterised by the formation of lamellipodia, adhesion to the substrate through FA, which can generate traction forces which then enables the cell to translocate. Another key difference between the two is that mesenchymal migration requires proteases such as matrix metalloproteinases (MMP) in order to create a pathway to migrate through the ECM. Amoeboid migration does not require proteases and relies on the ability of the cells to modulate their shape and squeeze through holes in the ECM.

Amoeboid and mesenchymal migration are interconvertible. There is strong evidence showing that the density and elasticity of the ECM can influence the type of cell motility. Denser ECM with non-linear elastic qualities favours mesenchymal migration whereas linear elastic non-adhesive ECM promotes amoeboid migration. The intracellular switch between amoeboid and mesenchymal migration is governed by the small GTPases Rac and RhoA. Rac activation promotes actin polymerisation and reduces
contractility through actin regulators, Arp2/3 and WAVE2. It also promotes lamellipodia formation, MMP activity, MMP production, and consequently mesenchymal migration. On the other hand, RhoA activation triggers ROCK-dependent myosin II activation, actomyosin contractility, inhibition of Rac, polarisation of ezrin and blebbing amoeboid movement (Bergert et al., 2012; Petrie et al., 2012; Sahai and Marshall, 2003; Sanz-Moreno et al., 2008; Wolf et al., 2003).

2.3.3.2 Collective cell migration

Collective cell migration occurs when cells migrate together. This type of migration occurs during embryogenesis, development, angiogenesis and wound healing. In cancer, particularly carcinomas, collective migration occurs at the tumour border. High substrate stiffness enhances collective cell migration. Since often the tumour stroma is stiffer than normal tissue stroma, this may serve to encourage tumour invasion (Ng et al., 2012). Collective cell migration requires maintenance of cell-cell junctions, a feature that is lost in single cell migration. The cells in the anterior part of the migrating sheet or strand form lamellipodia and pseudopodia protrusions and generate traction and contractility using FA and myosin-II. The rear of these cells maintains cell-cell contact through cadherins and tight junction proteins and exerts pulling forces on the cells behind them (Friedl et al., 2012; Ng et al., 2012). Intact cell-cell junctions maintain the paracrine signalling required for co-ordination and movement of the cell group. A study in drosophila border cells showed that increasing Rac activation over the basal level in just a single cell was able to stimulate collective migration of an entire cell cluster (Wang et al., 2010). The signalling for collective migration is highly complex and uses a blend of intracellular signalling commonly found in single cell migration and signalling gradients throughout the cluster which can determine migration behaviour (Bianco et al., 2007; Ng et al., 2012).

2.3.3.3 Breaching the matrix

Tissues are comprised of different cell types and the extracellular matrix (ECM). The ECM surrounds and acts as a scaffold for cells and interacts with them biochemically and mechanically, regulating tissue morphogenesis, differentiation, homeostasis and pathology. Apart from water and polysaccharides, the ECM is composed of a variety of fibrous proteins and
glycoproteins, including 28 types of collagen, elastin and fibronectin among others. These proteins form matrices which vary in their physical properties such as rigidity, porosity and topography. Since the ECM components are produced by the constituent cells, each tissue has a unique ECM composition, which reflects the cellular composition and requirements of the tissue. The ECM is continuously remodelled enzymatically, non-enzymatically and post-translationally by the cells it surrounds (Lu et al., 2012).

For amoeboid migration, cells need to deform themselves to traverse pores in the ECM. If cells are unable to deform their nucleus sufficiently the cell needs to break down the ECM proteolytically and remodel it to create a path in order to move in a mesenchymal fashion (Wolf et al., 2013). Digestion of the ECM occurs through cell surface expression or secretion of proteolytic enzymes such as serine proteases, cathepsins and MMPs. Degradation of the ECM can release pro-migratory signalling molecules bound to the ECM and produce ECM fragments which can independently regulate cell function such as migration (Giannelli et al., 1997; Page-McCaw et al., 2007).

In Section 2.3.3.2 it was mentioned that the tumour stroma is often stiffer than normal ECM, enabling faster migration. The stiffening of the network arises, for example, from increased collagen crosslinking. Collagen fibres are also remodelled from networks into thicker, more linear fibres along which cells can move (Condeelis and Segall, 2003; Levental et al., 2009; Provenzano et al., 2006). Insight into how MMPs remodel ECM came from an elegant study which showed that collagen degradation by membrane type 1-MMP (MT1-MMP) does not occur at the very anterior of the cell. Instead, collagen is degraded adjacent to the anterior of the cell and towards the cell rear. In these regions, MT1-MMP is colocalised with actin and β1-integrin, reminiscent of invadopodia, small protrusive structures that facilitate cell invasion. Collagen fibres that run perpendicular to the cells’ direction of movement are cleaved and the fragments are pushed forwards leaving an open track, facilitating the migration of other cells (Wolf et al., 2007).

While some cell types clearly favour one type of migration over another, the reality is that cells switch between different migration modes and use a combination depending on the environmental context (Bergert et al., 2012; Friedl and Wolf, 2010; Petrie et al., 2012).
2.3.4 Vimentin is a potent driver of cellular migration

The IF profile of cancer cells has been studied for over a quarter of a decade. The fact that IF expression profiles reflect the differentiated/transformed state of a cancer cell was first published in the mid-eighties (Günther et al., 1984). Vimentin has been associated with metastatic ability of cells for over 30 years (Hendrix et al., 1992; Ramaekers et al., 1983; Thompson et al., 1992). It is widely accepted that expression of vimentin confers an increase in migratory ability (Chung et al., 2013). Early suggestions that vimentin is important for cell motility came during observations of wound healing of rabbit corneas (SundarRaj et al., 1992) and later in vimentin KO mice whose fibroblasts had poor chemotactic migration and the mice themselves exhibited wound healing defects (Eckes et al., 1998; Eckes et al., 2000). Further studies provided evidence that vimentin is important for proper organisation of FA and sensing of chemotactic signals and directed cell migration (Eckes et al., 1998). Vimentin also regulates cell adhesion molecule organisation such as intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and β1 integrin. Vimentin KO peripheral blood mononuclear cells (PBMC) are unable to home to lymph nodes and transendothelial migration is severely impaired. Quantitative analysis of protein levels and organisation of vimentin is correlated with cell shape and migratory ability (Mendez et al., 2010).

Vimentin has a role in cell polarity, lamellipodia formation and formation of contacts with the ECM and invadopodia formation. There are numerous papers describing the effect of over-expression and down-regulation of vimentin on cell motility which have been reviewed extensively already (Chung et al., 2013). Instead, this section will focus on specific mechanistic aspects of vimentin and nestin regulation of cell motility (Figure 5).

2.3.4.1 Vimentin reorganisation and lamellipodia

Much research on the IFs has until recently focused on the filament networks themselves. The soluble, non-filamentous pool of IFs, which is necessary for subunit exchange and the reorganisation of filament networks, has largely been ignored. Non-filamentous vimentin subunits are important for lamellipodia formation, cell polarity and consequently cell motility. Vimentin IFs are inhibitory for lamellipodia formation and ruffling, but necessary for fibroblast locomotion. Upon Rac-1 activation, which is also a stimulator for FA formation, vimentin filaments disassemble in a S38
phosphorylation dependent manner and retract, independently of MT, from the cell periphery which facilitates actin-dependent lamellipodia formation. It was proposed that vimentin disassembly could act as a molecular clutch for actin-based motility and polymerised vimentin could act as a brake on lamellipodia formation (Helfand et al., 2011). Filamin A is a scaffold for vimentin and active PKCε enables vimentin phosphorylation at S6, S38 and S50. This recruits vimentin to the cell periphery, and regulates the surface expression and activation of β1 integrin (Kim et al., 2010a; Kim et al., 2010b). This extends the studies showing that PKCε-dependent phosphorylation of vimentin is important for β1-integrin trafficking to the cell membrane (Ivaska et al., 2005). Interestingly, PKCε also phosphorylates the tight junction protein zona occludins protein-1 (ZO-1) and regulates ZO-1 association with α5 integrin which in turn regulates lamellipodia formation and cell motility (Tuomi et al., 2009). ZO-1 also regulates Rac activity – a stimulator of lamellipodia formation. Perhaps vimentin IFs act as a preliminary scaffold for the recruitment of lamellipodia associated proteins to the cell periphery, at which point vimentin is no longer required for this process and is disassembled by phosphorylation and the non-filamentous vimentin retracts to the cell body.

2.3.4.2 Intermediate filaments act as focal adhesion signalling hubs

Considering one of the functions of IF is to provide resistance to mechanical stress and mediate mechanotransduction signalling it is natural that they are involved in FA composition and function. Studies on IF and FA have focused mainly on keratins and vimentin. Mechanical stress response at FA occurs through co-ordinate interactions of the actin, MT and IF cytoskeleton (Bordeleau et al., 2008). Keratins are able to regulate this through RhoA/ROCK regulation of actin dynamics (Bordeleau et al., 2012).

Vimentin filaments connect to integrins in FA through interactions with the linker protein plectin and β3 integrin in order to stabilise FA and modulate adhesion strength (Bhattacharya et al., 2009; Gonzales et al., 2001; Kreis et al., 2005). Since plectin acts as a cytoskeletal linker between actin, MT and IFs (Svitkina et al., 1996) it may act to link the IF and actin cytoskeleton at FA to provide initial stabilisation of the FA. In these experiments it appeared that vimentin stabilised FA dynamics, whereas others have shown that vimentin enhances FA turnover (Mendez et al., 2010). This may reflect differences in cell types and the FA proteins studied (Kim et al., 2010a). In
prostate cancer cells, vimentin knockdown increased Erk1/2 activation which was accompanied by a decrease in cell motility and invasion with no change in Akt levels. Interestingly, there was also a decrease in β4-integrin protein levels. Plectin and β4 integrin interact and it is thought that vimentin, plectin and β4-integrin interact to mediate cell motility and invasion (Burch et al., 2013).

Vimentin regulates VASP localisation to FA and acts as a scaffold for protein kinase G (PKG) and VASP, enabling PKG to phosphorylate and activate VASP (Lund et al., 2010). Vimentin also interacts with the scaffolding protein, receptor for activated C kinase 1 (RACK1), and FAK, which serves to regulate FAK expression and activation. Without RACK1 or vimentin, FA formation and cell adhesion was attenuated leading to impaired endothelial sprouting (Dave et al., 2013). Keratins also act as FA signalling modulators through PKCδ. K8/K18 regulates FAK activation and turnover at FAs, as well as modulating assembly of a FA localised signalling complex containing RACK-1-plectin-PKC-Src (Bordeleau et al., 2010). Not only can vimentin act as a signalling scaffold during migration, the very expression of vimentin can induce upregulation of migration-related proteins. A recent example describes the vimentin induced upregulation of Axl, a receptor tyrosine kinase involved in haptotaxis and signalling to Rac and Akt. Vimentin mediated migration in breast cancer cells was highly dependent on Axl upregulation, as without Axl, vimentin exhibited weak effects on migration (Vuoriluoto et al., 2010). How this vimentin-dependent upregulation of Axl occurs remains to be investigated.

As discussed previously in Section 2.1.3 heteropolymers of IFs can confer altered filament characteristics. Synemin acts as a bridge joining FX, IFs and the actin cytoskeleton (Uyama et al., 2006). It is also able to bind to α-actinin in membrane ruffles as well as several FA proteins, including talin, vinculin and zyxin. It inhibits recruitment of zyxin to FA, effectively blocking adhesion and promoting cell motility (Jing et al., 2005; Sun et al., 2008a; Sun et al., 2008b; Sun et al., 2010a). These results suggest that not only does synemin act as a connector between the ECM and the actin and IF cytoskeleton, but that it directly regulates FA composition in order to fine tune the balance between adhesion and cell migration.
2.3.4.3 Focal adhesions regulate intermediate filament formation

Not only do IF have a role in FA stabilisation and composition, FA themselves also regulate IFs. Recent work has shown that non-filamentous vimentin subunits are transported along MT to the sites of growing adhesions which catalyses the formation of vimentin filaments and stabilisation of FA which then enhances signal transduction (Lynch et al., 2013; Tsuruta and Jones, 2003). This is supported by earlier work describing transport of IF precursors along microtubules and the requirement for motor proteins dynein, kinesin in proper vimentin organisation (Helfand et al., 2002; Sakamoto et al., 2013; Shim et al., 2008). Further work showed that the plectin isoform, P1f, recruits and anchors vimentin precursors at mature FA, enabling de novo filament assembly leading to FA stabilisation which contributes to increased cell polarisation (Burgstaller et al., 2010).

One hypothesis regarding vimentin’s role in migration is that it is not directly involved in the act of migration but instead serves to maintain directionality of a cell that has already committed to migrate (Chernoivanenko et al., 2013). This view is supported by the work of Mendez et al., 2010 who show that vimentin disassembly in targeted regions induced membrane ruffling and stimulated the cells to change migration direction. The actual dynamics of vimentin filament organisation during cell locomotion remain to be elucidated. The studies presented here are just scratching the surface of the mechanisms involved in vimentin’s role in cell motility. It is clear that vimentin is important as a scaffolding protein at FA and throughout the cell, not just to provide mechanical support but also to interact with signalling molecules for their either proper activation and/or localisation. Most likely, there is a complex co-ordination of phosphorylation and dephosphorylation and interaction with linker proteins such as filamin and plectin with MT and actin. There is much work to do to understand vimentin’s role fully in cell motility. High-resolution live cell imaging and advanced image analysis and modelling of vimentin filament organisation during cell migration will greatly to help understand how the vimentin network influences migration.
2.3.5 Nestin as a regulator of cell migration and invasion

The first study to really try and elucidate a functional role for nestin in cancer showed that nestin promoted the migration and metastasis of PC-3 prostate cancer cells. They also noted that nestin expression increased in metastases of men who had undergone androgen deprivation therapy (Kleeberger et al., 2007). Androgen deprivation would appear to trigger some cells to revert to a more progenitor like state (Pfeiffer et al., 2011). While there are a number of studies providing correlative evidence for a role for nestin in cell migration (Section 2.2.5), real functional understanding is woefully limited.

![Diagram of cell migration and invasion](image)

**Figure 5:** Vimentin and nestin participate in many aspects of cell migration. This summary diagram demonstrates some of the separate and shared functions of vimentin and nestin in the organisation of cell motility signalling and organisation.
Nestin may regulate cell migration and invasion by several means. Downregulation of nestin in pancreatic cell lines corresponded to a redistribution of the actin network and affected cell morphology and retarded cell migration (Matsuda et al., 2011). This is particularly interesting since the nestin homolog synemin interacts with α-actinin, an actin-interacting protein known to bind actin, and connect it to FA via FA proteins such as vinculin and zyxin (Bellin et al., 2001; Sun et al., 2010a; Uyama et al., 2006). Synemin interacts with vinculin and talin directly and several of these interactions impact cell motility and adhesion (Pan et al., 2008; Sun et al., 2008b; Sun et al., 2010a; Uyama et al., 2006). These interactions are cell-type dependent, as synemin was not found to interact with vinculin in cultured astrocytes (Jing et al., 2007). Synemin and nestin have been described as homologs. Although they share little sequence similarity, they both share the features of having unusually short N-termini and long C-termini and they both require an IF binding partner to form filaments (Guérette et al., 2007). All the interactions that synemin has with FA related proteins are through its long C-terminus, and the length of the tail is believed to facilitate binding of IFs to other proteins. Consequently, nestin and synemin may have analogous functions in different tissues.

2.4 REGULATION OF INTERMEDIATE FILAMENTS BY POST-TRANSLATIONAL MODIFICATIONS

IFs are regulated on a transcriptional, translational and post-translational level. However, it is the primary focus of this section to describe the post-translational and assembly related regulation of IFs, with particular focus on vimentin.

One of the key regulators of IF function are PTMs. While often PTMs are thought of in the orthosteric sense, whereby the PTM directly confers a binding site for a protein for instance, there is increasing understanding that allosteric PTMs, those that confer conformational alterations, are equally important in regulating protein function. As such the combination of allosteric and orthosteric PTM regulation combined with the fact that many PTMs cross-talk, provides an immense array and depth of regulatory possibilities. This has been referred to as the ‘PTM code’ and is suggested as one reason as to how proteins can appear to regulate a diverse range of biological functions (Nussinov et al., 2012). Given the range of functions of
some IFs, such as nestin, this concept of a PTM code regulating its function could easily apply.

Eukaryotic phosphorylation is the addition of a phosphate group to a serine(S), threonine(T) or tyrosine(Y) residues on a protein. Phosphorylation is catalysed by a kinase, while protein phosphatases catalyse the removal of the phosphate group. Interestingly, kinases make up around 2% of the genome (Manning et al., 2002), in comparison there are approximately 50% fewer identified phosphatases which implies that phosphatases are less specific than kinases (Sacco et al., 2012). Sequential protein phosphorylation events can act as a form of signal amplification. Co-operative phosphorylation events will require that several phosphosites are modified before a signal is generated. Each of these types of multi-phosphorylation events can be considered as a threshold to regulate filament organisation or cell signalling events. While much of the focus on the next couple of sections is on the action of phosphorylation and kinases, dephosphorylation by phosphatases is equally important to maintain phosphoprotein homeostasis since hyperphosphorylation of IFs is a trigger for many diseases.

2.4.1 **Overview of intermediate filament phosphorylation**

Phosphorylation of IFs is arguably the most potent PTM regulator of IF stability, organisation and function. It is at least the most well studied of all IF PTMs. Almost all IFs are validated phosphoproteins, with the exception of syncoilin, a recently identified and very little studied IF. Considering the pervasiveness of phosphorylation in IF regulation, future studies may reveal a role for phosphorylation in syncoilin function. IFs were found to be phosphoproteins very early on (Lazarides, 1980) and it was noted that IF phosphorylation appeared to be a dynamic process (Robinson et al., 1981). However, the purpose of IF phosphorylation remained a mystery until a landmark paper correlated IF network organization with phosphorylation events in mitosis (Evans and Fink, 1982). Since then IF phosphorylation as a regulator of IF organisation and function is implicated in a whole host of cellular processes and pathologies (Hyder et al., 2008).

Phosphorylation on IFs typically occurs on the head and tail domains. Phosphosites have been predicted by mass spectrometry (MS) on the central helical rod domains, although these are unvalidated (Hornbeck et al., 2004). Phosphorylation is a transient PTM and regulates the dynamic assembly and
disassembly of IF filaments (Inagaki et al., 1987), in some cases causing complete disassembly into soluble fragments or, conversely causing protein aggregation (Heins and Aebi, 1994; Kasahara et al., 1993). Often the protein aggregation is a misregulation of phosphorylation processes which can lead to disease (Hyder et al., 2011). As such, control of phosphorylation needs to be tightly regulated in order not to disrupt cellular and tissue homeostasis.

The roles of phosphorylation in IF regulation are three-fold: IF assembly, protein-protein interactions and signalling, however, they are all interconnected. Phosphorylation on the vimentin head domains disrupts IF dimerisation (Aziz et al., 2009), and it reduces the affinity of IF head domains for the central rod domains, thus promoting disassembly (Gohara et al., 2001). The influence of phosphorylation is more complex, as it can also induce structural changes in the non-adjacent linker 2 and C-terminus (Pittenger et al., 2008). In the context of the discussion above, this type of phosphorylation event would be considered allosteric. It must be stressed that most of these structural studies have been conducted in in vitro systems and may not mimic the reality of in vivo vimentin assembly/disassembly. Examples of the other roles of phosphorylation as a regulator of protein-protein interactions and signalling will be covered in the next section with a particular focus on vimentin.

2.4.2 Vimentin phosphorylation

Vimentin has 23 identified phosphorylation sites on its N-terminus and C-terminus. The N-terminus phosphosites are primarily regarded to regulate vimentin organisation and protein-protein interactions. A comprehensive list of vimentin phosphosites, their function and associated kinases is presented in Figure 6 at the end of this section. The goal of this section is to highlight some established and emerging themes of vimentin phosphorylation. In particular it seems that certain phosphosites are specifically phosphorylated by one or two kinases, which is reflected in its very narrow function in vimentin regulation. Others such as S38, appear to be commonly phosphorylated by a large set of kinases, leading to a broad array of functions for those particular phosphorylation events.

While there is some research emerging on IF phosphorylation functions and interactions we are still very much at the discovery phase of finding out what each site does and to what physiological processes it contributes. In the
future it will be interesting to consider the added diversity brought by cell type specific phosphorylation functions. For instance nestin exhibits differential phosphorylation according to cell type, with some nestin phosphosites being CNS specific (Namiki et al., 2012). This could be another explanation as to why vimentin C-terminus functions have remained elusive - they simply have not been investigated under the right conditions.

The majority of the validated phosphosites on vimentin are serines. There are also five tyrosine phosphorylation sites on the vimentin head-domain that have been identified by several independent MS studies, but none have been validated \textit{in vivo} (Hornbeck et al., 2004). As such all of the discussion on the phospho-regulation of vimentin will concentrate on serine phosphorylation.

\subsection*{2.4.2.1 Signalling scaffolds and protein localisation}

The 14-3-3 proteins can be considered as central signalling hub for the signalling of a diverse range of cell functions. 14-3-3 proteins have high affinity for binding phosphorylated proteins (Yaffe et al., 1997). 14-3-3$\zeta$ binds phosphorylated vimentin at the N-terminus. Sequestration of 14-3-3$\zeta$ by vimentin leads to displacement of other proteins bound to 14-3-3$\zeta$, such as Raf-1 (Tzivion et al., 2000). 14-3-3$\epsilon$ binds to phosphorylated vimentin and acts as a bridge to bundle vimentin and GFAP in reactive astrocytes (Satoh et al., 2004). As described in Section 2.4.2.3 PI3K$\gamma$ mediates vimentin phosphorylation at S6 on the N-terminus which facilitates 14-3-3:vimentin binding (Barberis et al., 2009). Considering that vimentin does not contain classical 14-3-3 binding motifs it has been suggested that multiple phosphorylation sites would be requirement for stable 14-3-3 binding (Tzivion et al., 2001). Many kinases known to phosphorylate vimentin are also 14-3-3 binding proteins, suggesting that 14-3-3 binding to vimentin may regulate kinase binding to vimentin. An alternative view is that vimentin could sequester these kinases away from 14-3-3 perhaps acting as a checkpoint to control signal propagation (Margolis et al., 2006). Recent work shows that Akt phosphorylation of Beclin, an autophagy marker, can lead to vimentin sequestration of Beclin in a complex with 14-3-3$\epsilon$, tipping the balance between tumour-suppressive autophagy and tumourigenesis (Wang et al., 2012b). This interaction of vimentin, Beclin and 14-3-3$\epsilon$ is mediated by vimentin phosphorylation at S38. Whether this phosphorylation is Akt-dependent is unclear, although S38 is a known Akt target (Zhu et al., 2011).
IFs are well described as signalling scaffolds that are able to regulate the activation of signalling molecules, while being a target of regulation themselves. In smooth muscle cells, 5-hydroxytryptamine (5-HT) stimulation activates Cdc42 GTPase-activating protein (Cdc42GAP) and p-21 activated kinase (PAK). This induces PAK-dependent phosphorylation of vimentin at S56, which results in disassembly and reorganisation of vimentin and consequently the dissociation of the adaptor protein p130 Crk-associated substrate (p130Cas) from vimentin. Since p130Cas is a known PAK activator, the dissociation of p130Cas from vimentin acts as a feedback loop to facilitate PAK activation and vimentin disassembly. This regulates force generation in smooth muscle during contraction (Li et al., 2006; Li et al., 2009). 5-HT stimulation and consequent vimentin phosphorylation enhances the translocation of the serotonin transporter (SERT) to the plasma membrane in platelets and facilitates the association of SERT and vimentin at the plasma membrane (Ahmed et al., 2009).

In neuronal cells, vimentin phosphorylation and scaffolding functions contribute to the aggregation of the mutant Huntington polyQ protein (Htt) which is a characteristic of Huntington’s disease. Aggregation of the mutant Htt protein leads to the formation of aggresomes surrounded by a vimentin “cage” at centrosomes. Altered IP3R1 (inositol 1,4,5-trisphosphate receptor type 1) signalling is implicated in the pathogenesis of Huntington’s disease. IP3R1 signalling is negatively regulated by IRBIT (IP(3)R binding protein released with inositol 1,4,5-trisphosphate protein), which competes with IP3 (inositol trisphosphate) for binding to IP3R1. Vimentin phosphorylation at S38 and S71 by RhoA kinase (ROCK) stimulates perinuclear reorganisation of vimentin. This altered vimentin network organisation sequesters IRBIT. Without IRBIT regulating its activity, IP3R1 activity increases, leading to increased accumulation of mutant Htt, aggresome formation and neurodegeneration (Bauer et al., 2012).

Vimentin phosphorylation promotes axon growth and Schwann cell migration towards injured neurons. Phosphorylation of vimentin at S55 by Cdk1, an event first identified in mitosis (see Section 2.4.2.2), is upregulated in Schwann cells following nerve injury. Co-culture of injured Schwann cells with dorsal root ganglion (DRG) cells promoted DRG neurite outgrowth. This was due to Cdk1-dependent S55 phosphorylation of vimentin, which promotes interaction of vimentin with and stimulation of β1 and β3 integrin activation. This in turn leads to downstream effects on ERK1/2 signalling.
and focal adhesion kinase (FAK) localisation and activation (Chang et al., 2012; Chang et al., 2013). This could well be related to the scaffolding and retrograde transport of active ERK1/2 by soluble vimentin in injured neurons (Perlson et al., 2005; Perlson et al., 2006) and demonstrates that vimentin phosphorylation contributes to vimentin as a protein and signalling scaffold.

A growing body of research suggests that the filament precursors and soluble fragments also participate in scaffolding and signalling functions. Often they are involved in a positive feedback loop of regulation. Soluble vimentin interacts with activated MAP kinases (MAPK, Erk1/2, p38) to induce mast cell activation (Toda et al., 2012). In this case vimentin was phosphorylated on S55, S71 and S81 on the N-terminus. The degree of co-operative phosphorylation would be enough to solubilise vimentin extensively. Work in our lab has shown that during myogenesis, soluble nestin is a more efficient scaffold for Cdk5/p35 than filamentous nestin and that Cdk5 phosphorylates nestin on T316 to promote its own association with nestin further (Pallari et al., 2011). Vimentin is also a Cdk5 target at S56, and scaffolds Cdk5/p35 in GTP-stimulated neutrophils, although whether the interaction and regulation is reciprocal is unknown (Lee et al., 2012).

Together these studies demonstrate that the phosphorylation and assembly state of vimentin (and IFs in general) is important for protein interaction, localisation and transport and may have implications for disease.

2.4.2.2 Mitosis

Much of the original work on the function of vimentin phosphorylation comes from studies in mitosis. Vimentin is extensively phosphorylated and organised throughout mitosis and provides a comprehensive example of the co-operative phosphorylation events involved.

During prometaphase, as the cell enters mitosis and proceeds through to metaphase, vimentin is phosphorylated on S55 by the mitotic kinase CDK-1 (Chou et al., 2003; Tsujimura et al., 1994). This triggers recruitment of Plk-1 to vimentin and consequently phosphorylation on S71, S72 and S82 by ROCK, Aurora B kinase and Plk-1 respectively, enabling filament segregation between the two daughter cells (Goto et al., 1998; Goto et al., 2003; Yamaguchi et al., 2005; Yasui et al., 2001). This process is precisely co-
ordinated: while recruitment of Plk1 requires the prior phosphorylation of S55, Aurora-B and ROCK act independently to co-ordinate cytokinesis. However, neither is indispensible since depletion of either will cause mitotic defects. Plk-1 also acts to phosphorylate vimentin at the C-terminus S459 residue and facilitates endocytic vesicle fusion during M-phase, particularly the delivery of β1-integrin to the cleavage furrow.

Unlike N-terminus phosphorylation, C-terminus vimentin phosphorylation at S459 does not affect filament polymerisation, which is in accordance with data from our laboratory (Ikawa et al., 2013). It will be interesting to see how the Plk-1 mediated phosphorylation kinetics of the C-terminus are co-ordinated with the N-terminus phosphorylation kinetics. Phosphorylation at S82 is continuous until completion of mitosis. This particular site is proposed to be a memory phosphorylation site, one that is not so readily dephosphorylated and serves to maintain steady state phosphorylation and lower the threshold for other phosphorylation signals (Oguri et al., 2006). Vimentin is also phosphorylated by PKC at S33 and S50 during metaphase and anaphase (Takai et al., 1996). It is an interesting concept to consider that some phosphosites may have differing kinetics, adding a further dimension to IF PTM regulation.

2.4.2.3 Cell migration

Vimentin promotes cell migration. The exact dynamics of this have been little studied, until recently. Migration is a co-ordinated process involving formation of the leading edge, lamellipodia extension, adhesion, locomotion of the cell body and retraction of the trailing edge. Activation of two key effectors of migration, cell division control protein 42 homolog (Cdc42) and Ras-related C3 botulinum toxin substrate 1 (Rac1) causes tyrosine phosphorylation dependent collapse of the vimentin network (Meriane et al., 2000). As can be seen from Figure 6 almost nothing is known about the specific sites of vimentin tyrosine phosphorylation, it has merely been reported to exist in a few studies, several of which connect it to the activity of the migration effector PI3K (Barberis et al., 2009; Bouamrani et al., 2010; Meriane et al., 2000; Valgeirsdóttir et al., 1998). PI3Kγ-dependent phosphorylation of vimentin on S6 and S38, but not S72, induces vimentin filament reassembly and retraction from the cell periphery. In particular, inhibition of the phosphorylation of S6 and the adjacent serines impaired transendothelial migration, demonstrating that disassembly of the vimentin
network is important for proper cell motility. Since PI3K does not directly phosphorylate vimentin, it could act upstream through several effectors to induce vimentin phosphorylation, such as Rac, or Akt. Akt1 is a known scaffolding target of vimentin; it phosphorylates vimentin at S38, and facilitates cell migration. Inhibition of PI3K prevented the Akt1 phosphorylation of vimentin thereby confirming PI3K kinase as an upstream activator of Akt induced vimentin phosphorylation (Zhu et al., 2011). While the effect of S38 phosphorylation on the vimentin network was not shown, it can be assumed that vimentin assembly was disrupted thus enabling proper migration and tumourigenesis.

There is a gradient of vimentin organisation in motile cells, from primarily non-filamentous precursors in the distal regions of lamellipodia, with vimentin gradually becoming more organised further away from the lamellipodial edge towards the nucleus, suggesting that vimentin is dynamically organised during cell motility (Helfand et al., 2011). A cluster of serines at the N-terminus of vimentin, including S6, is phosphorylated in a PKCε-dependent manner which is important for integrin recycling (Ivaska et al., 2005). During cell spreading, vimentin is phosphorylated in a PKCε dependent manner at S6, S38, and S50. Filamin A, an actin cross-linker, forms a complex with activated PKCε and phosphorylated vimentin and this is required for vimentin reorganisation in spreading cells (Kim et al., 2010a). This has been further verified by studies showing that an organised vimentin network is antagonistic for lamellipodia formation, but is necessary for establishing cell polarity. Rac induced vimentin phosphorylation at S38 induces network disassembly and retraction from the lamellipodia prior to membrane ruffling (Helfand et al., 2011). The use of a photoactivatable Rac construct demonstrated that vimentin phosphorylation was not localised to the site of Rac activation, instead it spread rapidly throughout the cell. No other phosphosites were investigated in this study, but in light of previous examples, it is likely that S38 is not the only vimentin phosphosite involved.

Interestingly, inhibition of ROCK, a known vimentin kinase, and Rac, an upstream activator of another vimentin kinase PAK, has differing effects on vimentin solubility depending on whether cells were grown on soft or stiff substrates (Murray et al., 2013). These results are pertinent considering that malignant tissues exhibit increased ECM stiffness; as such vimentin turnover could be affected in malignant situations, potentially altering migratory
Review of the Literature

abilities. Considering that vimentin has been shown on numerous occasions to be essential for cell motility, these findings provide an interesting insight into the role of vimentin in motility. It is not just the presence of vimentin that is important, but also its assembly status. In fact vimentin disassembly was hypothesised to act as a molecular clutch which interacts with and modulates the actin machinery during cell migration, whereas assembled vimentin acts as a brake (Helfand et al., 2011).

One area that is little studied is the relationship between RhoA, ROCK and vimentin. RhoA is highly involved in migration signalling. Typically, most migration signalling pathways converge to Cdc42, RhoA and Rac, which are important for many aspects of cell motility and their ratios can influence speed and directionality of cell locomotion. ROCK is the downstream effector of RhoA and specifically phosphorylates vimentin on S38 and S71, leading to vimentin reorganisation (Goto et al., 1998; Komura et al., 2012; Lei et al., 2013; Nakamura et al., 2000; Yokoyama et al., 2005). Unlike many vimentin phosphorylation events, S71 phosphorylation by ROCK promotes filament assembly, more specifically to a more bundled almost hyper-assembly type status. A balance of ROCK and PP1 is required to maintain a continuous exchange of vimentin between the soluble and filamentous pool (Goto et al., 1998; Inada et al., 1999; Lei et al., 2013; Sin et al., 1998). A disassembled vimentin network plays a part in the formation of podosome rosette formation. FAK suppresses ROCK-dependent vimentin phosphorylation at S38 and S71 to promote rosette formation. On the other hand, SHP2 stimulates ROCK activity to promote vimentin phosphorylation and assembly which inhibits podosome rosette formation (Pan et al., 2011; Pan et al., 2013). This work supports earlier studies showing that vimentin is not necessary for invadopodia formation, although it is required for the elongation of mature invadopodia (Schoumacher et al., 2010).

The results from lamellipodia and podosome formation imply that vimentin is inhibitory for the formation of some of the structures required for cell motility, yet it is clear from many siRNA and animal KO studies that vimentin is essential for motility, and so the question is how to reconcile this ostensible paradox. Not only do cells need to form protrusions in order to move forward, the cell body needs to translocate which which requires disassembly of adhesion sites in the rear of the cell. It is known that the vimentin phosphorylation is important for the recycling of integrins which is important for adhesion disassembly (Ivaska et al., 2005). Another possibility
is that through its scaffolding function, vimentin filaments act to recruit the required molecules to the appropriate site i.e. lamella or podosome, once this function is fulfilled the filaments are no longer necessary and thus are disassembled and retract.

### 2.4.2.4 Organelle trafficking

As described in Section 2.2.6 vimentin is involved in the organisation and positioning of several organelles, including the endo- and exocytosis machinery. Vimentin phosphorylation regulates late endosome and recycling machinery, presumably by a scaffolding and release mechanism. The first indication of vimentin as a regulator of recycling endosomes comes from several years ago where a highly conserved N-terminal cluster of serines was found to be involved with recycling of β1 integrin. Vimentin phosphorylation at the S4,6,7,8,9 cluster regulates proper recycling of integrin containing vesicles and promotes cell migration. To date no kinase has been directly associated with phosphorylation of this cluster, although PI3K signalling and PKCε have been implicated (Barberis et al., 2009; Ivaska et al., 2005).

The Rab family of proteins are involved in all stages of endocytosis and exocytosis, with each having specific functions. Vimentin scaffolds Rab9, which is involved with late endosome trafficking to the trans-golgi network. In Niemann-Pick disease, sphingolipids accumulate in endosomes and inhibit PKC activity leading to decreased vimentin phosphorylation and increased vimentin insolubility. The increase of insoluble vimentin increases sequestration of Rab9 positive endosomes and inhibits LDL-cholesterol trafficking out of late endosomes (Walter et al., 2009). Rab7 is also a component of the late endosome pathway. In a similar study, Rab7a was found to interact directly with filamentous and soluble vimentin and regulates vimentin S38 and S55 phosphorylation and solubility. Since Rab proteins do not exhibit kinase activity, the increased vimentin phosphorylation presumably derives from activation of S38 and S55 specific kinases by the cargo which is carried by Rab7a endosomes. The effect of this increased phosphorylation on endosomal trafficking was not elucidated. However, it is likely that trafficking is perturbed in some way. Interestingly, CMT-2b Rab7a disease mutations also increased vimentin phosphorylation, providing another link to deregulation of vimentin assembly and disease (Cogli et al., 2013).
2.4.3 Dephosphorylation

Rearrangement of the vimentin cytoskeleton in a reversible phospho-dependent manner requires the co-ordination of kinases and phosphatases. Despite the wealth of research describing the importance of vimentin phosphorylation in vimentin assembly and function, remarkably little effort has been invested into understanding the role of dephosphorylation. Inhibition of dephosphorylation causes hyperphosphorylation and disassembly of IFs, suggesting that phosphorylation and dephosphorylation act in equilibrium to maintain filament organisation (Eriksson et al., 2004).
Protein phosphatase 2A (PP2A) is one of the first identified vimentin phosphatases which interacts directly with vimentin, important for filament reassembly (Turowski et al., 1999). Intracellular Ca\(^{2+}\) is important for vimentin dephosphorylation (Evans, 1989), most likely through Ca\(^{2+}\) activation of protein phosphatases. ROCK and protein phosphatase 1 (PP1) dynamically phosphorylate and dephosphorylate S71 in interphase (Inada et al., 1999). However, PP2A appears to be the more dominant protein phosphatase and colocalises with vimentin filaments prior to reorganisation. Whether it is there in an inactive state and needs to be activated prior to filament release remains to be seen, although it has been confirmed since that the action of PP2A is direct and not through inactivation of vimentin-associated kinases (Cheng et al., 2000).

2.4.4 Intermediate filament regulation by other post-translational modifications

While phosphorylation has been the primary PTM regulator of IF dynamics studied over the years, other PTMs are starting to get a look in. What has been emerging in recent years is that there is also cross-talk between PTM types, although this aspect of IF regulation is still very much in its infancy.

2.4.4.1 Intermediate filaments are sumoylation and acetylation targets

Keratins are sumoylated and the degree of sumoylation regulates keratin solubility. Keratin sumoylation is associated with stress-induced phosphorylation and ubiquitination, although exactly how remains to be studied (Snider et al., 2011). In the same report it was shown that vimentin is sumoylated. Although the functional significance of vimentin sumoylation is currently unknown, it is not unreasonable to assume that it may interact with phosphorylation to regulate vimentin organisation. Keratins are also acetylated, which can be upregulated in response to glucose stimulation. Interestingly, K8 acetylation directly modulates K8 phosphorylation which has consequences for K8 filament organisation and solubility (Snider et al., 2013a).

2.4.4.2 Phosphorylation and glycosylation cross-talk

A more established example of IF PTM cross-talk is that of glycosylation and phosphorylation. Glycosylation involves the addition of an oligosaccharide, or a single sugar molecule, to asparagine (N-linked), or serine and threonine
(O-linked) residues. Unlike acetylation and sumoylation, which occur on lysines, O-GlcNAcylation commonly occurs on the same, or adjacent to phosphorylatable serine or threonine residues. Thus glycosylation can compete with phosphorylation for the same residues. It can also stimulate phosphorylation and vice versa. This can give phosphorylation and glycosylation complementary and competing functions, known as the ‘yin-yang’ hypothesis (Comer and Hart, 2000; Hart et al., 1995; Hart et al., 2007; Hyder et al., 2011; Wells et al., 2004).

Vimentin has a GSK3 regulated O-GlcNAcylation site on S54, a site directly adjacent to the vimentin mitotic phosphorylation site S55 (Wang et al., 2007). During M-phase vimentin is increasingly glycosylated and phosphorylated by a complex involving Aurora B, O-GlcNAc transferase, O-GlcNAcase and PP1. In this case, vimentin phosphorylation on S55 was required in order for glycosylation on S54 to occur. Phosphorylation on vimentin S81 and S71 (which occur sequentially after S55 phosphorylation) was inhibited and induced by glycosylation respectively, stimulating filament disassembly which allows daughter cell segregation (Slawson et al., 2008). Thus, these data indicate a tightly regulated temporal PTM dynamics on vimentin during mitosis. Keratins, vimentin, lamins, nestin, GFAP, peripherin and NFs are all glycosylation targets (Chou et al., 1992; Dong et al., 1993; Ferraro et al., 1989; Grigelioniene et al., 1996; King and Hounsell, 1989; Korolainen et al., 2005; Slawson et al., 2008; Wrigley et al., 2002). Accordingly, studies of IF PTMs should take into account PTM cross-talk when considering PTM regulation.
2.5 SPHINGOLIPIDS

2.5.1 Sphingolipid biosynthesis and general signalling mechanisms

In the last 20 years it has become apparent that sphingolipids are not just the structural components of the plasma membrane that they were once thought to be. Sphingolipids compromise of a family of bioactive signalling molecules. They have roles in many aspects of biology including embryogenesis, angiogenesis, proliferation, survival, cell death, adhesion and migration. This breadth of functions is beyond the scope of this overview. Here, I will only describe the key roles of sphingolipids in cancer, cell motility and their relationship with the IFs.

The sphingolipid family describes a family of lipid molecules whose common feature is a sphingosine containing backbone. One of the key sphingolipids studied in this thesis, sphingosine 1-phosphate (S1P), is derived from the phosphorylation of membrane anchored sphingosine by sphingosine kinase (SK) 1/2. Another molecule of interest is sphingosylphosphorylcholine (SPC) which is derived from membrane anchored sphingomyelin (Breslow and Weissman, 2010; Nixon et al., 2008).

Once S1P or SPC is synthesised at the plasma membrane it is secreted by the cell and can act in an autocrine or paracrine manner. Signalling is achieved by the binding of the sphingolipid molecule to one of five S1P specific G protein-coupled receptors (GPCR; S1P1-5) to trigger downstream signalling cascades such as phospholipase C, MAPK, PI3K, Cdc42 and Rac as well as regulating intracellular calcium levels. The receptors are expressed in different ratios and combinations on different cell types, enabling versatility of cell type specific signalling and cellular responses. As with many things in cell biology it is a balance of factors that maintains homeostasis. The same is true for sphingolipids and this known as the “sphingolipid rheostat” (Chalfant and Spiegel, 2005). Maintenance of sphingolipid metabolism is achieved by regulation of the sphingolipid enzymes involved in synthesis and degradation. Deregulation of sphingolipid metabolism leads to cell stress and disease.
2.5.2 Sphingosine 1-phosphate (S1P) and sphingosylphosphorylcholine (SPC)

S1P is produced by platelets, among other cell types, and is enriched in the blood. S1P is present in human serum anywhere between 100-900nM and has a half-life of 15 mins (Hla et al., 2008; Murata et al., 2000a; Murata et al., 2000b). The range of concentrations described most likely comes from experimental differences. Additionally the bioactive pool of S1P is only in the 10 nM range which means that it will not saturate and overactivate the S1P receptors which have a dissociation concentration between 2-30 nM (Murata et al., 2000b). S1P concentrations in the body form a gradient, with the interstitial fluid containing considerably less S1P than the plasma. This gradient is purported to act as a chemotactic sensing system for immune cell trafficking as well as a mechanism for regulating cell activation (Hla et al., 2008; Pappu et al., 2007).

SPC is present in serum at around 130 nM (Liliom et al., 2001). Little else is known about SPC, whether it forms a gradient similarly to S1P, it’s half-life, or the exact receptors that it binds to. S1P and SPC are structurally similar and appear to have analogous functions through distinct signalling pathways. However, S1P is considered to be more potent than SPC by between 100 – 1000 times (Gonda et al., 1999; Murata et al., 2000a). It is possible that while SPC signalling appears to act in a similar manner to S1P it represents a pathway of sustained low level activation and has divergent biological functions.

2.5.3 Sphingolipids and cancer

Increased concentrations of S1P are found in some cancer patients. S1P kinase is upregulated and overexpressed in many cancers which can lead to increased S1P production (Pyne and Pyne, 2010; Shida et al., 2008). Increased SK1 expression is correlated with tumour grade and decreased patient survival. Importantly, for cancer cells S1P is pro-survival whereas ceramide is pro-apoptotic. In the context of the ceramide-S1P rheostat, a tendency to produce more S1P will lead to increased cell survival, through apoptosis- and chemotherapy- resistance. Thus, exploitation of this rheostat by manipulating SK is an approach for cancer therapy.
2.5.4 S1P and SPC regulation of migration

The sphingolipid S1P has paradoxical roles in cell motility which are related to the S1PR expression profile of a cell and which GPCR\(\alpha\) subunit is activated following stimulation (Sugimoto et al., 2003). For example if G\(i\) is activated, PI3K/Akt or Erk signalling pathways become activated, G\(q\) activation stimulates phospholipase C (PLC) signalling and G\(12/13\) stimulates Rho GTPase signalling mechanisms. Each S1P receptor has affinity for specific GPCRs, some of which overlap between receptors. However, the dose of S1P required for activation of the S1P receptor downstream signalling pathways is different (Okamoto et al., 1999). S1P receptor can also cross-talk with growth factor receptors to modulate signalling. S1P regulates cell motility in a range of cell types including, but not limited to, Chinese hamster ovary (CHO) cells, vascular smooth muscle, HEK293, fibroblasts and various cancers including melanoma and breast cancer (Takuwa, 2002). Thus the response of cells to sphingolipids is entirely context dependent. The situation is less clear for SPC, there are fewer articles regarding the role of SPC and migration, although it seems that the effects of SPC are similar to, but less potent than those of S1P (Takuwa, 2002). This section will concentrate primarily on S1P receptor 1-3 (S1P\(1-3\)) roles in migration since these are the mostly widely expressed S1P receptors (Figure 7) and the remaining two, S1P\(4\) and S1P\(5\), are confined to lymphoid tissues, platelets and the CNS, respectively.

2.5.5 S1P\(_1\) and S1P\(_3\) regulation of cell motility and invasion

S1P\(_1\) couples exclusively to G\(_i\). S1P\(_3\) is the other key chemotactic promoting receptor which functions through G\(_i\), G\(_q\) and G\(_{12/13}\). It is hypothesised that the pro-migratory functions of S1P\(_3\) arise from a preference for G\(_i\) based signalling. S1P activation of S1P\(_{1,3}\) generally causes increased calcium signalling, stimulation of Rac-Cdc42 and Ras-dependent Erk signalling pathways and MMP activation, which together act to promote cell motility and invasion (Brocklyn, 2010). S1P\(_1\) is understood to mediate S1P stimulated membrane ruffling and reduced actin stress fibres possibly through its activation of Rac (Lepley et al., 2005). PDGF stimulation can increase localised S1P production by facilitating SK translocation specifically to membrane ruffles whereby it increases FA formation through FAK and Src. This increased adhesion could act to stabilise lamellipodia and drive directed migration in mouse embryonic fibroblasts (MEF) (Rosenfeldt et al.,
2001). SK activation and translocation to lamellipodia is facilitated by filamin A which scaffolds SK and S1P1. Together with PAK1 they promote heuregulin- and S1P1-dependent lamellipodia formation and cell migration (Maceyka et al., 2008). S1P1 and S1P3 stimulation also activates integrin αVβ3, which stimulates FA formation through Rho in endothelial cells (Paik et al., 2001).

In MCF10a breast cancer cells S1P induces cell invasion via S1P3 coupling to Gq and Fos mediated transcriptional upregulation of active MMP9 (Kim et al., 2011b). This may or may not be linked to the observations that S1P treatment activates Stat3, a known MMP9 transcription factor, in prostate cancer cells (Sekine et al., 2011). Additionally S1P is reported to regulate MMP2 transcription via p38 MAPK signalling upregulation of the ZNF580 transcription factor with concomitant increases in endothelial cell migration (Sun et al., 2010b). In marrow-derived stromal cells S1P induced rapid actin stress fibre, lamellipodia and FA formation and increased migration. These were mediated through downstream RhoA and MAPK signalling. Interestingly they showed that MMPs were a central mediator of the cross-talk between RhoA and MAPK (Meriane et al., 2006). S1P also promotes translocation, activation and interaction of both MT1-MMP and p130Cas at membrane ruffles at the leading edge of endothelial cells (Gingras et al., 2008). This could be related to the work identifying Gq coupled S1P1,3 as the key regulators of MT1-MMP and MMP2 mediated migration (Devine et al., 2008; Langlois et al., 2004).
Figure 7: Sphingolipid-mediated signalling cascades that regulate cell motility and invasion. Thicker black arrows identify preferred signalling routes.
2.5.5.1 S1P2 regulation of cell motility and invasion

Generally, S1P2 is considered to inhibit cell migration. Similar to S1P3, S1P2 functions through G\textsubscript{i}, G\textsubscript{q} and G\textsubscript{12/13}. Inhibition of cell motility and chemorepulsion occurs in a Rho dependent, Rac inhibitory mechanism which is characterised partly by inhibited Rac-dependent membrane ruffling and actin stress fibre formation (Okamoto et al., 2000; Sugimoto et al., 2003). In melanoma cells, S1P2 treatment affects both lamellipodia formation and regulation of FA size. It also perturbs actin polymerisation. S1P treatment activates FAK through RhoA and also activates paxillin and β1 integrin, stimulating increased FA growth, halting cell motility through S1P2 (Yamamura et al., 2000). Interestingly the reverse effect was obtained in fibroblasts which additionally implicated ROCK activation in the signalling pathway that stimulated migration (Hashimoto et al., 2008). In vascular smooth muscle cells (VSMC), both G\textsubscript{q} and G\textsubscript{12/13} are required for Rac inhibition and Rho activation inhibition of migration through S1P2, independently of ROCK. Additionally PLC nor PKC were involved and the downstream mediators of this effect do not appear to have been identified at the time of writing (Takashima et al., 2008).

Rac is not always inhibited upon S1P2 stimulation by S1P. In the U118 glioblastoma cell line both Rac and RhoA are activated (Lepley et al., 2005). One explanation is that RhoA activation (> 10 fold) is sufficient to override any increase in Rac activity (< 3 fold) and still inhibit migration. The role of Rac in cell motility is rather complex. Large increases or decreases can completely abrogate movement, however, incremental shifts in activity can regulate a switch between persistent and directional cell migration (Pankov et al., 2005). It would be interesting to investigate specific cell motility parameters in more detail with regard to the effects of S1P2-mediated motility defects as these may reflect the balance of Rac and Rho activation. In contrast to the very clear inhibition of migration, S1P2 may have variable effects on cell invasion. S1P2 inhibits invasion in HeLa cells by global phosphorylation of ezrin radixin moesin (pERM) proteins and increased filopodia formation which increase cell-cell adhesion (Gandy et al., 2013).

Most of these studies assess global changes in Rac and Rho activity. However, it is now apparent that the effects of Rho and Rac (and pERM) on cell motility are highly dependent on their cellular localisation (Pertz, 2010). Exploration of the effects of sphingolipids on the spatial-temporal
activation/inhibition of Rac, Rho and Cdc42 using biosensors and computational multiplexing might resolve some of the apparent conflicts regarding sphingolipid cell motility signalling in the literature (Machacek et al., 2009; Welch et al., 2011).

These studies highlight the importance of cell context in sphingolipid migration. Cells tend to express several S1PR subtypes in varying amounts which will enable them to modulate their chemotactic response to S1P and SPC.

2.5.6 Sphingolipid regulation of intermediate filament assembly and migration

The first mechanistic link between the IFs and sphingolipids was between sphingosylphosphorylcholine (SPC) and keratins, specifically K8 and K18. SPC treatment induced metabolic energy dependent keratin reorganisation to the perinuclear compartment. This was SPC specific since S1P had no effect on keratin in these cells. The filament reorganisation was attributed to SPC induced K8 phosphorylation at S431, a key in vivo keratin phosphorylation site. K18 was phosphorylated by SPC at S33 and S56. The filament collapse increased cellular elasticity, independently of the actin and MT cytoskeleton, implying that keratin filaments alone are able to regulate cell elasticity. SPC was also found to strongly induce migration in a Boyden chamber model, an effect that could be attributed to the keratin mediated changes in cell morphology and elasticity. Since keratins are present in many cancers, it appears that keratin could contribute to cancer cell visco-elastic properties (Beil et al., 2003).

In PANC-1 cells, SPC induced K8 S431 phosphorylation and filament reorganisation is mediated by transglutaminase-2 (TGase-2). SPC increases TGase-2 expression with downstream effects on c-Jun N-terminal kinase (JNK) activation and increased cell migration. In this model TGase-2 forms a complex with the phosphorylated K8 and JNK in response to SPC (Park et al., 2011). Furthermore, the MEK/ERK signalling pathway is thought to be critical for SPC induced K8 phosphorylation at S431 and that this single phosphorylation event is sufficient for keratin reorganisation and cell migration. SPC mediated K18 S52 phosphorylation is MAP2K/ERK independent and is insufficient to induce keratin reorganisation (Busch et al., 2012).
Little has been studied with respect to vimentin and the sphingolipids until recently. A study in 3T3 cells demonstrated that ROCK and vimentin colocalise and when vimentin organisation is disrupted by depolymerisation of MT, ROCK translocates to the cell periphery. In the same study, S1P was utilised as a ROCK activator. This caused collapse of vimentin and induced ROCK translocation to the cell periphery (Sin et al., 1998). Vimentin is necessary for endothelial sprouting at the beginning of angiogenesis. Vimentin and activated calpains regulate MT1-MMP cellular localisation and S1P stimulates vimentin-dependent MT-MMP relocalisation to the cell membrane which is essential for angiogenesis initiation (Kwak et al., 2012). Further work showed that S1P treatment triggered interaction of vimentin, RACK1 and FAK in invading endothelial cells, this complex was important for cell adhesion and FA formation (Dave et al., 2013). To date the only IFs that have been linked to sphingolipids are keratins and vimentin.

### 2.6 Intermediate Filaments Fine Tune Cell Signalling

IF research has come a long way in the last three decades. From the initial studies showing that IFs are important structures for maintaining cellular integrity, our understanding of the function of IFs has evolved considerably. Neither vimentin nor nestin are essential for organism survival as demonstrated by the viability of the KO mouse models. While many other IF mouse models have overt pathologies associated with them, the lack of overt pathology demonstrated by the vimentin and nestin KO mice suggests that these IF proteins have more subtle roles to play in the cell. In the last decade, research involving nestin has exploded. As well as modulating vimentin dynamics during mitosis, it also has important functions in regulating the pace of muscle differentiation and in the regulation of cell motility. Vimentin too, has a wealth of roles in the cell, including quite diverse functions such as lipid homeostasis, organelle tethering and cell motility. What is clear is that many of these functions arise from their ability to scaffold signalling molecules and receptors, modulating their cellular localisation and activation state with knock-on effects on timing and magnitude of the downstream signalling.
3 OUTLINE OF THE STUDY

Our group has been instrumental in identifying nestin as a regulator of and signalling scaffold of Cdk5/p35 where it regulates the pace of myoblast differentiation and contributes to cell survival during oxidative stress (Pallari et al., 2011; Sahlgren et al., 2003; Sahlgren et al., 2006). A paper published by another group in 2007 identified nestin as a promoter of cell migration (Kleeberger et al., 2007). Considering our interest in and expertise with nestin, this study prompted us to investigate how nestin might regulate cell migration. We showed that nestin is an inhibitor of cell invasion, but had no effect on cell motility. Since the intermediate filaments are known to provide functional links to focal adhesions and have been implicated in integrin turnover, we characterised nestin’s role in regulation of focal adhesion and integrin turnover. Finally, we showed that nestin regulates extracellular matrix degradation and integrin mediated-cell invasion.

Another stronghold of our lab is the study of post-translational regulation of intermediate filaments, with particular emphasis on vimentin phosphorylation. A decade ago we showed that vimentin had a number of in vivo phosphorylation sites (Eriksson et al., 2004). Initially when this PhD thesis work commenced in January 2006, the intention was to create single mutations of these phosphosites, characterise and identify physiological functions for them. Specifically we showed that a small cluster of serines at the N-terminus of vimentin, S7,8,9 was critical for vimentin network organisation. To a lesser extent, another serine cluster in the N-terminus, S71,72 was identified as important for vimentin organisation. We utilised knowledge from our findings in a collaboration with the Törnqvist group for the final study. Here we showed that the sphingolipids S1P and SPC had dramatic effects on the vimentin cytoskeleton. Further investigation revealed that these sphingolipids stimulated ROCK dependent phosphorylation of vimentin specifically at S71 which dramatically inhibited cell motility.
4 MATERIALS AND METHODS

4.1 CELL CULTURE, TRANSFECTION AND TREATMENTS (I, II, III)

4.1.1 Cell lines and cell culture (I, II, III)

All cell lines being maintained prior to experiments were cultured in their respective media supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin at 37 °C in a 5% CO₂ humidified atmosphere and passaged every 2-3 days unless otherwise indicated.

The androgen independent prostate cancer PC-3 cell line was obtained from the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The MDA-MB435S were a kind gift from Dr James Fagin (University of Cincinnati, Ohio, USA). The MDA-MB-435S is of disputed origin. It was originally thought to be from breast ductal carcinoma, however, studies suggest it is derived from the same individual as the melanoma M14 cell line (Chambers, 2009; Rae et al., 2007). In spite of this we chose to continue to use it for biochemical reasons since it gives a strong and consistent response to S1P and SPC. The A172 glioma cell line was from ATCC and the DU145 cell line was a kind gift from Dr. He (VTT Technical Research Centre of Finland, Turku, Finland). PC-3, MDA-MB-435S, A172 and DU145 cells were cultured in RPMI-1640 (Sigma-Aldrich). MDA-MB-436S media was additionally supplemented with 1% non-essential amino acids (NEAA). Immortalized mouse embryonic fibroblasts (MEF) from vimentin wild-type (wt) and vimentin knockout (-/-) were a kind gift from Dr. Johanna Ivaska (University of Turku, Turku, Finland). The cervix carcinoma HeLa cell line was obtained from American Type Tissue Collection (ATCC). C643 cell line was a kind gift from Dr Nils-Erik Heldin (Karolinska Institute, Stockholm, Sweden). MEF, HELA and C643 cell lines were cultured in 4500 mg/l glucose containing DMEM. The breast adenocarcinoma MCF-7 cell line was obtained from ATCC and was cultured in 1000 mg/l glucose containing DMEM.

MDA-MB-435 and C643 cells were lipid starved prior to sphingolipid treatments by growing them overnight in medium containing 5% lipid-
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stripped FBS (III). Serum starvation was carried out by growing cells in medium without FCS (I).

4.1.2 Transfection (I, II, III)

4.1.2.1 Plasmid transfections (I, II, III)

For N-terminal serine cluster mutants, pcDNA4 vector with vimentin inserted between HindIII and KpnI sites was used. Other vimentin phosphorylation-site mutants were cloned in pCMV vector. Vimentin mutations were created with the Quik Change II site-directed mutagenesis kit (Agilent Technologies, USA).

mCherry empty vector (EV), vimentin wild-type (WT), vimentin S38A, vimentin S38D, vimentin S71A and vimentin S71D plasmids were kind gifts from Dr. Hong-Chen Chen (National Chung Hsing University, Taiwan) (Pan et al., 2011).

GFP and Nes640-GFP have been used previously (Pallari et al., 2011). GFP-zyxin and GFP-VASP were kind gifts from Dr. Maddy Parsons (King’s College London, UK) (Worth et al., 2010).

Transient transfections for wild-type MEF and vimentin -/- MEF, MCF-7, HeLa, PC-3, DU145 and A172 cells were done with Lipofectamine LTX (Invitrogen, UK) according to manufacturer’s instructions for the specific cell lines except for the PC-3 cell lines which required further optimization.

For the solubility assay MEF vim-/- cells were transfected by electroporation in OptiMEM I (Invitrogen, UK). Electroporation settings were 300 V, 1.07 ΩF in a 4 mm cuvette with Gene Pulser II (Bio-Rad, UK).

Plasmid transfections of C643 and MDA-MB-435S cells were carried out using electroporation at 220V, 975 ΩF in a 4 mm cuvette with Gene Pulser II (Bio-Rad, UK).

4.1.2.2 RNAi transfections (I, III)

Nestin and integrin RNAi transfections were performed using Lipofectamine RNAiMAX (Invitrogen, UK). Nestin was downregulated in in PC-3 and A172 cells by transfection of a 100 nM of pool of 4 flexitube siRNA
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oligos (Qiagen, Germany) against human nestin. β1-integrin was downregulated with 50 nM siRNA custom ordered (Eurofins MWG Operon, Germany) with the following sequence: 5’-AUGUAACACCGGUAGCA-3’. Allstars Negative Control siRNA (Qiagen, Germany) was used as a control.

Fluorescently tagged siRNAs were custom ordered (Eurofins MWG Operon, Germany) and the sequences used were identical to the Qiagen siRNA pool. Co-transfections were performed so that first the siRNA was transfected and 6 hours later plasmid DNA transfections were performed. Media was changed the following day. All assays were performed 48-72 hours post-transfection.

S1P2 transfections were performed with electroporation at 240V 375 ΩF using control and S1P2 siRNA (Ambion, USA).

4.1.3 Treatments (I, III)

FAK was inhibited for 24 hours prior to and during the relevant assays using 1 μM FAK Inhibitor II (Millipore, USA); DMSO was used as a control.

C643 and MDA-MB-435S cells were treated for the indicated timepoints and concentrations (II) with Sphingosylphosphorylcholine (SPC, Sigma-Aldrich, USA), D-erythro-sphingosine-1-phosphate (S1P, Enzo Life Sciences, USA), S1P2 antagonist JTE013 (Tocris Bioscience, USA), ROCK inhibitor Y27632 (Calbiochem, USA) or Blebbistatin (a kind gift from Dr. Matthias Nees (Turku Centre for Biotechnology, Turku, Finland) and their respective controls.

4.2 MICROSCOPY

4.2.1 Immunofluorescence labelling (I, II, III)

MEF, HeLa were either grown on glass coverslip and transfected directly. C643 and MDA-MB-435S cells were either grown on coverslips for 2 days prior to treatment, or transfected allowed to recover for 1 day and then split and grown on coverslips 1 day prior to treatment. PC-3 cells were usually transfected and then split and grown on glass coverslips or Millicell EZ-slide chambers (Millipore, USA). Cells were fixed 2 days post-transfection in 3.7 % PFA in PBS for 5 minutes at room temperature (RT). Cells were stained with one of the following protocols:
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Protocol 1 (MEF, HeLa (II), PC-3 (I)): cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min in RT, blocked with 1% BSA in PBS for 1 hour in RT. Then incubated with primary antibody in 1% BSA in PBS for 1 hour, washed 3x5 min in PBS, coverslips were incubated for 1 hour in RT with secondary antibody, washed 3x5 min in PBS, rinsed in distilled H$_2$O and mounted with Mowiol mounting media.

Protocol 2 (MEF, HeLa (II)): cells were incubated for 5 min in 100 mM glycine in PBS at RT, blocked with blocking buffer: 10% fecal calf serum (FCS), 0.2% saponin, 0.5% fish skin gelatin in PBS for 1 h in RT, incubated with primary antibody in blocking buffer, rinsed 3 times in washing buffer (blocking buffer without serum), incubated with secondary antibody for 45 min in blocking buffer, rinsed 3 times in washing buffer and once in distilled H$_2$O and mounted on microscope slides with Mowiol mounting media.

Protocol 3 (C643, MDA-MB-435S (III)): cells were permeabilised with 0.1% Triton-X-100 at RT. Blocking was carried out with 10% goat serum in PBS for 1 hour at RT. Then incubated with primary antibody in 1% BSA in PBS for 1 hour, washed 3x5 min in PBS, coverslips were incubated for 1 hour in RT with secondary antibody, washed 3x5 min in PBS, rinsed in distilled H$_2$O and mounted with Mowiol mounting media.

Primary antibodies used were: nestin (1:200, clone 10C2, Millipore, USA), vimentin (1:100, clone V9, Sigma-Aldrich, USA), $\beta$-tubulin (1:200, clone DM1B, Abcam, UK), integrin $\alpha$5 (1:25, clone P-19, Santa Cruz Biotechnology, Germany), phosphorylated Y397 FAK (1:25, Invitrogen, UK), talin (1:25, clone 8D4, Sigma-Aldrich, USA) and vinculin (1:100, clone 7F4, Millipore), vimentin (1:100, clone V9, Sigma-Aldrich, USA), vimentin (1:100, Covance, USA), vimentin pS71 (1:8, TM71, kind gift from Dr. Masaki Inagaki), Alexafluor-conjugated phalloidin (1:750, Invitrogen, UK).

Secondary antibodies used were species specific IgG conjugated antibodies: Alexafluor-conjugates for confocal (Invitrogen, UK) or ATTO-conjugates for STED microscopy.

4.2.2 Confocal microscopy and image analysis (I, II, III)

Confocal microscopy was done using a either Leica SP5 confocal microscope with 63X/1.4 plan-apochromat oil immersion, 2048x2048; or Zeiss LSM 710
with 63X magnification. Z-stacks were taken according to Nyqvists Theorem for optimal sampling (I, III).

For correlative electron microscopy samples were imaged with Zeiss LSM510 META laser scanning confocal microscope using 100X /1.4 plan-apochromat oil immersion objective (II).

All images were imported into ImageJ and Fiji (http://fiji.sc/wiki/index.php/Fiji) and only linear adjustments were made to their brightness and contrast.

For optimal images of focal adhesions, z-stack projections were made from between 4-10 stacks from the bottom half of the cell. In all other cases z-stack projections were made from z-stacks collected throughout the entire cell (I).

Image quantitation for filament mixing experiment was done with ImageJ (II). Cell areas where vimentin intensity in the cell was measured were labelled manually. Images were converted into 8-bit grayscale and autothresholded. Results for pseudocolor images were obtained using the following formula: \( P = \frac{(X/2+127)-(Y/2)}{2} \) where \( P \) is pixel intensity, \( X \) green channel intensity (both vimentins) and \( Y \) red channel intensity (human mutant vimentin). Results for histogram quantitation was calculated from areas after autothresholding with corresponding formula \( P = \frac{Y}{X} \).

### 4.2.3 Correlative electron microscopy (CLEM) and electron microscopy (II)

Cells were grown on plates with gridded glass bottom (MatTek Corporation, USA) and stained according to the protocols in Section 4.2.1 After confocal microscope imaging and writing down cell positions cells were fixed in 2,5% glutaraldehyde in cacodylate buffer (50 mM sodium cacodylate, 50 mM KCl, 2.5 mM MgCl, pH 7.8). Then plates were rinsed in cacodylate buffer and incubated for 1 hour in cacodylate buffer with 1% OsO4. Plates were washed 2x 3 minutes in cacodylate buffer and after that 3x 5 minutes in distilled water, then treated with 1% uranyl acetate 0.3 M sucrose in distilled water for 1 hour at 4 °C. Plates were washed 3x 3 minutes in distilled water. Samples were dehydrated in a 70%, 96% and twice absolute ethanol series for 1 minute each. Ethanol was removed and cells were embedded in EPON Epoxy resin for 2 h at RT, then baked for 14 hours at 60 °C. Coverslips were removed from embedded samples. Samples were cut with diamond
Materials and Methods

Microtome and previously with confocal microscope imaged cells were imaged with FEI Tecnai 12 transmission electron microscope.

For pure electron microscopy samples, coverslips were used and sample preparation was done as described above for CLEM except no immunostaining and confocal imaging was done and the sample preparation was started from glutaraldehyde fixation. Dehydration step included rinse in acetone in the end and EPON treatment was done on aluminium planchette.

4.2.4 Fluorescent recovery after photobleaching (FRAP) analysis (I,II)

For the FA FRAP analysis RNAi and plasmid co-transfected cells were plated on fibronectin (10 μg/ml) coated live cell imaging dishes (MatTek, USA) and allowed to adhere for 24-36 hours. The media was replaced with Leibovitz L-15 media (Invitrogen, UK) with 10% FCS before the experiment. FRAP experiments were performed using the Zeiss LSM780 confocal microscope. Cells were first imaged with 543 nm laser to visualize siRNA transfection and 488 nm to visualize FA. Photobleaching of the FA was done at 100% of the 488 nm laser with 20 iterations. Recovery was followed with low laser power every second until the intensity had reached a steady plateau. For each timepoint the bleached area intensity was normalized to a corresponding unbleached area and background signal. The recovery signal was fitted with ImageJ FRAP analyzer (I).

For the vimentin FRAP analysis FRAP was carried out with Leica SP5 confocal microscope (Leica, Germany, Wetzlar) using FRAP wizard from microscope software. Cells were transfected with mCherry-vimentin and the following day were lipid starved. Forty-eight hours post-transfection cells were imaged. Bar-shaped 2 μm wide regions were bleached and recovery was imaged for 15 minutes at 30 second intervals. FRAP-images were analyzed afterwards with ImageJ (measurement of intensity of the bleached spot and control area) followed by FCalc (by Rolf Sara - fitting of the recovery curve and calculation of half-recovery values).

4.2.5 Colocalisation analysis (III)

Colocalization analysis of ROCK1 and vimentin was done using the Fiji ImageJ software and associated plugins. 3072x3072 resolution images with 80 nm pixel size were used for the analysis. Subtract Background function
was used with 200 pixel rolling ball radius for each image. Manders colocalization coefficients and images showing colocalization were calculated with Colocalization Threshold. Zero-zero pixels were not included in the threshold calculation. Pearsons colocalization coefficients were calculated with Colocalization Test.

4.2.6  **Live cell microscopy (I, II, III)**

PC-3 cells were plated on glass bottom cell culture dishes (MatTEK, USA), the following day they were imaged for 4 hrs 10 mins using Leica SP5 confocal microscope and 10x air objective. Widefield images were taken every 10 mins. The resulting videos were analysed for individual cell speed, directionality and persistence using ImageJ MtrackJ plugin (Meijering et al., 2012) of 20 cells per video with 3 independent repeats. Statistical significance was determined using Graphpad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA) and unpaired t-test (III).

Random motility analysis of C643 and MDA-MB-435S cells. C643 and MDA-MB-435S cells were grown on 24 well plates to less than 50 % confluency. They were treated as indicated with S1P, SPC, JTE and Y27362 and were imaged using the Cell-IQ (CM Technologies, Finland). Use of this machine allowed continuous imaging of cells in many wells of a 24 well plate. Images were collected once an hour over a 20 hour period. The videos were analysed by manually tracking cell movements for individual cell speed, directionality and persistence using Manual Tracking plugin of 20 cells per video with 3 independent repeats. Rose plots and vector plots were made using the Chemotaxis and Migration Tool (Ibidi, Germany).

4.3  **SDS-PAGE AND WESTERN BLOTTING**

Whole cell lysates were prepared by lysing cells in laemmli buffer containing beta-mercaptoethanol. Proteins were resolved using SDS-PAGE and then transferred to either nitrocellulose or PVDF membrane using wet transfer. Antibodies used for western blotting were as follows:

- nestin (clone 10C2, Millipore, USA; clone MCA-4D11, EnCor Biotechnology, USA),
- vimentin (clone V9, Sigma-Aldrich, USA),
- actin (clone AC-74, Sigma-Aldrich, USA),
- β-tubulin (clone DM1B, Abcam, UK),
- integrin α5 (Millipore, USA),
- integrin β1 (clone N29, Millipore, USA),
- focal adhesion kinase (FAK) (clone 77, BD Transduction, USA),
- phosphorylated Y397 FAK (Invitrogen,
UK), Ki67 (Abcam, UK), (Clontech, USA), heat shock complex 70 (Hsc70, SPA-815, Stressgen, USA), vimentin pS6, pS38, pS71 (MO6, TM38, TM71, kind gift from Dr. Masaki Inagaki), ROCK1 Antibody (EP786Y, Novus Biologicals, USA) Blots were quantified using ImageJ, Graphpad Prism 5 (GraphPad Software Inc., USA) and analysed using the unpaired t-test.

4.4 MASS SPECTROMETRY (II)

MDA-MB-435S cells were used for the identification of S1P and SPC induced phosphorylation. Cells were treated with either 1 μM SPC for 1 hr, EtOH for 1 hr, 100 nM S1P for 30 minutes or HBSS for 30 minutes, an untreated control was also included. Cells were lysed in SDS buffer, sonicated, boiled and run on a 10% large SDS-PAGE gel with an IF preparation as a positive control (kind gift from a colleague). Gels were stained with Coomassie and destained with methanol and acetic acid.

To identify phosphorylation sites, in-gel digestion with trypsin, TiO₂ affinity chromatography and liquid chromatography-tandem mass spectrometry (LC-MS/MS) were performed as described in Imanishi et al., 2007 with some modifications. For LC-MS/MS analysis, an EASY-nLC 1000 nanoflow liquid chromatograph coupled to a Q Exactive mass spectrometer (Thermo Fisher Scientific) was used. Database search was performed against the Swiss-Prot (Homo sapiens) and the reversed sequence decoy database using Mascot 2.4 (Matrix Science) via Proteome Discoverer 1.3 (Thermo Fisher Scientific). Label-free quantification was performed using Progenesis LC-MS 4.0 (Nonlinear Dynamics).

4.5 INTERMEDIATE FILAMENT SOLUBILITY ASSAYS (I, II, III)

4.5.1 Simple intermediate filament solubility assay (I, II)

PC-3 cells were washed once with cold PBS and lysed on ice for 20 minutes in a Triton-X based buffer (pH7.5 25 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, 5 mM EGTA, 0.5% Triton X-100, 1:1000 protease inhibitor cocktail (Roche, Switzerland)). Lysates were centrifuged at 15000 xg for 45 minutes at 4 °C to separate the insoluble fraction from the soluble. All fractions were then boiled in laemmli buffer before SDS-PAGE and western blotting (Sahlgren et al., 2006).
4.5.2 Detergent extraction of vimentin (II)

Triton X-100 extraction with immunofluorescence microscopy was performed according to the method in (Correia et al., 1999).

4.5.3 Sequential ultra-centrifugation fractionation (II, III)

Fractionation of vimentin into different assembly states was necessary since our results suggested that S1P causes vimentin to become more insoluble. MDA-MB-435S cells were grown on 60 mm plates and treated with 100 nM S1P for 10 minutes. Following treatment cells were lysed in an NP-40 based fractionation buffer on ice (10 mM Tris 150 mM NaCl 7 mM EDTA 0.5 % NP-40). The buffer was different to that used in the simple fractionation since it does not contain MgCl$_2$ which is known to induce filament cross-linking and therefore insolubilisation. Protein concentration was normalised using Bradford assay. A sample of total lysate was taken and boiled in laemmli sample buffer. Remaining lysate was centrifuged at 500 xg, 4 °C for 5 minutes. Supernatant was moved to a new eppendorf tube and centrifuged at 5000 xg, 4 °C for 5 minutes. Supernatant was moved to a new eppendorf tube and centrifuged at 15000 xg, 4 °C for 30 minutes. Supernatant was moved to a new eppendorf tube and centrifuged at 2000000 xg, 4 °C for 30 minutes. During the centrifugation steps the pellets were kept on ice and lysed in laemmli sample buffer. After the last centrifugation step the supernatant and pellet were separated and lysed in laemmli sample buffer. The laemmli lysate samples were then boiled, separated by SDS-PAGE and blotted for vimentin (clone V9, Sigma, USA), vimentin pS71 (TM71, kind gift from Dr. Masaki Inagaki) and ROCK (Cell Signaling, USA).

4.5.4 Glutaraldehyde crosslinking of vimentin subunits (I)

Fractionation was performed as described in Section 4.5.3. However, different concentrations of glutaraldehyde in distilled water was added to the supernatant from the 200000 xg fraction as indicated. Reaction mixtures were incubated for 10 minutes at RT. Reaction was stopped by adding 0.1 M Tris-HCl pH 8.0. Samples were analysed by SDS-PAGE and western blotting. HeLa cells treated with 50 nM calyculin A for 30 minutes was used as a positive control.
4.6 MIGRATION ASSAYS (I, II)

4.6.1 In vitro migration assay (I, II)

All boyden chamber migration experiments were conducted with 6.5 mm-diameter Transwell Permeable Support inserts with 8 μm pore size. For the MDA-MB-435S and C643 experiments cells (II) were lipid-starved overnight before the experiment. 1 x 10^5 MDA-MB-453S cells or 5 x 10^4 C643 cells in medium containing 5% lipid-stripped FBS was added to the upper well and 10% lipid-stripped FBS and the sphingolipid to the lower well. MDA-MB-435S cells were allowed to migrate overnight and C643 cells for 6 hours. For the PC-3 experiments (III) cells were serum starved overnight. 5 x 10^4 cells in full media were added to the top chamber of a transwell insert (Corning, USA), full media containing 10 ng/ml human SDF1-α (Peprotech, USA) was added to the bottom chamber and cells were allowed to migrate for 24 hours. For all migration experiments cells that had not migrated were wiped from the top of the chamber. The chambers were fixed with 3.7 % PFA and stained with 0.1 % crystal violet (Reagecon, Ireland), rinsed twice with PBS, once with Milli-Q water and then allowed to dry. For the C643 and MDA-MB-435S migration experiments (II) the number of cells was counted from eight microscopic fields in each chamber. For the PC-3 migration experiments (III) the number of cells was counted manually from edge to edge. Statistical significance was determined using Graphpad Prism 5 (GraphPad Software Inc., USA) and unpaired t-test.

4.6.2 In vitro wound healing assay (II, III)

4.6.2.1 Manual method of wound healing and analysis (I)

Confluent monolayers of PC-3 cells were made either by wounded by scratching a pipette tip from the top to the bottom of the well. Images were taken of the whole length of the wound at the indicated time points using an Olympus GK2 inverted microscope coupled to a digital camera (Canon PowerShot A510) and analysed with PSRemote (Canon).
4.6.2.2 Random motility assay (I)

PC-3 cells were imaged on glass bottom cell culture dishes (MatTEK, USA), and widefield images were taken every 10 minutes for 4 hours 10 minutes using Leica SP5 confocal microscope and 10x air objective. The videos were analysed for individual cell speed, directionality and persistence using ImageJ MtrackJ plugin (Meijering et al., 2012) of 20 cells per video with 3 independent repeats (III).

4.6.2.3 Wound healing and analysis using the Incucyte ZOOM (III)

For live wound healing analysis and single cell tracking of vimentin mCherry mutants stable cell lines of the C643 cells were made of the mCherry-EV, mCherry-WT and mCherry S71A vimentin constructs, by electroporation and selection with geneticin. To boost the number of plasmid expressing cells, the stable cell lines were re-transfected with the appropriate plasmid. The following day cells were split and plated in duplicate on 96-well Essen Image Lock plates (Essen BioScience, UK) at 35000 cells per well and were allowed to adhere for 6 hours before lipid-starving overnight. Wounds were made using the Essen WoundMaker (Essen BioScience, UK) and one image was obtained using the Incucyte ZOOM (Essen BioScience, UK) prior to treatment. Cells were treated with S1P, SPC or the appropriate controls and wound closure was monitored by acquiring images every 2 hours over a 24 hour period with the Incucyte ZOOM. Analysis of wound closure was done using the Incucyte Scratch Wound Analysis module. For the single cell tracking 10 mCherry positive cells were chosen close to or at the edge of the cells for tracking, with 3 independent repeats. Cells were manually tracked using the ImageJ Manual Tracking plugin. The data from one side of the wound was inverted in order to reflect migration from the starting point into the wound. Analysis of directionality and velocity, rose and vector plots was done using Chemotaxis and Migration tool (Ibidi, Germany).

4.6.2.4 Random motility assay using the Cell-IQ (III)

C643 and MDA-MB-435S cells were grown on 24-well plates to less than 50% confluency. They were treated as indicated with S1P, SPC, JTE and Y27362 and were imaged using the Cell-IQ (CM Technologies, Finland). Use of this machine allowed continuous imaging of cells in many wells of a 24
well plate. Images were collected once an hour over a 20 hour period. The videos were analysed by manually tracking cell movements for individual cell speed, directionality and persistence using ImageJ MtrackJ plugin (Meijering et al., 2012) of 20 cells per video with 3 independent repeats. Rose plots and vector plots were made using the Chemotaxis and Migration Tool (Ibidi, Germany).

4.7 INVASION ASSAYS (I)

4.7.1 Inverted invasion assay

Transfected PC-3 cells were serum starved overnight in 0 % FCS containing media. The matrigel coated chambers were made as follows. Growth-factor reduced Matrigel (BD Biosciences, USA) was supplemented with 5 μg/ml human fibronectin (GIBCO Invitrogen, USA) and combined with 250 nM nestin siRNA (Qiagen flexitube siRNA) and Lipofectamine RNAiMAX (Invitrogen, UK) in 0 % RPMI-1640 and allowed to set at 37 °C for 1 hour. The 8.0 μm pore transwell inserts (Corning, USA) were inverted and 3 x 10^4 cells were added to the opposite face of the filter and allowed to adhere. Inserts were transferred to 0.5 % FCS containing media and 10 % FCS containing media was added to the top of the matrix. Cells were allowed to invade for 6 days, every 2 days media was changed. To maintain downregulation 100 nM siRNA with Lipofectamine RNAiMAX were added throughout the system during the media changes. Inserts were fixed with 3.7 % PFA, permeabilised (3 % Triton-X, 2 mM MgCl₂, 5 mM EGTA in PBS) and stained with Alexafluor488-conjugated phalloidin (Invitrogen, UK). Inserts were visualised with Leica SP5 Matrix, z-intervals of 1.8 μm, 20x magnification, 1.0 optical zoom, 1048x1048 resolution from 8 randomly chosen fields. The distance invaded was measured using BioImage XD (University of Jyväskylä and University of Turku, Finland) (Kankaanpää et al., 2012) and statistical significance was determined using Graphpad Prism 5 (GraphPad Software Inc., USA) and unpaired t-test.

4.7.2 In vitro invasion assay

The in vitro invasion assay was performed similarly to the migration assay. The key difference was that growth factor reduced matrigel-coated boyden chambers were used (BD Biosciences, USA). Statistical significance was determined using Graphpad Prism 5 (GraphPad Software Inc., USA) and
either the unpaired t-test or the one-way ANOVA with Bonferroni correction.

4.7.3 Function blocking invasion assay

To block integrin function, cells were briefly serum-starved and then the appropriate antibody was incubated with the cells for 30 minutes at 37 °C and the matrigel boyden chamber invasion assay was performed as described in Section 4.7.2 with the exception that the integrin function blocking antibodies were added to the media in the upper chamber. The antibodies used for integrin function blocking were: α3 clone P1B5 (1:200, clone P1B5, Millipore, USA), α5 (1:200, clone JBS5, AbD Serotec, UK), αVβ3 clone LM609 (1:100, clone LM609, Millipore, USA) and rabbit anti-mouse IgG (1:200, Invitrogen, UK).

4.8 INTEGRIN ASSAYS (I)

4.8.1 Integrin clustering assay

Cells were grown on Millicell EZ-slide chambers (Millipore, USA). Integrin α5 (1:500, clone JBS5, AbD Serotec, UK) was added to the media and the cells incubated at 37 °C for 30 minutes. The antibody was washed out and an Alexafluor488-conjugated mouse IgG antibody (Invitrogen, UK) was added to induce clustering for 15 minutes at 37 °C. Cells were fixed in 3.7 % PFA and mounted with Mowiol. To analyse clustering quantitatively, images were taken with Leica SP5 confocal microscope with 63x oil objective. Z-stacks were collected randomly from throughout the sample (approximately 20 cells per sample, 3 independent repeats) according to the specifications determined by Nyquist’s theorem for optimal sampling. To quantify cluster number, volume and intensity per cell, images were thresholded, segmented and analysed using BioImageXD (University of Jyväskylä and University of Turku, Finland) (Kankaanpää et al., 2012). All images were analysed using the same threshold and segmentation parameters. The results were analysed using GraphPad Prism 5 (GraphPad Software Inc., USA) and the unpaired t-test.

4.8.2 Flow cytometry of integrin surface expression

For integrin surface expression analysis, cells were fixed with 3.7 % PFA, washed with Tyrode’s buffer (0.1 % glucose, 0.1 % albumin, 140 mM NaCl,
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2 mM KCl, 12 mM NaHCO₃ pH 7.4, 5 mM HEPES pH 7.4, 1 mM MgCl₂) and incubated with primary and then either Alexafluor488- or Alexafluor633-conjugated secondary antibodies. Primary antibodies for flow cytometry were: α5 (1:100, clone JBS5, AbD Serotec, UK), β1 (1:100, CD29-FITC, Beckman-Coulter, France), active-β1 (1:100, clone 12G10, Abcam, UK). The mean fluorescent intensity of 10⁴ cells was analysed with BD FacsCalibur and BD CellQuest software (BD Biosciences, USA).

To measure the level of activated integrins over time transfected cells were plated onto 5 µg/ml human fibronectin (Invitrogen, USA) coated plates and collected over the indicated time points and fixed in 3.7 % PFA and then processed for flow cytometry analysis as described above. The results were analysed using GraphPad Prism 5 (GraphPad Software Inc., USA) and statistical significance was determined using 2-way repeated measures ANOVA.

4.8.3 Integrin recycling assay

The integrin recycling assay was based on a modification from the assay as described in Arjonen et al., 2012; Ivaska et al., 2002; Roberts et al., 2001. Serum-starved cells were surface labelled with 0.5 mg/ml EZ link-sulfo-NHS-SS biotin (Pierce, Thermo Scientific, USA) in Hanks Balanced Salt Solution (HBSS, GIBCO Invitrogen, USA) at 4 °C for 30 minutes. They were returned to 37 °C to allow internalisation of biotin. Any remaining surface biotin labelling was cleaved with 60 mM sodium 2-mercaptoethanesulphonate (MESNA, Sigma-Aldrich, USA) in 50 mM Tris-HCl and 100 mM NaCl at 4 °C for 30 minutes and free sulphyl groups were quenched with 100 mM iodoacetamide (Sigma-Aldrich, USA) in PBS on ice for 15 minutes. To stimulate recycling cells were returned to serum-containing media and incubated at 37 °C. Cells were collected at the indicated time points and underwent another round of MESNA and iodoacetamide treatment and were then lysed (1.5 % Triton-X-100, 200 mM NaCl, 75 mM Tris-HCl pH 7.5, 7.5 mM EDTA, 7.5 mM EGTA, 1.5 mM Na₃VO₄, 15 mM NaF, protease inhibitor cocktail (Roche, Switzerland). Samples were normalised for protein concentration using the Bradford assay and incubated overnight at 4 °C on α5 integrin (5 µg/ml, clone VC5, BD Pharmingen, USA) coated maxisorp (Nunc, Thermo Scientific, USA) plates. ELISA was performed with biotin-HRP antibody (1:1000, Cell Signaling, USA), 1-step slow TMB-ELISA reagent (Pierce,
Thermo Scientific, USA) and 2M H$_2$SO$_4$ as the stop solution. The absorbance was measured with MultiSkan (Thermo Scientific, USA). The results were analysed using GraphPad Prism 5 (GraphPad Software Inc., USA) and statistical significance was determined using 2-way repeated measures ANOVA.

4.8.4 **Integrin trafficking assay**

The internalisation assay was performed in a similar manner to the recycling assay described above. However, after the cells were surface labelled with biotin, cells were returned to 37 °C to allow for internalisation and samples were collected at the indicated timepoints and underwent MESNA and iodoacetamide treatment (as described in the integrin recycling assay) and were then lysed. The remaining part of the assay continued as described in the recycling protocol. To measure for endocytosis as opposed to net trafficking, recycling was blocked with 0.6 nM primaquine bisphosphate (Sigma-Aldrich, USA).

4.8.5 **Integrin degradation assay**

Serum-starved cells were surface labelled with 0.5 mg/ml EZ link-sulfo-NHS-SS biotin (Pierce, Thermo Scientific, USA) in HBSS on ice for 30 minutes. Cells were washed once with sterile PBS and then incubated for 0 hours, 12 hours and 18 hours. Samples were lysed and subjected to ELISA as described in Section 4.8.3.
5 RESULTS AND DISCUSSION

5.1 NESTIN AND CELL INVASION (I)

The intermediate filaments and their role in cell motility have been of interest to this lab for some time (Ivaska et al., 2007; Nieminen et al., 2006). So when a paper was published showing that nestin could have a role in cancer cell motility, our interest was piqued (Kleeberger et al., 2007). We chose to emulate some of the key methods in the Kleeberger et al., 2007 paper in order to build upon their findings. The PC-3 cell line has the unique characteristic of being the only prostate cancer cell line in common use to express nestin and vimentin, meaning that nestin is primarily filamentous, a form that is important for its role as a signalling scaffold. When we tried to recapitulate the migration and invasion results we were exceptionally surprised to get the opposite results to Kleeberger et al., 2007. In an effort to dissect these further, we performed wound healing and random motility assays which showed that nestin downregulation did not affect any motility parameters. However, our results in PC-3 cells showed very clearly that nestin is an inhibitor of cell invasion, not a promoter as had been previously shown (Fig. 7; Fig. S4A-E, I).

5.1.1 Nestin regulates pFAK localisation and focal adhesion turnover

As described in Section 2.3.4 vimentin connects to FA and forms a scaffold with FAK and RACK1 in endothelial cells (Bhattacharya et al., 2009; Dave et al., 2013; Gonzales et al., 2001; Kreis et al., 2005). FAK also influences cell invasion (Chan et al., 2009; Hauck et al., 2002; Hsia et al., 2003). Since nestin and vimentin are binding partners, we investigated whether nestin had any influence on FAK localisation or activity. We showed that nestin downregulation does not affect FAK activation nor its downstream signalling (Fig. 1E-H, I). Immunofluorescence imaging showed that upon nestin downregulation phosphorylated FAK (pFAK) accumulated in punctate structures at the cell periphery which strongly resembled FA. This accumulation indicated a change in FA turnover which was confirmed by fluorescent recovery after photobleaching (FRAP) of the late FA marker vasodilator-stimulated phosphoprotein (VASP; Fig. 2, I). We hypothesised that nestin could act as a regulatory signalling scaffold for FAK and regulate its localisation and activation in a similar way that vimentin is able to. We performed co-immunoprecipitation of nestin and FAK with limited success.
(data not shown). It is well known that the IF proteins are primarily insoluble, as our studies (Fig. 9, II; Fig. 5 III) and others have shown, which may explain our co-IP problems. Vimentin and FAK are known to colocalise at FA (Bhattacharya et al., 2009). Vimentin also regulates FA organisation, while FAK protein levels regulate vimentin organisation through ROCK-1 (Lynch et al., 2013; Pan et al., 2011; Tsuruta and Jones, 2003). Co-labelling of vimentin, nestin and pFAK showed little pFAK and nestin colocalisation when analysed with BioImageXD, however, pFAK did colocalise with vimentin. Although no quantitation was done in this case, it seems that upon nestin downregulation, FAK colocalisation with vimentin was dramatically reduced indicating that nestin mediates FAK:vimentin interactions without altering vimentin organisation (Figure 8).

As described in Sections 2.1.3 and 2.2.1 nestin regulates vimentin stability. Furthermore, IF heteropolymers and isoforms also regulate IF protein:protein interactions. As such it would not be surprising if nestin modulates vimentin signalling interactions. These results were not included in the final article because confirmation of this hypothesis would require more detailed analysis than we were able to do in the time. However, this is an intriguing aspect of vimentin and nestin regulation and warrants further study.
5.1.2  Nestin regulates integrin expression and organisation at the cell surface

Integrins are an integral component of FA and IFs are known to connect and tether integrins at the cell surface (Section 2.3.4). Although we did not see any overt downstream signalling changes for FA upon nestin downregulation, integrin function is also regulated through alterations in protein expression and organisation at the cell membrane. Using antibodies that recognise the extracellular domain of integrins we employed flow cytometry analysis to identify whether integrin expression on the cell surface was altered by nestin downregulation (Fig. 3, I). This revealed that in resting cells nestin downregulation leads to an increase in the amount of $\alpha_5$ and active $\beta_1$ on the cell surface. Western blot analysis demonstrated that the total protein levels of these integrins in the cell remains unchanged,
suggesting that nestin affects the localisation of integrins within the cell (Fig. S2D). Intriguingly, when we performed an adhesion assay, there was no discernable change in adhesion upon nestin downregulation, despite the changes in adhesion (data not shown). However, this assay measured absolute numbers of adhered cells and did not take into account alterations in, for example, cell spreading, which is also mediated by integrins.

Since integrins are mediators of cell adhesion, spreading and motility we wanted to address how the integrin expression levels on the cell surface changed during cell adhesion and spreading. To do this, cells were detached and then plated onto fibronectin-coated plates and then fixed and labelled over the course of 8 hours. The extracellular labelling of integrins was analysed by flow cytometry. Both integrin α5 and active β1 cell surface levels were consistently elevated throughout the time of the assay (Fig. 3, I). One key difference was that α5 surface expression was higher than the control from the start of the experiment. Active integrin β1 levels started from the same point and diverged over the course of the assay. Where the control cells saw an immediate decline in active β1 on the cell surface, in nestin downregulated cells active β1 increased for 30 minutes before declining. This would seem to reflect a nestin dependent change in the cell surface dynamics and activation state of β1 during cell spreading and adhesion. Interestingly, there were no changes in cell adhesion.

5.1.3 Integrin relocalisation to the cell membrane is independent of trafficking or degradation mechanisms

The flow cytometry results would suggest that targeting of integrins to the cell membrane is altered. Although nestin has not been directly connected to integrin trafficking, vimentin is known to regulate integrin trafficking, in particular recycling of β1 integrin (Ivaska et al., 2005). We embarked on a series of experiments to identify whether nestin affects trafficking of integrins. We chose to use α5 as the primary target since this was the most consistently affected during the time course assay. We hypothesised that nestin downregulation could either increase integrin recycling back to the cell surface, or that nestin downregulation would delay endocytosis from the membrane, or both. Net trafficking assays, recycling assays and endocytosis assays revealed absolutely no effect on α5 trafficking (Fig. 4, I). The recycling assay was especially tricky to perform since our PC-3 cell line did not appear to recycle integrins at all. The assay and timepoints used are
well described in the literature and have been used very successfully with a number of cell lines. Since the trafficking assay, which measures both endocytosis and recycling, failed to show any changes upon nestin downregulation we chose not to pursue this any further.

Upon ligand binding integrins are internalised where they either directed to the endosomal recycling pathway or for degradation. We used a modified version of the endocytosis assay to determine if nestin affects integrin degradation. These results showed very little alteration in integrin degradation upon nestin downregulation (Fig. 4, I).

An alternative explanation as to how integrin protein levels are increased on the cell surface could be that there are changes in their transcription or their translation. Since we did not observe an increase in the total integrin levels (Fig. S2D, I), this implies that the increase in integrin protein levels on the cell surface is not due to transcription and is the result of the altered cellular targeting of the existing integrin pool.

5.1.4 Nestin modulates integrin clustering at the cell surface

Although we saw increased integrin protein levels at the cell surface but no changes in downstream signalling or trafficking, we looked to see whether integrin organisation at the cell membrane was affected since this is also known to regulate integrin function (Maheshwari et al., 2000; Van Slambrouck et al., 2009; Wang et al., 2013). Integrin clustering is a process whereby integrin heterodimers cluster together on the cell surface. It is required for integrin ligand-binding, and can be stimulated through binding of a ligand such as fibronectin, or by the use of a well-characterised antibody recognising the extracellular domain of the integrin. The degree of integrin clustering can be used as a measure of integrin activation. We used a modified version of integrin clustering assays previously described (Jokinen et al., 2010). The volume, number and the intensity of the clusters in each cell was analysed. The images acquired and the analysis clearly show increases in the size and number of clusters (Fig. 5, I). In addition the organisation of the clusters on the membrane appeared to be more ordered in nestin downregulated cells (Fig. 5, I). Since we saw changes in pFAK localisation in the cell and pFAK is known to regulate integrin clustering (Comisar et al., 2011; Hood and Cheresh, 2002) we repeated the assay and included a FAK inhibitor (Fig. 5E-G, I). Inhibition of FAK had a negligible effect on
clustering. Future work could investigate whether downstream signalling following integrin clustering is affected upon nestin downregulation.

Currently we do not know why nestin regulates clustering. It is possible that as a result of nestin’s role as a scaffolding protein that it could directly or indirectly affect localisation of integrin activators, such as talin and FAK (Lawson and Schlaepfer, 2012). This would affect integrin activation and consequently, integrin clustering. A recent paper describes a spatial model of integrin clustering (Welf et al., 2012). The authors show that a small pool of active integrin is required to initiate integrin clustering. This then triggers a positive feedback loop to increase integrin binding and activation. In this context it is plausible that the increase in integrin activity because of nestin RNAi (Fig. 3C, I) could trigger a positive feedback loop which would in turn affect integrins clustering.

5.1.5 Nestin inhibits matrix degradation

Our data showing alterations in FAK localisation, integrin organisation and activation would suggest effects on cell motility. We performed several 2D cell motility assays, including a boyden chamber assay, wound healing and tracking of single cell motility (Fig. S3, I). We found that nestin had no effect on cell motility in any of these assays. Use of several types of invasion assay showed that nestin downregulation increases cell invasion, suggesting that nestin itself is inhibitory for cell invasion (Fig. 7A-C, I). Considering the number of studies that suggest that nestin is pro-migratory (Ishiwata et al., 2011a), we were very surprised by these results.

Since invasion requires proteolysis of the ECM we delved deeper and used a matrix degradation assay to quantify whether nestin downregulation increases the amount of ECM degradation. We were able to show that not only does nestin downregulation increase matrix degradation it is also FAK activity dependent (Fig. 7D,E I). Previous studies show that FAK recruitment to lamellipodia promotes invasion, while FAK can negatively regulate the vimentin cytoskeleton to promote podosome rosette formation (Hsia et al., 2003; Pan et al., 2011). There are also indications that vimentin is involved in the elongation of invadopodia (Schoumacher et al., 2010). Quantification showed that neither invadopodia size nor number changed upon nestin downregulation (Fig. S4G-I, I). This would suggest that the relocalisation of pFAK was not involved in the formation of invadopodia.
Not all invadopodia that form are proteolytically active invadopodia. Targeting of MT1-MMP to invadopodia is dependent on MT1-MMP association with a FAK-p130cas complex situated at focal adhesions (Wang and McNiven, 2012). Additionally, integrin activity is also important for invadopodia stability, maturation, MT1-MMP accumulation at invadopodia and matrix proteolysis (Beaty et al., 2013; Branch et al., 2012). MMPs too are able to regulate integrin localisation and turnover (Gálvez et al., 2002; Shi and Sottile, 2011). Nestin knockdown increases β1 integrin activity and triggers pFAK relocalisation, which may have repercussions for MT1-MMP recruitment to invadopodia and subsequently cell invasion. Western blot analysis showed that MT1-MMP expression levels were only slightly affected by nestin downregulation, however, the effects on MT1-MMP activity and localisation are currently unknown. Future studies could investigate whether nestin knockdown affects MT1-MMP recruitment to invadopodia and MT1-MMP activity.

5.1.6 Nestin regulates the integrins required for invasion

The integrins are major participators in cell adhesion, motility and invasion. β1 activity and integrin organisation on the plasma membrane correlates with cell invasion (Pellinen et al., 2012; Van Slambrouck et al., 2009). Additionally different integrin classes have different effects on FA, protrusion formation and cell behaviour (Morgan et al., 2009). For instance β1 integrins promote small FA and protrusions, αV on the other hand promote large FAs. This is due to their distinct interactions with the Rho signalling pathway with downstream consequences for cell motility (Schiller et al., 2013). Integrins and IFs interact and are able to reciprocally regulate each other. Since we showed clearly that nestin regulates the surface expression of α5 and active β1 integrin and integrin clustering in a spatio-temporal manner (Fig. 3, 5, I), we wanted to see if the increase in invasion in nestin-downregulated cells was integrin dependent. Integrins α3, α5 and β1 were inhibited with function-inhibiting antibodies and an invasion assay was performed which demonstrated that nestin can regulate which integrins are required for invasion (Fig. 7F, I). Co-transfection of siRNA against nestin and β1 integrin or treatment with a FAK inhibitor showed that the increase in invasion upon nestin downregulation was β1 integrin and FAK activity-dependent (Fig. 7G,H; Fig. S4G, I).
This study describes nestin as an inhibitor of invasion, which is contrary to a number of reports (Ishiwata et al., 2011b; Kleeberger et al., 2007; Matsuda et al., 2011). A key difference between ours and others’ reports lies in the experimental design. All of the other reports utilise short hairpin RNA (shRNA) and stable transfections which represent a long-term downregulation. Our work utilises transient downregulation using small interfering RNA (siRNA) oligomers. The difference between ours’ and others’ results may reflect the difference between short term and long term downregulation of nestin. It is possible that long term downregulation causes cells to switch phenotype with regard to the mechanisms they use for migration and invasion. Nestin has very cell and developmental/ transformation stage specific effects. During myogenesis it regulates the pace of differentiation whereas it promotes muscle regeneration which involves the acquisition of motility. However, nestin expression is also correlated with inhibition of acinar- ductal metaplasia (Miyamoto et al., 2003; Pallari et al., 2011; Vaittinen et al., 2001). These results invite caution when generalising nestin’s cellular functions across cell types.

Since nestin is unable to form homopolymers and must co-polymerise with another IF protein, such as vimentin, GFAP or desmin, nestin’s effects on cell motility may be a result of the effects it exerts on the other filament networks. As discussed in Section 2.1.3, IFs that can only form heteropolymers are able to alter the properties of the IF network that they integrate into such as mechanical rigidity, filament stability and protein-protein interactions. Additionally, the different IF subtypes also exhibit different viscoelastic properties which relate to their function in different cell types (Plodinec et al., 2011; Schopferer et al., 2009). It is known that metastatic cancer cells are much softer than non-metastatic cells and this softness allows a greater mechanical deformation of the cell enabling it to invade through the ECM (Cross et al., 2007; Cross et al., 2008; Plodinec et al., 2012; Swaminathan et al., 2011). In astrocytoma cells, those with expressing high nestin and little GFAP have a higher motility than those with GFAP alone. In these cells, one could assume that nestin is co-polymerising with vimentin, so is nestin “softening” the vimentin network to enhance motility and invasive potential? In those cells with higher GFAP and less nestin, is the GFAP network contributing to a more rigid and less
pliable cell which is less able to move (Chou et al., 2003; Mendez et al., 2010; Rutka et al., 1999)?

In continuation from the above discussion, nestin may also act to modulate the vimentin heteropolymer network through changes in interactions with signalling partners. Something similar has been observed for GFAP; incorporation of GFAP isoforms in different ratios confers varying protein binding properties on the GFAP filaments which would be otherwise impossible with homopolymeric GFAP filaments (Nielsen et al., 2002; Perng et al., 2008). Nestin has a very long C-terminus which has been postulated to act as a bridge between IF filaments and actin, and as protein-protein binding site. As such incorporation of nestin into vimentin (and desmin) filaments would facilitate a broader range of signalling interactions and interactions with other IF and actin filaments than would be possible with vimentin filaments alone. This flexibility in protein-protein interactions may be important during the process of malignant transformation as the cell changes morphology and phenotype quite drastically in order to become motile and invasive.

While this study does not present one clearly defined mechanism as to how nestin regulates cell invasion, we do identify 3 novel avenues by which nestin could regulate cancer cell invasion and migration (Fig. 8, I). Dissecting the dynamics of each of these is worthy of a separate article in their own right. This study provides a foundation for the study of nestin in the regulation of FA and integrin dynamics during cell invasion.

5.2 ANALYSIS OF VIMENTIN PHOSPHORYLATION SITES (II)

Phosphorylation is an essential mechanism by which vimentin function and organisation is controlled (Section 2.4.2). Our laboratory has been active in the use of mass spectrometry and other tools to identify functional vimentin phosphorylation sites (Eriksson et al., 2004; Kochin et al., 2004). Following this work, it was one of the initial goals of my PhD thesis work to create functionally mimetic or functionally inactive phosphorylation mutants in order to characterise these vimentin phosphorylation sites for effects on vimentin organisation and function. We used site-directed mutagenesis to introduce amino acid substitutions for the phosphorylatable serines (Eriksson et al., 2004). Serines (S) were substituted to alanines (A) to render
them unphosphorylatable or aspartates (D) to mimic constitutive phosphorylation. Both untagged and GFP-tagged vimentin constructs were used, however, it was soon discovered that GFP tagged vimentin is unable to polymerise in a vimentin null background (MCF7 and MEF -/-). We tried C-terminus and N-terminus GFP-tagged vimentin WT. However, only a very small proportion of cells expressed filamentous vimentin. Clearly, GFP-tagged vimentin requires an endogenous vimentin network with which to integrate. This observation has also been reported in the literature (Yoon et al., 1998) Unfortunately the inability to use a fluorescent tag severely hampered the use of live cell imaging in this work which would have allowed us to study vimentin phosphomutant dynamics in real-time.

5.2.1 The N-terminal cluster and S71 are key regulators of vimentin organisation

Obvious changes in the vimentin organisation were observed in vimentin N-terminal cluster mutants (S4,6,7,8,9 A & D) and S71,72A (Figure 9). None of other mutants had visibly obvious structural alterations, although this is not to say that they are functionally irrelevant. However, the wealth of phosphomutants generated necessitated that we initially characterise those mutants with the most obvious morphological defects (Table 1, II). The most striking structural changes came with the N-terminal cluster mutants which had a range of morphologies from small and large aggregates to enlarged hollow spheres. Similar morphologies have been shown for the Alexander Y242D mutation in GFAP (Snider et al., 2013b). The presence of aggregates was confirmed using correlative light electron microscopy (CLEM; Fig. 8, II). Mutations of individual sites (S4 and S6) of the cluster did not have any

![Figure 9: Vimentin phosphosites.](image)

Serines (S) or tyrosines (T) were replaced with either unphosphorylatable alanine (A) or phosphomimicking aspartate (D) residues. The phosphosites that resulted the most prominent vimentin assembly alterations are highlighted by coloured boxes.
effects on the vimentin structure, only S7,8,9D gave similar morphologies to the large cluster mutants (Fig. 2C-H, 8 II). The vimentin N-terminal cluster has been implicated in the recycling of β1 integrin and inhibition of cell motility and transmigration through PKCe and PI3K dependent mechanisms (Barberis et al., 2009; Ivaska et al., 2005). However, they did not observe the range of vimentin morphologies that we did. We were also able to demonstrate that the N-terminal cluster mutants and S71,72A mutations were able to disrupt the endogenous vimentin WT network in HeLa and MEF cells (Fig. 4,5, II).

**5.2.2 N-terminal cluster may regulate cytoskeletal interactions**

Further characterisation of the mutants was performed using stimulated emission depletion microscopy (STED) which revealed vimentin organisation irregularities in phosphomutant transfected cells that were imperceptible at normal resolution. (Fig. S1, II). This highlights the advantages of using super-resolution methods to investigate IF organisation. Quantification of the integration of endogenous mutant vimentin with wild-type vimentin in aggregates in MEF cells revealed that the S4,6,7,8,9 phosphosites have a role in vimentin assembly/disassembly (Fig. 5, II). Considering that actin and MT are known to regulate IF network organisation, one of the most interesting observations of this paper is that S4,6,7,8,9 vimentin phosphosites colocalise with actin and tubulin (Fig. 7, II). This suggests that these phosphosites might be important in binding cytoskeletal linker proteins such as plectin, kinesin and dynein. This part of the study could be extended by investigating the effects of actin and MT destabilising compounds such as, cytochalasin D and nocodazole, on the vimentin phosphomutant phenotypes.

We have created a significant library of vimentin phosphomutants and characterised them in resting cells. This manuscript demonstrates clearly in interphase cells that S7,8,9 of the vimentin N-terminus are the most critical phosphorylation sites for vimentin assembly. If they cannot be phosphorylated then vimentin structure is dramatically perturbed.

One caveat to this approach is that phosphorylation is a highly dynamic PTM and the introduction of phosphomutants into vimentin null cells represents an artificially forced state of phosphorylated and dephosphorylated vimentin. Additionally phosphorylation can occur in a
sequential fashion and the use of single phosphomutations means that we cannot properly dissect the temporal aspects of vimentin phosphorylation. Use of phosphoantibody labelling in conjunction with expression of the phosphomutants could reveal whether the phosphomutants alter the phosphorylation of other vimentin phosphosites.

Another limitation to this approach is the use of phosphomutants in the first place. A study comparing phosphomimetic mutants to in vitro phosphorylated and WT α-synuclein showed that in vivo phosphorylation increased conformational flexibility of α-synuclein that could not be replicated by the use of phosphomimetic mutations (Paleologou et al., 2008). Particular problems are evident with phosphomimetic mutants that cannot bind to adaptor proteins in the same way a phosphorylated site would. This is quite pertinent considering we saw effects on tubulin and actin cytoskeletal networks. Additionally phosphomimetic mutations do not carry the same negative charge of a phosphorylated residue which will have different effect on the chemical environment surrounding that residue and its subsequent interactions (Dephoure et al., 2013). As such, caution must be employed when interpreting data solely derived from phosphomutant studies, particularly those that rely on structural observations.

As discussed in Section 2.4.2 vimentin phosphorylation is important in many homeostatic and pathological states. A good addition to this paper would have been to find some physiological role for some of these phosphorylation sites. Attempts were made to employ high-content imaging for migration studies. However very low transfection efficiencies and an inability to use fluorescently tagged vimentin constructs in a vimentin null background hindered this approach. Additional approaches that could be employed would be in vitro assembly assays of the mutants to see how assembly is perturbed. Our work involves the painstaking characterisation of a series of vimentin phosphomutants and their impact on vimentin network assembly in interphase cells. The next stage is to identify important cellular processes such as migration and proliferation where these phosphomutants have impact. An alternative approach would be to utilise known extracellular regulators of cell motility and identify how the vimentin network is affected and use our knowledge of the phosphomutants assembly characteristics to identify which phosphosites are most likely affected. This approach was applied in the third paper of this thesis (III).
**5.3 SPHINGOLIPID REGULATION OF VIMENTIN ASSEMBLY AND MIGRATION (III)**

### 5.3.1 Sphingolipids regulate vimentin organisation

The initial observations with western blot arose from a difference in the way our two laboratories handle samples for western blot. While our lab makes direct laemmli lysates, our collaborators lyse the cells in a buffer, centrifuge them and then make laemmli lysates. This centrifugation step revealed that less vimentin remained in the soluble fraction, indicating that the sphingolipids were affecting vimentin organisation. Sequential fractionation and immunofluorescence confirmed the alterations in vimentin organisation and showed that the vimentin network reorganised to the perinuclear space (Fig. 3A, B III). Interestingly, FRAP analysis revealed that S1P increased the speed of vimentin turnover (Fig. 3C, III). Although statistical analysis showed that these results were not significant, it is likely that including more cells in the FRAP experiments would then show that the filament turnover is significantly altered upon S1P treatment.

Sphingolipid induced effects on vimentin organisation appears to be a general phenomenon since we saw vimentin reorganisation in all the cell lines (< 5) that we tested. Albeit there was temporal variance between cell lines; in some, such as the MDA-MB-453S cells it was very rapid (< 10 minutes), and in the MDA-MB-231 cell line it was much slower (> 4 hours). Several studies investigating effects of sphingolipids on IFs use high sphingolipid concentrations (1 μM - 15 μM) (Beil et al., 2003; Busch et al., 2012; Sin et al., 1998). Considering that S1P and SPC are found at physiological concentrations of 10 – 100 nM (Section 2.5.2), we felt these were excessive and not physiologically relevant. Consequently we performed a dose response analysis (data not shown) to identify the lowest concentrations at which we still obtained effects on the vimentin filament network. These concentrations have been used throughout this work.

### 5.3.2 S1P and SPC induce rapid S71 vimentin phosphorylation

Considering that phosphorylation is one of the key regulators of vimentin organisation, we used mass spectrometry analysis to identify potential vimentin phosphorylation sites regulated by sphingolipid treatment. MDA-MB-435S cells were treated with S1P, SPC and their respective controls and
sent to Dr. Susumu Imanishi for mass spectrometry analysis. He identified several phosphorylation sites affected by sphingolipid treatment including S38, S41 and S71 (Fig. 1A, Fig. S1, Table S1 III), which were validated using site-specific phosphoantibodies (Fig. 1B, III). Of note, S1P treatment caused a 3-fold increase in S71 phosphorylation whereas for SPC the increase was closer to 1.5-fold (Fig. 1B, III). We saw no changes in S38 phosphorylation levels, consequently we chose not to study it any further. Analysis of the phosphorylation kinetics by western blot and immunofluorescence revealed that S1P induced a rapid S71 phosphorylation (< 10 minutes) which persisted for over 2 hours in MDA-MB-435S cells. However, in C643 cells the phosphorylation was induced within 10 minutes but was considerably weaker and shorter lived. This suggests that the magnitude of the effect of sphingolipids on vimentin phosphorylation are cell-type specific. We cannot rule out that a higher concentration of S1P required for a stronger response in these cells. SPC treatment induced a more gradual increase in S71 which persisted for more than 4 hours (Fig. 1C, III).

5.3.3 Serine 71 is the primary vimentin site that regulates chemotactic migration

Both sphingolipids and vimentin have very prominent roles in cell migration. With our cell models, we found that both S1P and SPC inhibit migration of C643 and MDA-MB-435S cells by 80 – 90 % (Fig. 2, III). Sphingolipids are also upstream activators of RhoA and ROCK, which both regulate cell migration and incidentally ROCK phosphorylates vimentin at S71. Thus, we hypothesised that sphingolipids activate ROCK through RhoA, which stimulates vimentin phosphorylation and reorganisation leading to inhibited cell migration.

In order to test this hypothesis we utilised mCherry-tagged vimentin phosphomutants. Since C643 and MDA-MB-435S already contain vimentin, proper expression of these mutants was not an issue. Boyden chamber migration experiments using empty vector (EV), vimentin wild-type (WT) and the nonphosphorylatable S71A mutant demonstrated that S71A was able to partially rescue the S1P and SPC induced migration defects (Fig. 2D, III). Interestingly, S71A on its own exhibited elevated cell motility compared to WT suggesting that basal S71 phosphorylation restricts cell motility to some extent. When cells are treated with S1P and SPC, they tend to round up, expression of S71A did not prevent this. Since the sphingolipids affect
numerous pathways that regulate actin polymerisation and actomyosin contractility, we used blebbistatin an inhibitor of non-muscle myosin IIA to prevent actomyosin contractility (data not shown). Blebbistatin rescued sphingolipid induced cell rounding. This contrasts with a report stating that SPC induced cell rounding is entirely keratin dependent (Beil et al., 2003). However, both the C643 and the MDA-MB-435S cell lines do not express keratins (Alix-Panabières et al., 2009; Iyer et al., 2013; Sellappan et al., 2004). These results imply that the sphingolipid induced cell motility defects are both vimentin and actin dependent. Further assays could confirm to what extent the actin-dependent reorganisation affects cell motility.

We also employed a wound healing assay using the C643 cell line to test whether S71A could rescue the defect in cell motility (data not shown). The S71A mutant had no effect on cell motility, neither did it rescue the sphingolipid induced effects on wound healing. Single cell tracking of mCherry-vimentin expressing cells revealed that not only did S71A not rescue the sphingolipid effects on cell motility; it actually had the same effect as the sphingolipids. This is possibly due to the fact that induction of S71 phosphorylation is not as strong in C643 cells as it is with the MDA-MB-435S cells. The key difference between the wound healing assay and the boyden chamber is that the boyden chamber assay involves migration of cells towards a chemotactic gradient. The wound healing assay on the other hand involves the migration of cells into the wounded area with no chemotactic stimulation. The difference between these migration results could indicate that vimentin phosphorylation affects chemotactic sensing ability. As described earlier in Section 2.3.4.2, vimentin is involved in the organisation of adhesion receptors on the cell surface. As such, vimentin phosphorylation may also regulate the organisation of other receptors, such as those involved in chemotactic sensing on the cell surface. Additionally, these results indicate a divergent cell-type specific regulation of vimentin network dynamics and cell migration.

5.3.4 **S1P and SPC act through the S1P2 receptor**

The sphingolipids potentially act through 5 G-coupled receptors (S1P1-5). One study showed that both MDA-MB-435S and C643 primarily express S1P2 and very little of the other receptors (Balthasar et al., 2006). Use of the S1P2 inhibitor JTE013 rescued the sphingolipid-induced cell morphology, vimentin reorganisation and migration effects (Fig. 4C-E, III). Boyden
chamber migration assays showed that both S1P and SPC dramatically inhibit chemotactic migration. Single cell motility analysis revealed that both S1P and SPC treatment dramatically affect the velocity at which cells move. This fits with the understanding that Rac is important for cell velocity and low Rac activation is a characteristic of S1P2 mediated sphingolipid signalling (Okamoto et al., 2000; Steffen et al., 2013; Sugimoto et al., 2003). S1P2 inhibition rescued the velocity defects back to control levels. Importantly, both JTE013 treatment and S1P2 downregulation prevented sphingolipid induced vimentin S71 phosphorylation (Fig. 4A, III), confirming that both S1P and SPC act through S1P2 to regulate vimentin phosphorylation. This is in agreement with previous work suggesting that signalling through S1P2 is inhibitory for migration (Section 2.5.5.1).

5.3.5 Inhibition of Rho-associated kinase (ROCK) prevents vimentin phosphorylation and sphingolipid effects on migration

In order to test our hypothesis further we utilised the well established ROCK inhibitor Y27632. Inhibition of ROCK was able to rescue all sphingolipid induced effects on cell morphology and motility as well as preventing vimentin S71 phosphorylation (Fig. 5, III). ROCK has been established in several studies as the specific, and currently only, identified kinase for vimentin S71. A study in 3T3 cells demonstrated that ROCK and vimentin colocalise and when vimentin organisation is disrupted by depolymerisation of MT, ROCK translocates to the cell periphery. In the same study, S1P was utilised as a ROCK activator. This caused collapse of vimentin and induced ROCK translocation to the cell periphery (Sin et al., 1998). However, no direct connection was made between S1P treatment, ROCK and vimentin phosphorylation.

5.3.6 Sphingolipid treatment induces increased ROCK and vimentin interaction

To provide additional evidence that ROCK phosphorylates vimentin following sphingolipid treatment we embarked on a series of experiments to identify whether ROCK interacts with vimentin. Since S71 phosphorylation varies with different sphingolipid treatments, we performed colocalisation analysis on vimentin and ROCK to see whether sphingolipid treatment affects their proximity to one another and at what timepoints. The colocalisation analysis was performed by treating cells with sphingolipids for the indicated timepoints, fixing and labelling the cells with vimentin and
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ROCK antibodies. Single stack confocal images were used for the analysis which was performed using the ImageJ colocalisation plugin. The Pearson correlation coefficient was used for the quantification. The Pearson correlation coefficient describes the overlap between images (colocalisation). It is presented as a number ranging from -1 to 1, where -1 is no overlap and +1 is perfect image overlap. The Pearson correlation coefficient was used because it can be used on images that have varying intensity such as those presented in Fig. 6A, B (III). Interestingly we observed increased colocalisation even after 10 minutes S1P treatment and 30 minutes SPC treatment (Fig. 6A, B, III), which reflect the phosphorylation kinetics previously observed (Fig. 1C, III).

As noted earlier in this thesis, immunoprecipitation studies with IFs are particularly tricky due to their insoluble nature. A dot blot assay was employed to test whether there is a possible interaction between vimentin and ROCK. Vimentin purified using a IF preparation protocol developed in our laboratory was immobilised on nitrocellulose membrane. The membrane was incubated with the lysate from cells stimulated with S1P and SPC and probed with an anti-ROCK antibody and an appropriate HRP-conjugated secondary antibody. Binding of ROCK to the vimentin was identified with enhanced chemiluminescence. The preliminary results indicated that S1P stimulation increases ROCK interaction with vimentin (data not shown). In vitro phosphorylation could also be employed to confirm whether ROCK directly phosphorylates vimentin.

5.3.7 Sphingolipids regulate vimentin through ROCK-mediated vimentin phosphorylation

The sphingolipids are versatile signalling molecules which have pro-oncogenic effects. Their effects on cell motility are dependent on the S1P receptor profile of a cell. Downstream targets of the sphingolipids, S1P and SPC, are the IFs, vimentin and keratin, whose networks are dramatically reorganised following sphingolipid treatment. It was identified that phosphorylation was the key mediator of sphingolipid induced keratin network reorganisation (Beil et al., 2003; Busch et al., 2012; Park et al., 2011). Considering that vimentin has roles in migration, we sought to identify how sphingolipids, S1P and SPC may affect the vimentin network. Here we show that the anti-migratory effect of the S1P2 receptor is a result of ROCK-mediated phosphorylation on vimentin S71 (Fig. 7, III). We also show that S1P and SPC affect cell rounding in an actin-dependent
manner. This may also regulate cell motility (Figure 10). This study provides novel insight into how extracellular cues can regulate vimentin network architecture and cell motility.

Figure 10: Sphingolipids stimulate ROCK-mediated vimentin phosphorylation and reorganisation which inhibits cell migration. Changes in cell shape are mediated by actin reorganisation which may have consequences for cell motility.
CONCLUDING REMARKS

The intermediate filaments are the most diverse and enigmatic protein family belonging to the cytoskeleton. Long gone are the days where they were thought to be merely structural proteins. As is evident from this thesis the IFs regulate many processes throughout almost every stage of a cell’s existence. Their functions depend highly on the organisation of the filament network, which governs many of the interactions with other members of the proteome.

Deciphering a function for nestin has proven challenging. It is difficult to work with biochemically and, by its very nature, it is expressed transiently in only very specific situations. When this thesis was embarked upon there was very little in the literature describing its presence and function in cancer. The wealth of studies that have emerged in the last few years regarding nestin and cancer indicate that it has multifaceted roles in cancer, in some situations it may prove pro-migratory and invasive and in others, such as the study presented here, indicate that can act as a brake on invasion. Our studies provide novel data showing that nestin affects FA formation and turnover, integrin dynamics at the cell surface and integrin-mediated cell invasion. The mechanisms behind these effects have not been revealed, however, this study provides considerable insight into the different methods by which nestin regulates the motility machinery. Considering the transient and temporal nature of nestin expression, these results suggest that nestin provides a way for a cell to fine-tune the bi-directional signalling between the cell and its environment. Most likely this occurs by modulation of the polymerisation and signalling characteristics of its IF heteropolymerisation partner. As such its role in migration and invasion may be very closely tied to specific stages of malignant transformation. Future work on the role of nestin in cell motility would do well to keep in mind the nature of the cell lines and their origins used, as this may be the key to understanding nestin’s Janus-like role in motility signalling and cancer progression.

It is intriguing that while vimentin is almost ubiquitously expressed in many cancers, the understanding of how it contributes to motility is just starting to be understood. Thanks to the work of others, we now know that the state of assembly of the vimentin network in different regions of a migrating cell can have great impact on how that cell moves. While actin will most likely
Concluding Remarks

remain the principal cytoskeletal element governing cell motility, recent studies indicate that vimentin has a supporting role ensuring the correct recruitment and targeting of signalling molecules in the migrating cells. Our work involved the painstaking characterisation of a series of vimentin phosphomutants and their impact on vimentin network assembly in interphase cells. These results demonstrate that S7,8,9 of the N-terminal vimentin cluster are the most critical sites regulating vimentin structure. The other vimentin phosphosites studied had a milder structural phenotype. Despite the extensive knowledge regarding vimentin phosphorylation sites, not much is known about non-mitotic extracellular stimuli that induce vimentin phosphorylation. We identified and characterised the effect of the sphingolipid regulation on vimentin phosphorylation and assembly during cell motility. Our results show that sphingolipid treatment induces specific and rapid phosphorylation of vimentin at S71, which causes rapid vimentin network organisation and inhibition of cell migration. Using small molecule inhibitors and siRNA we showed that these effects on vimentin phosphorylation and migration are channeled through the S1P2 receptor and ROCK. This work develops ideas about vimentin responses to sphingolipid treatment that have been hinted at 16 years previously in the literature.

It is not enough to understand the internal signals that govern IF regulation. Cells are extremely sensitive to their environment. We already have a great understanding of the external cues that affect cell motility and signalling in other fields and it is now time to apply that to understanding the environmental regulation of vimentin and nestin networks and signalling functions. Considering the difficulty of working with and manipulating IFs it will not be easy. However, there are now a great number of in silico and imaging tools that can be exploited. Acquiring this knowledge will give us a greater understanding of the role of vimentin and nestin in tumourigenesis and metastasis, and may yield new molecular and chemical tools by which we can target them.
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Turku, April 2014
Claire Hyder

“Life is not like water. Things in life don’t necessarily flow over the shortest possible route.”
-Haruki Murakami
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