Trafficking and signalling of Syndecan-4 during wound healing

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A dissertation submitted to the University of Sheffield in partial fulfilment of the requirements for the degree of Doctor of Philosophy

2020

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Abstract

Wound healing defects affect over 2.2 million patients and cost £4-5 billion per year in the UK. Improper healing often progresses to chronic ulceration with excruciating pain, tissue necrosis and frequently limb amputation. Upon injury, syndecan-4 receptor binds fibronectin from damaged blood vessel activating PKCα and Rac1 at the leading edge of fibroblasts mediating cell polarisation, cytoskeletal rearrangement, redistribution of receptors and migration to wound site.

Syndecan-4 wild type MEFs form vinculin-containing focal adhesion when stimulated with heparin-binding fragment of fibronectin that forms the syndecan-4 ligand. In contrast, syndecan-4 knockout MEFs fail to respond, supporting previous reports where knockout mice suffered delayed healing due to migration defect establishing the importance of these molecules during the healing response. Syndecan-4 enables fibroblast to detect changes in the environment and regulates the redistribution of integrin to provide a physical link between ECM and cytoskeletal contractile machinery. Syndecan-4 regulation is still unclear hence, understanding syndecan-4 trafficking will be a key step in elucidating how fibroblasts polarise and migrate in response to wounding.

Measuring syndecan-4 uptake in fibroblasts using biotinylated and fluorophore conjugated antibody demonstrated involvement and colocalisation of the receptor with caveolin and clathrin within 30 minutes of internalisation. Syndecan-4 receptor engagement activates PKCα to mediate receptor downstream signalling including Rac1 activation and localisation to the leading edge of migrating fibroblasts. The role of PKCα on syndecan-4 uptake was tested by RNAi-mediated knockdown of PKCα and BIM-I treated cells demonstrating attenuated receptor uptake suggesting that PKCα activity is required for endocytosis. Mutation of the PKCα-binding motif of syndecan-4 blocked syndecan-4 endocytosis, demonstrating that it self-regulates. Syndecan-4 fab fragment failed to trigger receptor uptake, despite binding, suggesting that clustering is required for receptor internalisation.

In conclusion, data demonstrates that syndecan-4 clustering is required for caveolin- and clathrin-dependant endocytosis of syndecan-4 in a PKCα-dependant manner.

Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic work.

Acknowledgements

First, I would like to express my deepest appreciation and gratitude to my supervisor Dr. Mark Bass for his continuous guidance and support throughout my time in the lab and afterwards. It was an honour and a privilege being in the lab. Thank you for believing in my potentials.

I would like to thank my advisory team: Dr. Kai Erdmann and Dr. Anestis Tsakiridis for their advice, reassurance and constructive critique throughout my PhD.

To the previous members of the Bass lab Martina, Rebecca, Alaa, Bret and Keely, thank you for sharing your cultural backgrounds, research ideas and most importantly spreading positive and creative energy in the lab. I would like to thank current members of the lab Dr. Aimee Paskins and Amani Alharbi for all the "science" fun we had and for crying together when encountering massive infection in tissue culture. Wishing you all success in your future careers.

I would like to thank Dr. Darren Robinson and Dr. Nick Van Hateren from Microscopy Facility for training and technical support. Also, would like to say thank you to the flow cytometry core facility at the Medical School of the University of Sheffield for sample analysis and technical support.

I want to thank my better half Walaa and my courageous son Wesam (Sam) for brightening up my life. I would like to welcome baby Bassem (Bass) who was born halfway through my writing up.

Finally, I would like to thank my family for their unconditional love and support throughout my educational journey away from home since 2010. I am finally home!

Thank you to all the members of the healthcare team for putting their lives on the line during the exceptional time of COVID-19 outbreak to ensure that we have a healthy and prosperous life. I would like to thank the Saudi Arabian Cultural Bureau in London and King Abdulaziz University for funding.

Lastly, I dedicate this work to my grandmother, blessed her soul, who suffered chronic wounds and inspired me to carry on research in the field of cellular trafficking during wound healing. Thank you for shining down on my path through my PhD journey. May you rest in peace.

Abbreviations

50K	Recombinant fragment of fibronectin comprising type III repeats 6-10
ALIX	Alpha-1,3/1,6-mannosyltransferase interacting protein X
AP-2	Adaptor protein-2
ARF6	ADP Ribosylation Factor 6
Arg-rich CPP	Arginine-rich cell-penetrating peptides
BAR	Bin/Amphiphysin/Rvs
BIM-I	Bisindolylmaleimide I
BSA	Bovine serum albumin
Dab2	Disabled homolog 2
DMEM	Dulbecco's Modified Eagle's medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ERK	Extracellular signal-regulated kinases
ESCRT	Endosomal sorting complex require for transport
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
GAG	Glycosaminoglycans
GAP	GTPase activating protein
GDI	Guanosine nucleotide dissociation inhibitor
GDP	Guanosine disphosphate
GEF	Guanine nucleotide exchange factors
GPI	Glycosylphosphatidylinositol
GTP	Guanosine trisphosphate
GTPase	Guanosine triphosphatase
H/0	Recombinant fragment of fibronectin comprising type III repeats 12-15
HFF	Human foreskin fibroblast
HSPG	Heparan sulfate proteoglycans
INF-γ	Interferon- gamma
MEF	Mouse emberyonic fibroblasts
MiTMAB	Myristyl trimethyl ammonium bromides
PACSIN2	Protein kinase C and casein kinase substrate in neurons protein 2
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PDZ	Post synaptic density protein (PSD95), Drosophila disc large tumour suppressor (DlgA), and Zonula occludens-1 protein (Zo-1)
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKCα	Protein kinase C-alpha
R8	Octa-arginine
Rac1	Ras-related C3 botulinum toxin substrate 1
RGD	Arginine-Glycine-Aspartate tripeptide sequence
Rho	Ras homolog
RNAi	RNA interference
SDS	Sodium dodecyl sulphate
siRNA	Silencing RNA
SNX	Sorting nexin
Sulfo-MBS	Sulfo-3-maleimidobenzoyl-N-hydroxysulphosuccinimide ester
TAE	Tris-acetate EDTA
TBS	Tris-buffered saline
TGF-β	Transforming growth factor beta (TGF-β)
TIF	Telomerase immortalised fibroblast
α-SMA	alpha-smooth muscle actin

Chapter 1: Introduction

1.1 Wound healing and defects

Wound healing is a complex dynamic process that involves the coordination of multiple overlapping phases: haemostasis, inflammation, proliferation and wound remodelling (Velnar et al., 2009). Upon tissue injury, haemostasis phase starts with the coagulation cascade activation leading to platelets aggregation and clot formation to minimise blood loss. During platelet aggregation, growth factors, including platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β), are released not only to promote clot formation and vasoconstriction but also to start up the inflammatory phase (Deuel et al., 1991; Postlethwaite et al., 1987; Seppa et al., 1982). Immune cells, including neutrophils and macrophages, are recruited to wound site to clear any pathogens, foreign bodies and damaged tissue matrix. At the end of this phase, fibroblasts can be observed at the wound site marking the start of the proliferation phase. Fibroblasts recruitment at the injury site is triggered by chemoattractant released during the inflammatory phase, including PDGF and TGF-β, by platelets and immune cells to induce fibroblast proliferation and differentiation. Early observations described fibroblasts movement in tissue culture to be dependent on the orientation of deposited collagen where they move via a process known as contact guidance (Stopak & Harris, 1982). However, few years later, a study demonstrated that fibroblasts spread and migrate better on fibronectin suggesting that in the wound bed, cells migrate on fibronectin fibres instead of collagen (Wojciak-Stothard et al., 1997). Therefore, early arriving fibroblasts to wound bed might secret ECM facilitating the migration of other fibroblasts (Hsieh & Chen, 1983).

Tissue repair begins in the proliferation phase as fibroblast proliferation, migration and formation of granulation tissue occur with deposition of new extracellular matrix (ECM). Chemoattractant release and fibroblast engagement with ECM via integrin surface receptor induce cell differentiation into myofibroblasts and enable cell migration (Deuel et al., 1991; Xu & Clark, 1996). Differentiated myofibroblasts express α-smooth muscle actin (α-SMA) empowering these cells to contract (Darby et al., 1990; Gabbiani et al., 1971; Schmitt-Gräff et al., 1994). The coordination of contracting myofibroblasts in wound edges aids myofibroblasts in pulling both edges together resulting in wounds closure. The final phase is wound remodelling which includes a realignment of collagen fibres and newly secreted matrix resulting in the formation of new epithelium. This phase could last anywhere between 1-2 years or even more as normal wound healing requires a delicate balance between matrix deposition and degradation (Velnar et al., 2009; Witte & Barbul, 1997; Yamakawa & Hayashida, 2019).

Any disruption in the mentioned steps could result in wound healing defect. Wound healing defects affect patients worldwide and a study in the UK alone showed that the number of affected patients (over 18) was 2.2 million with an annual cost of £4.5-5.1 billion (Guest et al., 2017, 2015). Several risk factors contributed to this number such as ageing, obesity, diabetes and chronic diseases. Chronic ulceration arises from improper healing and patients often develop painful, odorous ulcers, inflammation and tissue necrosis eventually leading to limb amputation (Guo & DiPietro, 2010; Sen et al., 2009). The recruitment of immune cells to wound bed is important but recruited fibroblasts demonstrate a central role in the process of wound healing. Fibroblasts are versatile cells capable of proliferation, differentiation and migration to wound. Changes in the surrounding environment are detected by fibroblast from interacting with fibronectin from damaged blood vessel at the dermis layer. Therefore, understanding the impact of fibroblast on wound healing by studying the changes in the intact skin during acute injury is key to develop a therapy for patients with chronic wounds.

1.1.1 Structure of human skin

The skin forms a barrier around the body protecting it from extreme changes in the external environment. The human skin is composed of multiple layers: epidermis, dermis and hypodermis. Figure 1.1 shows the structural layers composing the human skin. The epidermis is the outer protective layer composing of stratified squamous epithelium. The avascular layer undergoes cornification to replace the outermost cells that have been shed to maintain haemostasis (Eckhart et al., 2013). The epidermis is populated by group of cells including keratinocytes, melanocytes, Langerhans cells and Merkel cells (Boulais & Misery, 2008). Keratinocytes undergoes cornification in which cells commit to terminal differentiation leading to the formation of outermost layer of skin making keratinocyte the predominant cell type of that layer (Eckhart et al., 2013). Melanocytes can be found at the basal layer of the epidermis and produce melanin which play a role in skin pigmentation (Cichorek et al., 2013). Langerhans cells are antigen presenting cells which plays a role in microbial defence (Kubo et al., 2009). Merkel cells are located in the basal epidermal layer and thought to have sensation function by interacting with intraepidermal nerve endings in specialised structures known as tactile discs (Kashgari et al., 2018; Bichakjian & Johnson, 2007).

The dermis is located underneath the basement membrane of the epidermis and subdivided into the uppermost papillary layer (loose areolar connective tissue) and the lower reticular layer (dense irregular connective tissue). The dermis represents the vital layer of the skin where blood, lymphatic vessels and nerve endings can be found, as well as unstimulated fibroblasts that populate this layer maintaining ECM in a collagen-rich environment (Greiling & Clark, 1997). Lastly, the hypodermis layer

which is composed of adipose tissue and function as a cushion connecting the previous two layers with the bone structure underneath (Watt, 2014).

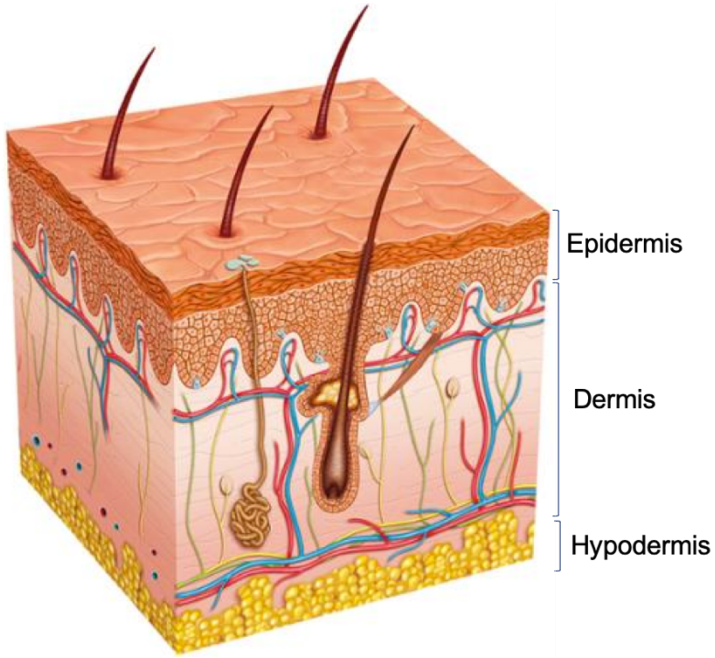


Figure 1.1 The structural layers of human skin. The epidermal layer is the outermost layer protecting the dermis from extreme changes in the environment. The vital dermal layer contains blood, lymphatic vessels and inactive fibroblasts in a collagen-rich environment populating and miniating this layer. The hypodermis is an adipose layer which provides support for the previous two layers.

Routinely, inactive fibroblasts maintain the dermis layer by secreting collage and ECM, where collagen is the major constituent. Upon acute injury, fibronectin leakage and growth factors release, including PDGF and TGF-β, shift the surrounding environment and stimulate fibroblasts activation and differentiation via surface receptors (integrins and syndecans). The differentiated myofibroblasts have increased mechanical force and contractibility due to increased α-SMA expression. The clustering of migratory myofibroblast expressing α-SMA around wound edges and contraction is essential for proper wound closure (Darby et al., 1990; Hinz et al., 2001; Tomasek et al., 2002). Hence, understanding the regulation between fibroblast surface key receptors syndecan-4 and integrin is essential in order to comprehend the role of fibroblast in the wound healing process. Each surface receptor will be discussed below.

1.2 Syndecan-4 and integrins

1.2.1 Introduction to syndecans

Syndecans are a family of specialised type I transmembrane receptors with 4 distinctive heparan sulphate proteoglycan (HSPG) members: syndecan-1, syndecan-2, syndecan-3 and syndecan-4.

Syndecans are expressed in all body tissues and some tissues show some level of specificity by expressing multiple syndecans which could suggest a unique function for some syndecans.

Syndecan-1 (CD138) was the first member of the family that was isolated from rat livers (Kjellen et al., 1981) and murine mammary gland epithelial cells (Rapraeger & Bernfield, 1983). The function of syndecan-1 was thought to be bridging between components of ECM and intracellular actin-containing cytoskeleton, hence the name "syndein" which derives from the Greek meaning "to bind" (Saunders et al., 1989). Genetically, syndecan-1 was the first syndecan that was cloned, and other members of the family were isolated and cloned afterwards (Saunders et al., 1989). Syndecan-1 is predominantly expressed in the surface epithelium of keratinocytes and mesenchymal cells and have been used previously as a marker for plasma cells (Bernfield et al., 1999). Syndecan-1 has been reported to play part in infection, inflammation, wound healing and tumours (Ishikawa & Kramer, 2010; Stepp et al., 2002).

Syndecan-2 (fibroglycan) has been isolated from lung fibroblast and liver and reported in many cell types such as endothelial (mostly), neuronal and fibroblasts in addition to cancer-derived cells (Fears et al., 2006; Kim et al., 1994). Syndecan-2 plays a key role in angiogenesis as its deficiency in zebrafish affected vascular development and rescue with human syndecan-2 restored angiogenic sprouting (Chen, Hermanson & Ekker 2004). It is thought that Syndecan-2 modulates integrin interaction with ECM affecting cell proliferation, adhesion, cytoskeletal organisation and migration (Kusano et al., 2000; Utani et al., 2001). The upregulation of syndecan-2 in tumours and abnormal angiogenesis makes syndecan-2 a potential therapeutic target for cancer (Chen et al., 2004; Park et al., 2002).

Syndecan-3 (neuronal syndecan or N-syndecan) is primarily found in neural cells of the nervous system and has been isolated from neonatal rat brain, chick embryo limb buds and rat newborn Schwann cells (Carey et al., 1992; Chernousov & Carey, 1993; Gould et al., 1992). Syndecan-3 is upregulated during foetal development as demonstrated in rodents and chicks (Gould et al., 1992, 1995). It is thought to be essential for oligodendrocyte differentiation and myelin formation in the central nervous system (Chernousov & Carey, 1993). Syndecan-3 is suggested to play a role in cortical neural cell migration as in vivo deletion in mice resulted in defective and reduced cells at the cerebral cortex (Hienola et al., 2006).

Syndecan-4 (amphiglycan or ryudocan) has been isolated from chick embryo and rat microvascular endothelium (Baciu et al., 1994; David et al., 1992; Kojima et al., 1992). Syndecan-4 is expressed in

many cell types including fibroblasts, epithelial and vascular endothelial cells. Syndecan-4 promotes many cellular events including cell spreading, focal adhesion, cell migration during wound healing. Fibroblast dysfunction is commonly seen in chronic wounds (Wall et al., 2008) limiting recruitment of fibroblasts to the injury site, and delaying in healing. Accumulated evidence has demonstrated that syndecan-4 is strongly upregulated after wounding and tissue repair suggesting a key role of this syndecan in the wound healing process (Echtermeyer et al., 2001; Gallo et al., 1996, 1994). In vivo experiments showed that syndecan-4 deletion in mice resulted in delayed wound healing due to defective cell migration (Echtermeyer et al., 2001). Likewise, syndecan-4 knockout fibroblasts failed to respond to fibronectin leading to impaired cell migration (Bass et al., 2011) establishing the importance of syndecan-4 to the healing process. Previous work in the lab investigated syndecan-4-dependant signalling to initiate wound healing by regulating integrin and other mediators including caveolin. However, syndecan-4 regulation upon wounding was not investigated, hence understanding syndecan-4 trafficking during wound healing will be a key step to improve cell migration and recruitment of cells to the injury site in order to promote healing.

1.2.2 Structural domain of syndecan-4

Syndecan-4 is a type I transmembrane receptor with a large extracellular domain composing of heparan sulphate chains covalently attached to the protein core. Each of the structural domains plays a role in cellular cues. Figure 1.2 shows the structural domains of syndecan-4. The extracellular domain is not conserved and is highly variable among syndecans. All syndecans contain covalently linked glycosaminoglycan (GAG) at the ectodomain with modification sites at the N-terminus for heparan sulphate and chondroitin sulphate chains (Lindahl et al., 2015; Rapraeger et al., 1985). Syndecan-4 has heparan sulphate modification where other syndecans including syndecan-1 and syndecan-3 can have both heparan sulphate and chondroitin sulphate (Shworak et al., 1994).

The heparan sulphate of syndecan-4 is responsible for cell interactions with ligands. Syndecan-4 ectodomain can interact with various distant and diluted ligands (can reach up to 500 nm from solid boundary) including fibronectin and heparin-binding growth factors, including fibroblast growth factor, to modulate cell to cell or cell to matrix interactions (Bass & Humphries, 2002; Roper et al., 2012). The formation of focal adhesion is contingent on syndecan-4 ectodomain interaction with fibronectin and heparin-binding domain via the heparan sulphate chain. This interaction requires the involvement of integrin surface receptor, which provides a linkage between intracellular and extracellular environment to mediate an effect, demonstrating that focal adhesion formation is functionally linked to syndecan-4. Hence, syndecan-4 engagement through ectodomain correlates with integrin

expression and activity (Morgan, Humphries & Bass 2007). In vitro study reported similar regulation of integrin surface expression using syndecan-1 demonstrating a direct connection between both surface receptors (Beauvais et al., 2004).

Shedding of syndecan-4 ectodomain is known to contribute to receptor signalling where intact GAG chains were found in ECM. Shed GAG chains maintain their capability to bind extracellular ligand including basic fibroblast growth factor and mediate syndecan-4 regulation of growth factor-mediated events (Elenius et al., 1992). Shedding of syndecan-4 ectodomain can occur constitutively or under physiological stimulation including acute skin assault. During acute skin or tissue injury, shed syndecan-4 ectodomain is found in dermal and inflammatory fluids of affected patients suggesting that shedding occurs in a regulated manner (Subramanian et al., 1997). Shedding of syndecan-4 ectodomain can be achieved by proteolytic cleavage, cellular stress and activation of several intracellular pathways including PKC activation (Jalkanen et al., 1987; Subramanian et al., 1997).

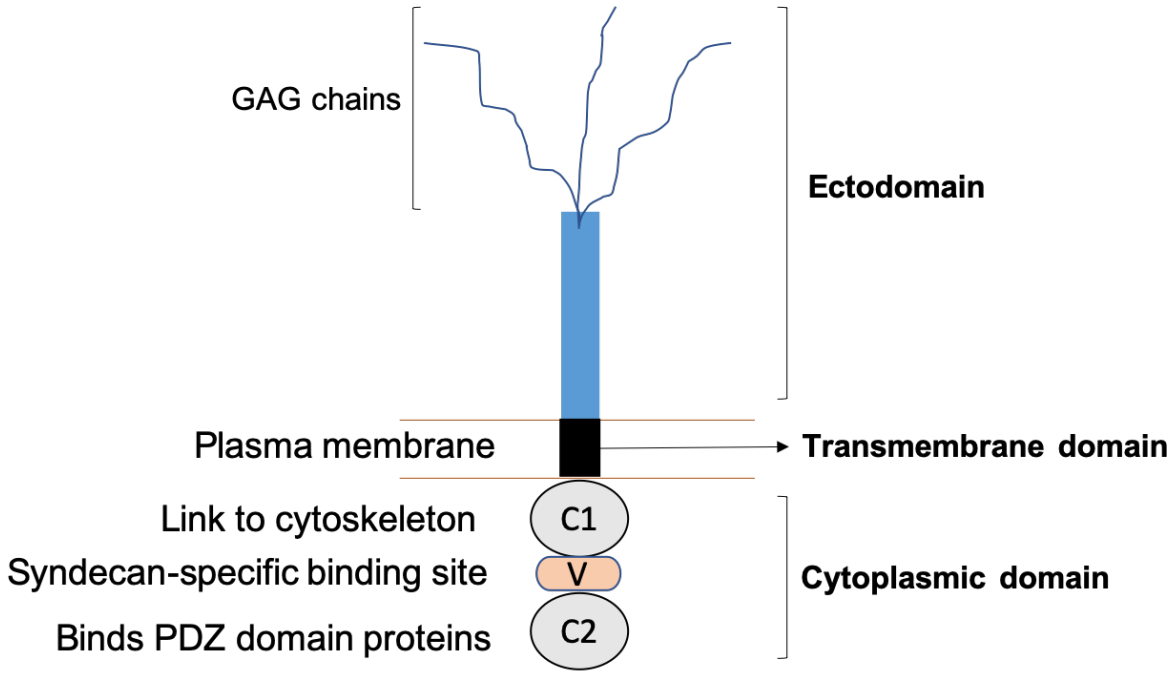


Figure 1.2 Structural domains of syndecan-4. The ectodomain of syndecans is composed of heparan sulphate proteoglycan enabling cells to reach distant ligands. The anchoring transmembrane domain is required for receptor dimerisation. The cytoplasmic domain consists of 2 conserved regions, similar in all syndecans, separated by syndecan-specific variable region. (C1=conservative region 1, V=variable region, C2=conserved region 2, GAG=glycosaminoglycan).

The transmembrane domain is conserved among all the syndecans, unlike syndecans ectodomain. The membrane-spanning domain connects extracellular to the cytoplasmic domain. It plays role in anchoring syndecan-4 to the membrane forming a detergent-resistant dimer as well as inducing

receptor oligomerisation due to the GxxxG dimerisation motif (Choi et al., 2005; Dews & MacKenzie, 2007). The transmembrane domain has been demonstrated to contribute to the overall function of syndecan-4 as well as other syndecans (Choi et al., 2005).

The short cytoplasmic domain of syndecan-4 is subdivided into two highly conserved regions: membrane-proximal conserved region-1 (C1) and membrane distal conserved region-2 (C2) regions, similar in all syndecans, separated by a syndecan-specific variable region (V). The cytoplasmic domain is critical for cytoplasmic interaction with cytoskeletal proteins and cell signalling (Oh et al., 1998). C1 region has been shown to bind cytoskeleton proteins, adaptors and kinases. The phosphorylation of syndecan-4 by Src kinase has been demonstrated to regulate cell adhesion and migration (Morgan et al., 2013). Src kinase is a member of Src kinase family: a non-receptor tyrosine kinase plays a role in multiple signal transduction pathways involved in cell adhesion, proliferation and survival. C2 region contains EFYA sequence at the C-terminus that is capable of binding PDZ domain-containing proteins including syntenin, which is proposed to act as an adaptor linking syndecans to cytoskeletal proteins (Grootjans et al., 1997).

The V region of the cytoplasmic domain is syndecan specific, it could suggest a specific function for each member of the syndecans. The V region of syndecan-4 is involved in regulating receptor interaction with the cytoskeleton to promote cell adhesion and migration (Tkachenko et al., 2005). V region of syndecan-4 contains LGKKPIYKK sequence to which phosphatidylinositol 4,5 bisphosphate (PIP2) can bind as well as the catalytic domain of protein kinase C α (PKCα) regulating PKCα activity (Oh et al., 1997). Other syndecans are incapable to regulate the activity of PKCα due to the absence of LGKKPIYKK sequence. Synthesised oligopeptides corresponding to syndecan-4 cytoplasmic domain demonstrate that in the presence of PIP2, syndecan-4 cytoplasmic domain forms an oligomeric complex which binds and potentiates PKCα highlighting the unique oligomerisation feature of syndecan-4 that is missing in other syndecans (Lee et al., 1998). The oligomerisation property of syndecan-4 has been demonstrated both in vivo and in vitro proposing that oligomerisation can be positively regulated by PIP2 or negatively by posttranslational modification (Lee et al., 1998; Oh et al., 1998, 1997a). The interaction of oligomeric syndecan-4 with PIP2 and PKCα has been suggested to localises both intermediates to focal adhesion. The conformation of syndecan-4 cytoplasmic domain contributes to receptor-mediated function as collaborative studies agree that under physiologic pH, syndecan-4 cytoplasmic domain forms a stable dimer via peptide ionic interaction (Koo et al., 2006; Lee et al., 1998; Shin et al., 2001). Another unique feature of the syndecan-4 cytoplasmic domain is the formation of compact interwind dimer with a twisted clamp shape at the V region. The twisted clamp

of syndecan-4 cytoplasmic domain has a cavity in the centre of the dimeric interface regulating the enzymatic activity of PKCα (Lee et al., 1998; Shin et al., 2001).

1.2.3 Syndecan-4 cytoplasmic interactions

1.2.3.1 Syndecan-4 interactions with PKCα

PKCα is a serine threonine kinase expressed in all tissues and has a contributing role in many cellular processes including cell proliferation, differentiation and motility (Bass et al., 2007; Newton, 1995). PKCα can bind syndecan-4 directly or following PIP2 binding to the V region through the LGKKPIYKK motif. PIP2 mediates syndecan-4 binding to PKCα to promote receptor downstream signalling, an attracting research area to explore effector proteins and function. Hence, some groups have investigated the role of syndecan-4 binding to PKCα via PIP2 demonstrating that binding to the syndecan-4 cytoplasmic domain is dependent on the phosphorylation of intracellular Serine 183 (Ser^{183}) residue of syndecan-4 using rat fat pad capillary endothelial cells (Horowitz & Simons, 1998; Murakami et al., 2002). The phosphorylation of Ser^{183} has reduced the ability of PIP2 to induce syndecan-4 oligomerisation suggesting destabilisation of the syndecan-4 cytoplasmic domain which can be demonstrated by lower PKCα activity. This could mean that oligomerisation of syndecan-4 cytoplasmic domain is essential to mediate syndecan-4-mediated cues.

In addition to PKCα, PKCδ can regulate syndecan-4 via the phosphorylation of Ser^{183} (Horowitz & Simons, 1998). Endothelial cell expressing dominant-negative PKCδ has demonstrated a lower level of syndecan-4 phosphorylation compared to those expressing dominant-negative PKCα (Murakami et al., 2002). Similarly, the activity of PKCα has increased in endothelial cells expressing dominant-negative PKCδ suggesting that PKCδ controls the activity of PKCα in a syndecan-4 dependant manner. Collectively, the interaction between PKCα and syndecan-4 cytoplasmic domain is critical in mediating syndecan-4 dependant regulation of focal adhesion formation.

1.2.3.2 Syndecan-4 regulation of Rho GTPases

Syndecan-4 receptor engagement with ECM regulates several Rho-family GTPases that signal downstream to regulate cellular processes and other receptors, including integrins during cell migration. GTPases are a large family of guanine nucleotide binding proteins (G proteins) that act as molecular switch binding guanine triphosphate and hydrolysing it to guanine diphosphate. The family can be further divided into Ras, Rab, Ran, Arf and Rho families and they participate in many cellular processes including trafficking of cytoplasmic vesicles (Rab and Arf) as well as rearranging actin stress fibre (Rho) (Takai et al., 2001). Upon stimulation, the GTPases family proteins are bound to either GTP

and GDP with high affinity and cycle between active (GTP-bound) or inactive (GDP-bound) states. The process of switching from active to inactive state is tightly controlled by guanine-nucleotide exchange factor (GEF) and GTPase activating protein (GAP). In GEFs, GDP nucleotide is exchanged for GTP (GTP-bound) switching the status of GTPase protein to "active", while GAPs hydrolyse GTP (GDP-bound) to inactivate it. Figure 1.3 summarises the cycle of activating and inactivating Rho GTPases through GEFs and GAPs to mediate cellular signalling. Among the Rho family proteins are Rac1, RhoA RhoG and Cdc42 that control the dynamics of the actin cytoskeleton, which will be discussed below.

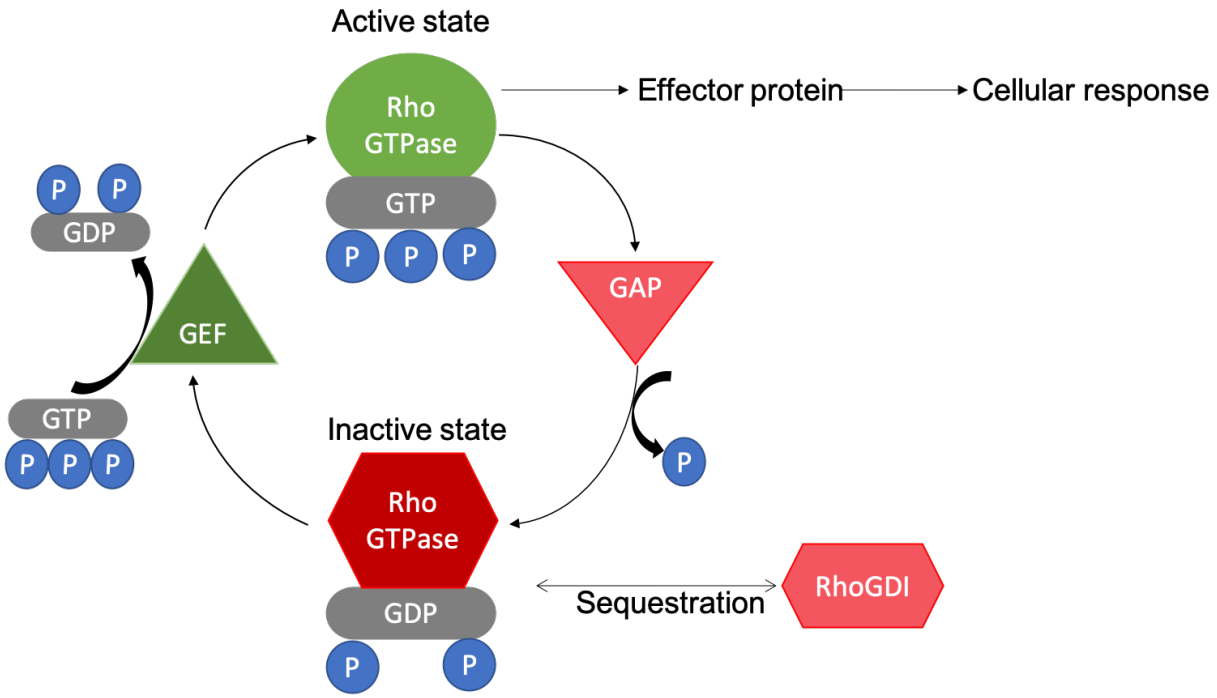


Figure 1.3 Rho family GTPase regulation. Rho family GTPases are regulated by GEF and GAP by cycling between GTP-bound status to mediate downstream signalling or inactive GDP-bound state. Rho GTPases activation is catalysed by GEF through the exchange of GDP for GTP. GTP-bound Rho GTPase activates downstream signalling to exert cellular response. Inactivation occurs through GAP by which GTP is hydrolysed to GDP inhibiting Rho GTPase activity. RhoGDI contributes to the regulation of Rho GTPases activity by sequestering active Rho GTPases.

Syndecan-4 receptor engagement with fibronectin has demonstrated to trigger the activation of PKCα promoting cell migration upon wounding (Bass et al., 2007). Syndecan-4 dependant activation of PKCα has been shown to regulate Rac1 activation, an event that plays a role in forming membrane protrusion (Bass et al., 2007; Matthews et al., 2008). The transient activation of Rac1 activity was not seen in PKCα-binding mutant demonstrating that PKCα is situated upstream of Rac1 and that PKCα activation is essential for Rac1 activation and localisation at the leading edge of migrating fibroblast. Stimulating PKCα-suppressed fibroblasts (using siRNA) with ECM failed to activate Rac1, similar to fibroblasts treated with PKCα-inhibitor affecting cellular migration. PKCα-dependant activation of Rac1 has been

demonstrated to maintain persistent migration in fibroblasts. Interestingly, syndecan-4-null fibroblasts can migrate on fibronectin-coated matrix similar to syndecan-4 wild type indicating that syndecan-4 loss does not affect the ability of migration but affects the persistence. High and delocalised Rac1 has been observed in syndecan-4 null fibroblasts demonstrating the role of syndecan-4 in localising Rac1 in cell migration (Bass et al., 2007). The role of Rac1 in wound healing has been reported to cause a delay in cutaneous wound closure due to reduction in migrating fibroblast to wound bed in Rac1-deficient mice (Liu et al., 2009).

The role of RhoA has been demonstrated to play a role in promoting focal adhesion and membrane protrusion (Bass et al., 2008; MacHacek et al., 2009; Nobes & Hall, 1999). Following the activation of Rac1 upon syndecan-4 engagement, RhoA undergoes a cycle of inhibition and reactivation that plays an essential role in modulating cell migration. Syndecan-4-dependant activation of PKCα phosphorylates the sequestering molecule Rho GDP-dissociation inhibitor (RhoGDI; Rho GTPase down-regulator) releasing RhoA to the cytosol for further modulation by GAPs and GEFs (Dovas et al., 2010; Koo et al., 2006). An event that is likely to play a role in focal adhesion stabilisation and contraction. Similarly, PKCα phosphorylation of p190RhoGAP (Rho GTPase down-regulator) leads to RhoA release from membrane-bound to the cytosol leading to RhoA inhibition then reactivation (Bass et al., 2008; Dovas et al., 2010). The inactivation of RhoA from p190RhoGAP play a role in membrane protrusion. Loss of RhoA has been documented to cause reduced cell migration and persistence (Jackson et al., 2011). The effect of PKCα on RhoA has been assessed by our group using PKCα-binding mutant demonstrating blockade of focal adhesion interrupting the cycles of RhoA inhibition and reactivation which affected actin stress fibres (Bass et al., 2007, 2008).

RhoG is generally involved in Rac1 activation, cytoskeletal reorganisation for cell migration, morphological changes including membrane protrusion similar to those triggered by Rac1 and endocytic pathways (El Atat et al., 2019; Gauthier-Rouvière et al., 1998). Engagement of syndecan-4 by fibronectin causes the release of RhoG from RhoGDI in a PKCα-dependant manner (similar to RhoA), an event that has been demonstrated by the lab previously (Bass et al., 2011). This interaction causes a transient wave of RhoG activation that plays a part in membrane protrusion of fibroblast as well as modulating integrin function. The importance of RhoG in the healing process has been evaluated in RhoG knockout mice. Post punch wounds, RhoG knockout mice have demonstrated a delay in wound closure due to defective cell migration to wound site impacting myofibroblast contractions at wound bed. Persistency of cell migration in vitro has been shown to be dependent on signalling starting from

syndecan-4 activation to RhoG as the persistent migration in RhoG knockdown and knockout cells were compromised when compared to control knockdown and wild type.

RhoG has been reported to mediate the internalisation of some receptors, like integrin, via multiple endocytic mechanisms including dynamin, caveolin and micropinocytosis in a syndecan-4-dependant manner. PKCα-dependant activation of RhoG has been demonstrated to trigger caveolar endocytosis of integrin upon stimulation of fibroblasts with a soluble syndecan-4 binding fragment of fibronectin (H/0). Syndecan-4 receptor engagement with H/0 has resulted in a rapid increase of RhoG activity within 10 minutes correlating with the disappearance of integrin from the cell surface in total internal reflection fluorescence microscopy images suggesting that RhoG has mediated integrin endocytosis in a syndecan-4 dependant manner (Bass et al., 2011).

Syndecan-4-dependant activation of RhoG initiates membrane ruffling during fibroblast growth factor receptor 1 signalling in endothelial cells promoting macropinocytic internalisation of the activated receptor. A group study has identified syndecan-4 and fibroblast growth factor receptor 1 as regulators of MAPK driving macropinocytosis of fibroblast growth factors 1 in a RhoG-dependant manner. This outcome is plausible in syndecan-4 knockout endothelial cells as it showed enhanced activation of MAPK enzymes extracellular signal-regulated kinases 1 and 2 (ERK 1/2) with slower deactivation of the mention enzymes meaning that syndecan-4 and RhoG control the rate of internalised receptor in the macropinosome (Elfenbein et al., 2012). Figure 1.4 summarises syndecan-4 receptor downstream signalling upon receptor engagement with fibronectin.

1.2.3.3 Syndecan-4 interactions with PDZ- and focal adhesion proteins

Syndecan-4, as well as all syndecans, contain EFYA sequence at the membrane distal C2 region enabling binding to postsynaptic density 95, disc large, and zonula occludens (PDZ)-domain-containing proteins including synectin and syntenin in addition to non-PDZ-containing proteins like α-actinin and syndesmos (Gao et al., 2000; Grootjans et al., 1997; Zimmermann et al., 2001). EFYA motif adds to the diversity of syndecan-4 as it shows to mediate syndecan-4-dependant signalling and protein-protein interactions including cytoskeletal rearrangement, cell adhesion and migration. Other protein interaction with syndecan-4 may modulate its function enabling the receptor to bridge ECM to the cytoskeleton.

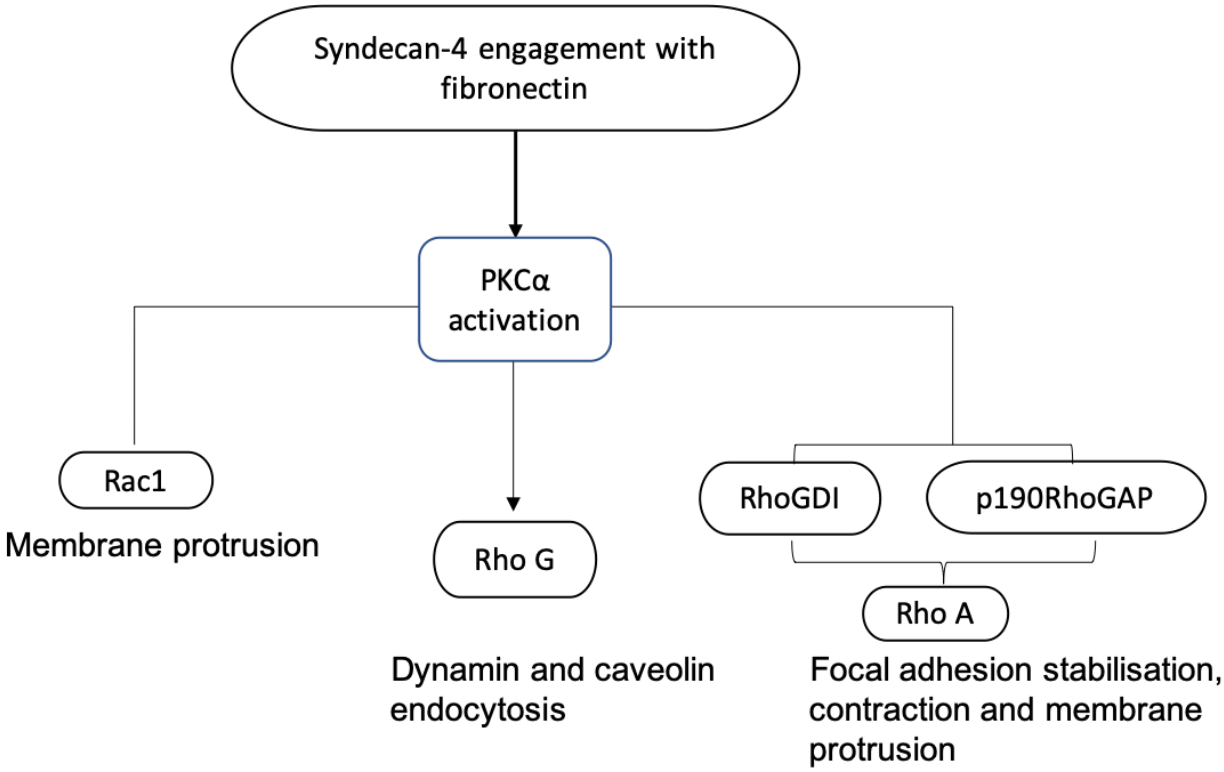


Figure 1.4 Syndecan-4 receptor engagement and downstream signalling to Rho family GTPase regulation.

Synectin is a PDZ-containing protein that is thought to play a role in syndecan-4 clustering and assembly of the signalling complex. The interaction of the two is known to affect actin cytoskeleton of migrating cells via binding Rho GTPases with high affinity. Synectin and syndecan-4 form a ternary complex when the receptor is not engaged with fibronectin leading to synectin binding to RhoGDI1 enhancing RhoGDI1 sequestering and suppressing of Rho GTPases. Subsequently, receptor engagement with fibronectin leads to syndecan-4-dependant activation of PKCα which phosphorylates RhoGDI1 releasing Rho GTPases as discussed above (Elfenbein et al., 2009). The deletion of synectin has affected syndecan-4-dependant signalling leading to decreased cell migration due to the high mislocalised Rac1 (Chittenden et al., 2006; Elfenbein et al., 2009). Similarly, syndecan-4 with EFYA mutation (lacking PDZ-binding region) has demonstrated reduced cell motility due to inability of fibroblasts to bind synectin PDZ-sequence (Tkachenko et al., 2006).

Another PDZ-containing protein is syntenin which is involved in regulating cell adhesion, focal adhesion stabilisation, turnover via modulating integrin endocytosis and recycling (Morgan et al., 2013). Syntenin can directly bind the phosphorylated cytoplasmic domain of syndecan-4 and PIP2 to modulate integrin. At focal adhesion, syntenin mediates integrin trafficking and switch surface expression of α5β1-integrin (by endocytosis) to αVβ3-integrin (increase recycling) in an Arf6-dependant manner, a small GTPase involved in vesicular trafficking, recycling and actin cytoskeleton organisation (Van Acker et al., 2019). The upregulation of αVβ3-integrin leads to focal adhesion stabilisation and inversely the

upregulation of α5β1-integrin destabilises focal adhesion and overall cell migration (Morgan et al., 2013).

Syntenin has been demonstrated to contribute to the biogenesis of exosome. Exosomes are vesicles consisting of cytoplasmic materials produced in endosomal compartment which can be fused with plasma membrane releasing its content to neighbouring or distant cells (David & Zimmermann, 2016; Trams et al., 1981). Syntenin is proposed to facilitate exosome biogenesis via coordination with syndecan and cytoplasmic ALG-2 interacting protein X (ALIX), accessory protein member of the endosomal sorting complex require for transport machinery (Baietti et al., 2012; Zimmermann et al., 2005). In fact, syntenin and ALIX are equally important and has demonstrated a direct effect of syndecan exosome as the knockdown of either one has resulted in lower exosomal syndecan. Overexpression of syntenin have resulted in increased syndecan and ALIX containing exosomes; however, the overexpression has no effect when ALIX was knockdown suggesting that ALIX is required for vesicle secretion. Furthermore, introducing a mutation in ALIX binding site of syntenin has lower exosomal syndecan confirming the involvement ALIX, syntenin and syndecan in exosome formation and release. The role of syndecans in exosomes is still central as the depletion of syndecans can result in low exosomal accumulation of syntenin and ALIX (Baietti et al., 2012; Roucourt et al., 2015).

Protein-protein interactions influence the function syndecan-4 providing more diversity to the receptor. For example, syndecan-4 can provide a link between ECM and cytoskeletal components such as α-actinin, actin crosslinking protein, to regulate cytoskeleton in an integrin-independent manner. α-actinin has been suggested to play a role in the assembly of focal adhesion components. Syndecan-4 knockout cells have demonstrated significant alteration in the cytoskeletal organisation with altered α-actinin distribution which has been restored upon rescue with syndecan-4 suggesting that α-actinin interaction with the syndecan-4 cytoplasmic domain may be important (Greene et al., 2003; Okina et al., 2012). Syndesmos is a syndecan-4 binding protein that has been reported to interact with syndecan-4 cytoplasmic domain (V and C1 regions) to maintain focal adhesion (Baciu et al., 2000). Syndesmos colocalises with syndecan-4 at focal adhesion and has been documented to bind focal adhesion adaptor, paxillin, an event that is triggered by PKC activation (Denhez et al., 2002). Syndecan-4 specific interaction with PDZ and cytoplasmic protein have demonstrated a variety of methods to activate the syndecan-4 receptor and show critical role in wound healing by regulating focal adhesion, cytoskeletal rearrangement and overall cell migration.

1.2.4 Introduction to integrins

Integrins are type I transmembrane heterodimeric receptors consisting of two subunits α and β. Integrins are non-enzymatic mechanosensing molecules functioning mechanically by providing a physical linkage between ECM and cytoskeleton, and biochemically by sensing the strength of cell-matrix adhesion and rigidity in order to respond (Hynes, 2002). Integrin, in cooperation with other receptors, is involved in many cellular processes including focal adhesion formation, cytoskeletal organisation and cell migration. There are 18 α- and 8 β-subunits giving rise to 24 distinctive dimerised integrin receptors in vertebrates suggesting not only ligand and tissue specificity but also functional specificity of integrins. For example, one of many studies demonstrated that α5β1-integrin, similar to αVβ3-integrin, is highly expressed in activated endothelial cells during the formation of new blood vessels (angiogenesis), but it is poorly expressed in normal quiescent endothelial cells (Bader et al., 1998; Brooks et al., 1994; Clark et al., 1996; Kim et al., 2000). A wide range of ligands that are recognised by integrin is summarised in (Stupack & Cheresh, 2002; Takada, Ye & Simon, 2007).

Integrin structural domain consists of ligand binding head with legs at the ectodomain for each subunit, transmembrane spanning domain and the short cytoplasmic tail. α-integrin subunit has α-I domain linked to 7 repeats of β propeller domain, thigh supported by 2 calf domains, transmembrane domain and short cytoplasmic domain. β-integrin subunit has β-I domain linked to plexin semaphorin integrin, hybrid, 4 repeats of epidermal growth factor, β-tail at the ectodomain, transmembrane domain and longer cytoplasmic domain containing NPxY/F motif. Figure 1.5 shows the structural domain of the integrin. Both α and β subunits are stabilised by a salt bridge keeping both cytoplasmic domains inactive (Campbell & Humphries, 2011; Gauthier & Roca-Cusachs, 2018; Xia et al., 2004).

Integrin crystal structures have demonstrated three confirmations of the receptor: inactive, intermediate and active. When integrin is inactive, it adapts a bent over structure with the headpiece bent over toward the membrane with closed structure and the salt bridge stabilises the cytoplasmic chains. While the activate confirmation results in high binding affinity of the receptor to its ligand and extension of the headpiece in open conformation with separation of the cytoplasmic domain. Between the two confirmations, there is a proposed intermediate confirmation where the ectodomain is extended but headpiece is closed (Kim et al., 2003; Vinogradova et al., 2004). Figure 1.6 summarises the conformational changes of integrin upon ligand activation.

Integrin activation and subsequent signalling is bidirectional. Signalling can be initiated from receptor engagement with ECM like fibronectin (outside-in signalling) leading to integrin conformational

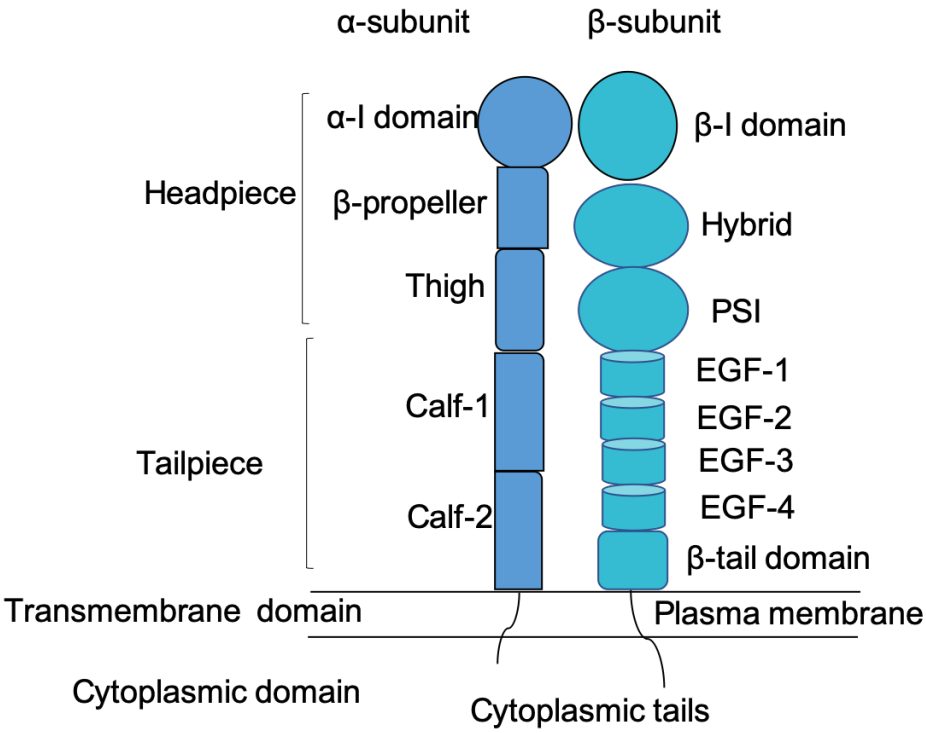


Figure 1.5 Structural domain of integrin. Integrin surface receptor is composed of α and β subunits. The activation of the large ectodomain can promote adhesion in addition to the short cytoplasmic domain that is involved in recruiting cytoskeletal proteins mediating actin stress fibre reorganisation. Hence, integrin can mediate bidirectional signalling providing a physical link between ECM and cell cytoskeleton during cell movement.

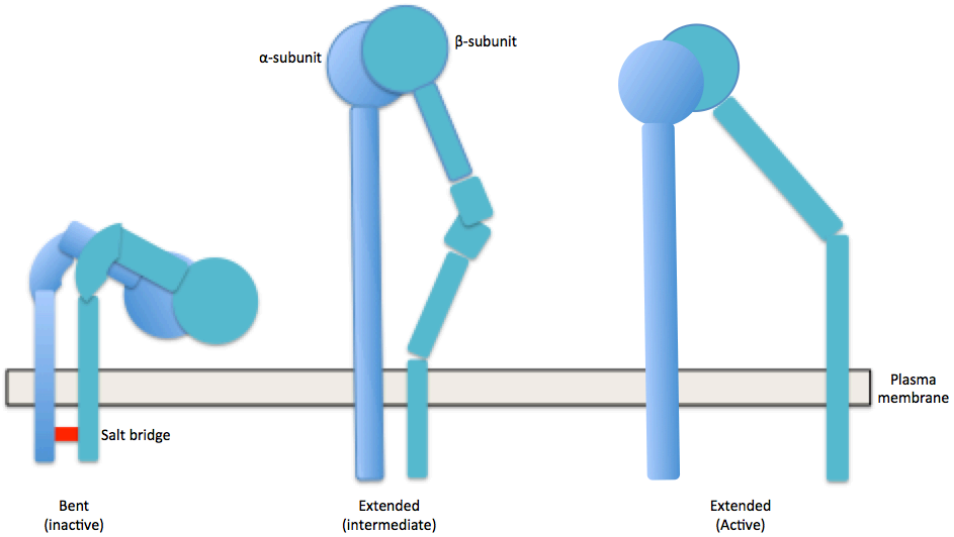


Figure 1.6: Integrin changes conformation and affinity to ligands during activation. In the inactive state, both subunits are closely located near the plasma membrane with closed head configuration. The ectodomain is extended at the intermediate state, yet the head is still closed. In the active state, the cytoplasmic domain of both subunits is separated leaving the head in an open configuration with high affinity to ligands.

changes to high affinity for ligand transmitting the signal from ECM to the cytoplasmic domain to form focal adhesion. Binding of intracellular adaptor proteins including talin to membrane-proximal NPXY sequence of β-integrin subunit initiates inside-out signalling to result in the dissociation of the salt bridge linking integrin subunits. After the dissociation of the salt bridge, talin is associated with actin

cytoskeleton inducing the binding affinity of integrin to ligands (Hynes, 2002). This demonstrates the critical role of talin in integrin activation and conformational changes which has been documented upon knockdown of the adaptor protein (Tadokoro et al., 2003). Talin can induce integrin conformation in the absence of integrin ligand, mechanical force or clustering to modulate integrin binding affinity (Ye et al., 2010).

The critical function of αVβ3-integrin and α5β1-integrin has been demonstrated in focal adhesion maturation, turnaround (as explained above) and angiogenesis (Brooks et al., 1994; Morgan et al., 2013; Rocha et al., 2018). Overall integrins participate in providing a physical link between the intracellular cytoskeletal contractile machinery and ECM generating traction force for migrating fibroblasts. Deletion of either subunit of integrin is associated with lethal effect; for example, deletion of β1-integrin in mice results in foetal death even before vascular development (Carlson et al., 2008; Mamuya et al., 2017). Moreover, β1-integrin excision from mice endothelial cells has led to abnormal vascular development by embryonic day 8.5 with severe vascular defects and death by embryonic day 11.5. Deletion of α-integrin is associated with extensive vascular and mesodermal defects leading to death (Yang et al., 1993) and deletion of αV-integrin subunits lead to premature death as a result of severe cerebral haemorrhage and placental defects (Bader et al., 1998; McCarty et al., 2002). Therefore, understanding the coordination between syndecans and integrin is crucial to develop our understanding of many cellular processes.

1.2.5 Cooperation of syndecan-4 with integrin

Syndecan-4 and integrin can act as receptors for ECM and the coordination of both is critical to promote focal adhesion and cell migration (Bass et al., 2007; Oh et al., 1997b; Woods et al., 2000). Fibronectin release from injured blood vessel stimulates fibroblast polarisation and migration toward site of injury by the regulation of syndecan-4 and integrin. Although fibronectin can activate both receptors, each has a different binding site on fibronectin. αVβ3- and α5β1-integrin bind Arginine-Glycine-Aspartate tripeptide sequence of fibronectin (refers to as RGD motif), but the affinity of α5β1-integrin is increased by binding an additional site adjacent to RGD motif containing Phe-His-Arg-Ser-Asp sequence (refers to as PHSRN motif) (Danen et al., 1995; Pierschbacher & Ruoslahti, 1984). The combination of both motifs is known as cell binding domain to which the binding of α5β1-integrin results in high binding affinity that is required for cell adhesion. Syndecans can bind to Type III repeats 12-15 of fibronectin while Type III connecting region (IIICS) of fibronectin is thought to contain GAG binding site or facilitate the binding of syndecans. Integrin binding motif is composed of Type III repeats

6-10 of fibronectin. (Mostafavi-Pour et al., 2001). Figure 1.7 shows fibronectin structure and binding sites for both receptors.

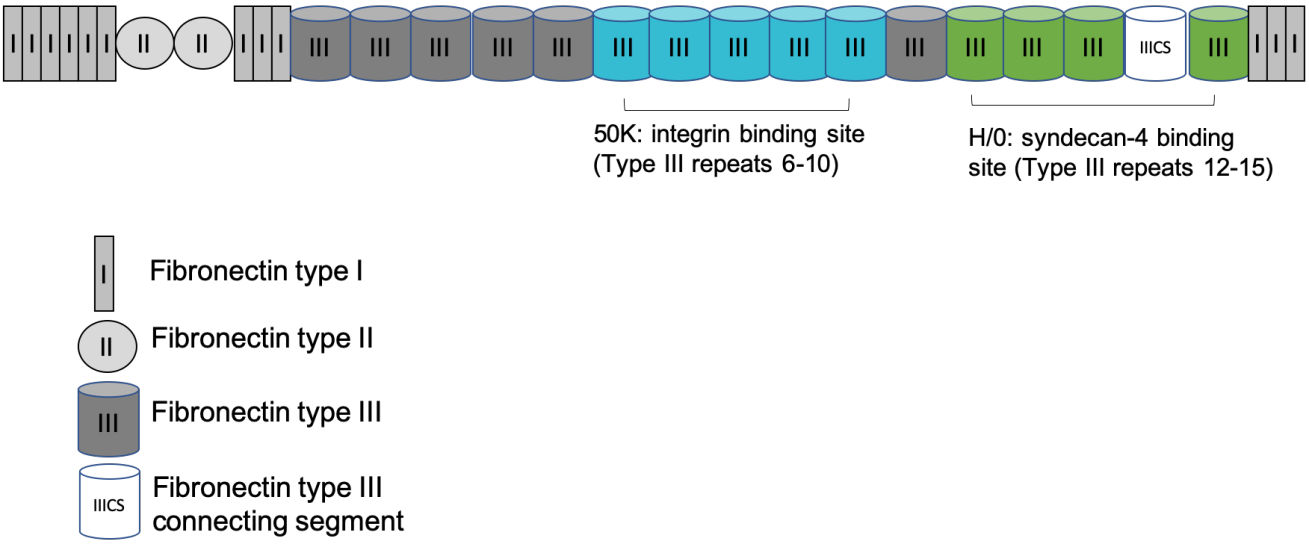


Figure 1.7: Structure of fibronectin and binding sites. Fibronectin is composed of 3 types of fibronectin repeats: 12 repeating units of Type I, two repeating units of Type II and 15 repeating units of Type III. Integrin binding motif is composed of Type III repeats 6-10, while syndecan-4 is composed of Type III repeats 12-15.

Both integrin and syndecan-4 co-interact together and both are required for efficient wound healing despite the conflict in the literature. For example, fibronectin engagement with integrin alone is not sufficient to promote integrin clustering or focal complex formation (Bass et al., 2007a; Bass et al., 2007b; Hotchin & Hall, 1995; Morgan et al., 2007). During cell spreading, integrin engagement can mediate cell spreading following fibronectin engagement but vinculin-containing focal adhesion and actin cytoskeleton rearrangement require additional signalling that is generated by syndecan-4 receptor activation (Bass et al., 2007a; Woods & Couchman, 1994). Therefore, understanding the relationship and interaction between syndecan-4 and integrin is essential to modulate cellular cues including cell spreading, migration and wound contraction during wound healing.

1.2.5.1 Syndecan-4 regulates integrin during wound healing

Both syndecan-4 and integrin in addition to other mediators are found at focal adhesion recruiting other proteins during wound healing. It has been reported by our lab that syndecan-4 initiates the process of wound healing by receptor engagement with fibronectin and regulates the internalisation of α5β1-integrin in a PKCα, Rac1 and Rho-dependant mechanism. Receptor engagement leads to PKCα binding to the V region of syndecan-4 cytoplasmic domain activating PKCα. The activation of PKCα results in the activation Rac1 and Rho GTPases (explained above in 1.2.3.1). Syndecan-4-dependant activation of RhoG and PKCα triggers the internalisation of α5β1-integrin by dynamin and caveolin-

dependant manner. PKCα phosphorylates RhoGDI and p190RhoGAP inhibiting RhoA that cycles between RhoA suppression followed by reactivation to allow membrane protrusions and stabilise focal adhesion (see above).

Syndecan-4 regulates surface expression of integrin for stabilisation and disassembly of focal adhesion through Arf6, an event that is essential for cell migration. Syndecan-4 phosphorylation by c-Src (a member of Src family kinases involved in cell movement and proliferation) has demonstrated to drive the endocytosis and degradation of α5β1-integrin. This phosphorylation leads to Arf6 inhibition and syntenin binding to the syndecan-4 cytoplasmic domain (explained above) mediating α5β1-integrin endocytosis, degradation and upregulation of αVβ3-integrin at the cell surface during syndecan-4-regulated-integrin trafficking to stabilise focal adhesion (Morgan et al., 2013). The role of syndecan-4 phosphorylation on cell migration has been demonstrated in fibroblasts spread on fibronectin with non-phosphorylatable syndecan-4 mutant showing blocked cell migration, while syndecan-4 phosphomimetic mutant showed induced migration, an event that is tightly controlled by establishing the role of syndecan-4 as integrin regulator rather than a co-receptor as demonstrated in early studies (Morgan et al., 2013). Arf6 activity during integrin trafficking is regulated by syndecan-4 phosphorylation for efficient cell migration. Cell migration has been decreased in fibroblasts expressing syndecan-4 wild type, phosphorylatable and non-phosphorylatable syndecan-4 upon expressing Arf6T157A (fast-cycling and constitutively active mutant mediating α5β1 upregulation and αVβ3 suppression). Cell migration in the phosphomimetic mutant has been restored upon rescue with Arf6 establishing syndecan-4 phosphorylation as a control point in the regulating integrin trafficking and adhesion dynamics in an Arf6-dependant manner during cell migration (Morgan et al., 2013).

1.3. Endocytosis of surface receptors

Endocytosis is a mean of internalising materials from extracellular to intracellular compartment via forming a vesicle surrounding the materials to be endocytosed. The vesicle is then pinched off to undergo endosomal trafficking and sorting to determine the fate of internalised material. Endocytosis is essential to maintain not only membrane homeostasis but also regulates receptor availability, ligand accessibility and signalling including integrin, which is involved in cell migration. Syndecan-4 regulates cell migration and stress fibre organisation by endocytosis to switch the type of integrin on cell surface. For example, syndecan-4 receptor can regulate cell motility by activating PKCα and RhoG triggering α5β1-integrin endocytosis and upregulating αVβ3 recycling in order to stabilise focal adhesion and promote directional motility (Morgan et al., 2013). Clathrin and caveolin endocytosis are two heavily

studied endocytic pathways that regulate cellular signalling and understanding the two mechanisms could help in developing migration- and delivery-based therapeutics for chronic wounds.

1.3.1 Clathrin-mediated endocytosis

Clathrin-mediated endocytosis is referred to as receptor-mediated endocytosis and it involves recruitment of adaptor protein (AP-2) and clathrin subunits (triskelia). Assembly of clathrin triskelia is guided by AP-2 forming a lattice-like structure at the inner side of the plasma membrane localising lattice formation to specific sites at the plasma membrane. AP-2, cargoes and clathrin lattice interact with PIP2 and synaptotagmin (type I transmembrane trafficking protein) at plasma membrane. This interaction is stabilised by epsin and AP180/CALM which are proteins involved in forming membrane curvature, linking clathrin vesicles to the plasma membrane and regulating coated pit formation, respectively. As more triskelia assemble, additional proteins are recruited to maintain a small circular-like curvature at the site of assembly known as clathrin-coated pits. Bin/Amphiphysin/Rvs- (BAR) containing proteins including endophilin, SNX9 and amphiphysin are recruited to bind clathrin and AP-2 to maintain membrane curvature. Dynamin is recruited and bind PIP2 causing further constriction of the mature clathrin-coated pit which leads to scission of the nascent clathrin-coated vesicle to be internalised and trafficked within the cell (Chen et al., 2011; Doherty & McMahon, 2009; Kumari et al., 2010; Sigismund et al., 2008; Young, 2007). Figure 1.8 summarises clathrin-mediated endocytosis.

1.3.1.1 Clathrin-mediated endocytosis of Integrin

Integrin heterodimers have been reported to be regulated by the two mentioned endocytic pathways. Integrin cytoplasmic domain or ectodomain can interact with a ligand promoting integrin internalisation via clathrin endocytosis. Integrin internalisation via clathrin-mediated endocytosis involves recruiting adaptor proteins at the plasma membrane for vesicle internalisation (as explained above). AP-2, Dab2 and Numb are common clathrin-mediated adaptor proteins have been reported to bind cytoplasmic NPxY motif of β-integrin to mediate endocytosis by this route. Clathrin-mediated endocytosis of integrin endocytosis is regulated by the adaptor proteins AP-2 and Dab2. A study has proposed that Dab2 is dependent on AP-2 to regulate integrin internalisation (Teckchandani et al., 2009). For example, AP-2 and Dab2 independent knockdown has resulted in increased α1- and β1-integrin surface expression which has been increased further when both adaptors were combinedly removed demonstrating that Dab2 requires AP-2 (Moreno-Layseca et al., 2019; Teckchandani et al., 2009).

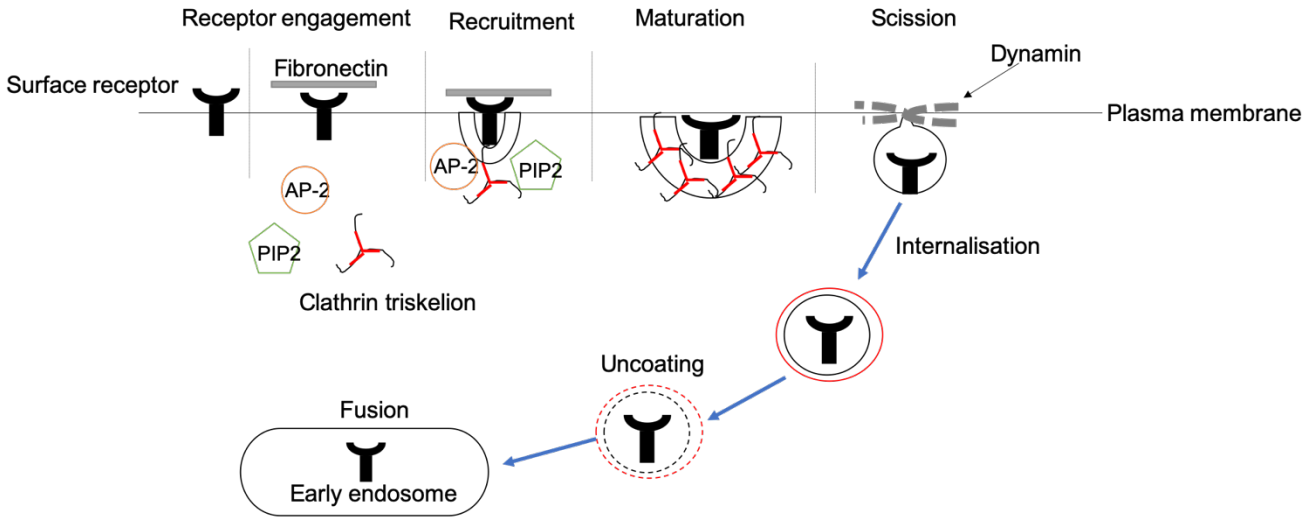


Figure 1.8: Summary of clathrin-mediated endocytic mechanism. Engagement of receptor with a ligand initiates the recruitment of adaptor protein-2, PIP2 and clathrin triskelia forming clathrin-coated pits. The clathrin-coated pits undergo maturation and stabilisation via BAR domain proteins by promoting cytoskeletal reorganisation to maintain membrane curvature. Dynamin assembles at the neck of the invagination and applies constriction to mature clathrin-coated vesicle releasing it for intracellular trafficking. The clathrin-coated vesicle is uncoated and fused to a sorting early endosome where it will be directed further for recycling, lysosomal degradation or modification in trans-Golgi network. (AP-2=adaptor protein-2).

Additionally, surface expression of α1-, α2-, α3- and β1-integrin has been increased in Dab-2 deficient cells while α5- and αV-integrin has been not changed suggesting a regulatory role of clathrin and Dab-2. (Teckchandani et al., 2009; Yu et al., 2015). Dab-2 is localised to the mid region of migrating cells hence associates with clathrin and AP-2 to bind inactive and unbound β1-integrin to ECM or actin cytoskeleton facilitating cell migration. The mechanism of regulating cell migration via clathrin-mediated endocytosis of integrin has been demonstrated by Dab-2 removal in mouse endothelium and human umbilical vein endothelial cells spread on collage IV and vitronectin resulting in blockade of cell migration on collagen IV indicating a specificity role of Dab-2 by mediating the trafficking of collagen-bound integrin not vitronectin (Orlandini et al., 2008). This can be explained by the type of integrin involved as collagen IV is a ligand for α1β1-integrin while vitronectin is a ligand for αVβ5-integrin demonstrating that clathrin-mediated endocytosis can regulate integrin trafficking and therefore involves in cell migration (Nishimura & Kaibuchi, 2007; Teckchandani et al., 2009; Upla et al., 2004).

Numb is another adaptor that regulates clathrin-mediated endocytosis and its function has been demonstrated by colocalising with AP-2 at clathrin-coated pits, early and late endosomal compartments (Santolini et al., 2000). Numb function has been investigated in integrin demonstrating its critical role during cell migration by regulating directional integrin trafficking in migrating cells (Nishimura & Kaibuchi, 2007). Moreover, Numb can bind and internalise α5-, αV and β1-integrin at the leading edge of migrating cell while Dab2 regulates α1, α2 and α3. However, Numb knockdown has

affected cell migration causing impaired directional migration which results from reduced endocytosis of β1- and β3-integrin (Moreno-Layseca et al., 2019; Nishimura & Kaibuchi, 2007; Teckchandani et al., 2009; Yu et al., 2015). In addition to Numb and Dab2, the cytoplasmic tail of integrin containing NPXY motif can interact with a plethora of adaptor proteins including talin. A mutation within the intracellular domain of β3-integrin tail has abolished the binding of Numb and talin to integrin implying that they may not bind to integrin at the same time (Calderwood et al., 2003). This conclusion could be true as a study has shown that no clear interaction between the two adaptor protein has been observed (Nishimura & Kaibuchi, 2007). There are some reports suggest that Numb could recruit some adaptors, including Dab2, to focal sites which may contribute to integrin internalisation and recycling. Dab2 binds the same motifs as Numb regulating integrin-based cell adhesion and spreading. Numb is preferentially localised behind lamellipodia and trailing edge, while Dab2 is localised to the apical surface suggesting coordination between the two adaptors to regulate integrin internalisation and recycling during cell migration (Morris & Cooper, 2001).

1.3.2 Caveolin-mediated endocytosis

Caveolae are hydrophobic flask-shaped, invagination of plasma membrane rich in cholesterol, sphingolipids, and oligomeric caveolin. Caveolin-1 and caveolin-2 are ubiquitously expressed and required for caveolae formation (Fra et al., 1995). However, several in vivo and in vitro studies have reported absence of caveolae structure in endothelial and epithelial cells of caveolin-1 knockout mice. Caveolin-2 expression disappears when caveolin-1 is knockout or knockdown demonstrating its key role in caveolae formation (Drab et al., 2001; Fra et al., 1995; Glenney, 1989; Razani et al., 2001). Caveolin-3 is limited to muscle cells, including smooth, cardiac and skeletal muscle (Tang et al., 1996; Way & Parton, 1995), and its expression has not changed in caveolin-1 knockout mice when compared to caveolin-1 and caveolin-2. Ultrastructural studies have introduced additional proteins required for caveolae formation and stabilisation refers to as cavins: cavin-1, cavin-2, cavin-3 and cavin-4 (Liu & Pilch, 2008). Similar to caveolin-1, cavin-1 has been demonstrated to have an essential role in caveolae biogenesis. Mammalian cells and zebrafish have demonstrated lower caveolae formation when cavin-1 is perturbed (Hill et al., 2008). Cavin-1, cavin-2 and cavin-3 are ubiquitously expressed while cavin-4 is muscle specific (Bastiani et al., 2009; Gustincich et al., 1999; Hill et al., 2008; Izumi et al., 1997). Cavin-2 and cavin-3 are thought to play a role in recruiting PKC to the site of caveolae. Since cavin-4 is muscle specific, it has been demonstrated to play a role in skeletal muscle differentiation (Bastiani et al., 2009). PKCα is one of the proteins that has been co-purified with caveolae suggesting to be involved in caveolin-protein ligand interaction (Oka et al., 1997). Caveolar endocytosis has been implemented in regulating cell signal transduction, mechanosensing and cholesterol metabolism.

Upon receptor engagement, oligomeric caveolin-1, cholesterol, sphingolipids, PIP2 and cavins, are recruited to form and stabilise the flask-shaped invagination. Actin stress fibre and filamin (actin-binding protein) interact directly linking oligomeric caveolin-1 at the cell surface to the cellular cytoskeleton (Stahlhut & Van Deurs, 2000). Budding of caveolae is mediated by actin reorganisation, dynamin and PKCα activity (Le Lay & Kurzchalia, 2005; Smart et al., 1995). Similar to clathrin-mediated endocytosis, dynamin is recruited at the surface of the flask-shaped invagination applying further constriction pinching off caveosome, neutral pH compartment with no lysosomes, to the intracellular compartment for vesicular trafficking (Pelkmans et al., 2004). The internalisation of caveolae is sensitive to cholesterol depletion and dynamin inhibition (Kumari et al., 2010). Figure 1.9 summarises caveolin-dependant endocytosis.

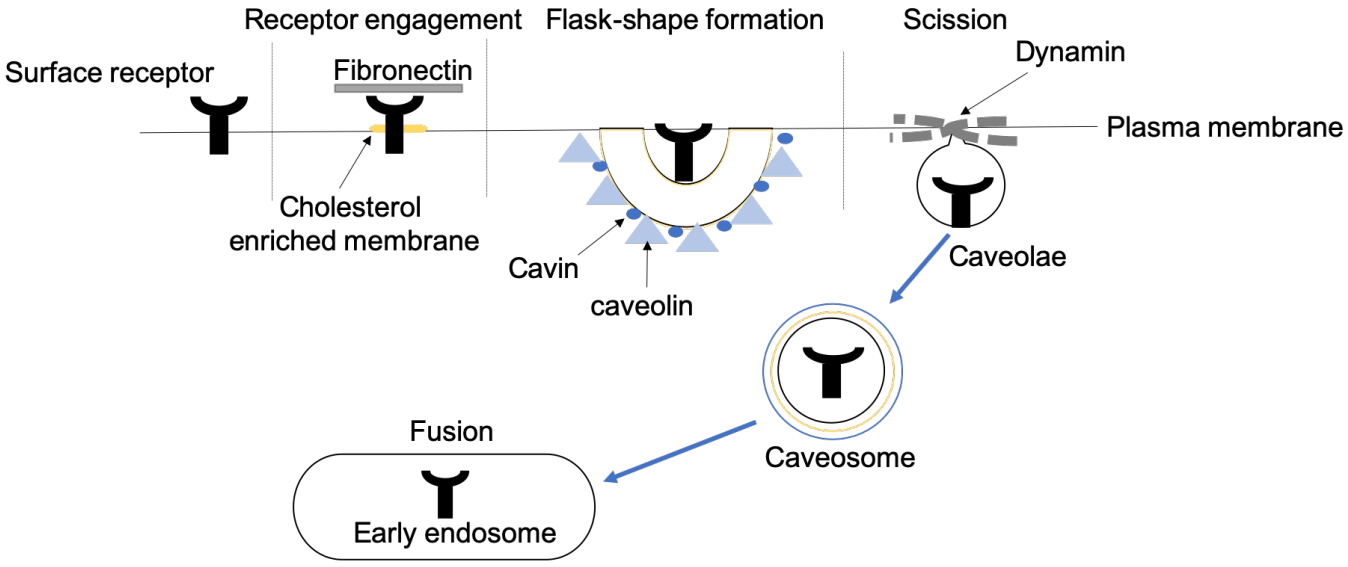


Figure 1.9: Summary of caveolin-dependant endocytic mechanism. Engagement of receptor with a ligand creates cholesterol-enrich flask-like invagination at the plasma membrane containing caveolin and cavin. Dynamin assembles at the neck of the invagination and applies constriction pinching off nascent caveolae for intracellular sorting. Caveosmoe is fused to a sorting early endosome where it will be directed further for recycling, lysosomal degradation or modification in trans-Golgi network.

1.3.2.1 Caveolin-mediated endocytosis of integrin

Integrin and focal adhesion machinery have been associated with caveolin-mediated endocytosis to regulate integrin-based cell processes including cell spreading and migration. Numerous studies in the literature have documented the role of caveolin in regulating integrin surface expression of α5β1 and αVβ3 integrin during cell migration of endothelial cells (Bass et al., 2011; Brooks et al., 2012; Gálvez et al., 2004; Morgan et al., 2013). α5β1 and αVβ3 integrin subunits have been associated with caveolae (Shi & Sottile, 2008). Syndecan-4 play a key role in regulating α5β1-integrin endocytosis by caveolar endocytosis and requires the activity of PKCα and Rho family GTPases to internalise caveolae

containing α5β1-integrin (mentioned earlier). This is a direct link between syndecan-4 and α5β1 integrin internalisation by caveolar endocytosis (Bass et al., 2011). Switching integrin surface expression from α5β1-integrin to αVβ3-integrin in order to stabilise focal adhesion is tightly controlled by syndecan-4 dependant activation of Arf6 (Bass et al., 2011; Morgan et al., 2013).

1.3.3 Clathrin- and caveolin-independent endocytosis

This is a wide area in research where many endocytic pathways can fit including the actin cytoskeletal-dependant macropinocytosis. Stimulating fibroblasts with growth factors released during skin injury (including platelet-derived growth factor) can induce cell migration by circular membrane ruffling macropinocytic mechanism recruiting and triggering integrin endocytosis by a mechanism that is not-caveolin nor clathrin-dependant (Gu et al., 2011; Mellström et al., 1988).

Cell surface proteins that are anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI) can be internalised via clathrin- and caveolin-independent mechanism (Bamezai et al., 1992; Sabharanjak et al., 2002). GPI-anchored proteins have been found in all tissue and cells examined in mammals (Chatterjee & Mayor, 2001). α2β1-integrin is an example of integrin that has been associated with a lipid raft rich in GPI (Mellström et al., 1988; Upla et al., 2004). Due to the nature of GPI-linked proteins, it is postulated that those proteins are internalised differently compared to transmembrane linked proteins. Some studies have investigated the endocytic pathway of multiple proteins anchored to GPI by using fluorescently labelled ligand to detect internalised target revealing that GPI-anchored proteins have been detected in the same endocytic compartment as an internalised marker of fluid phase or recycling endosome (Chatterjee & Mayor, 2001; Mayor et al., 1998). While other reports have demonstrated that GPI-anchored proteins are internalised directly to Golgi (Nichols et al., 2001). However, other research groups have demonstrated that labelled GPI-anchored proteins are internalised in caveolin- and clathrin-independent manner named (GPI-anchored proteins enriched early endosomal compartment) but not to Golgi (Sabharanjak et al., 2002). The internalisation of multiple GPI-anchored protein has been demonstrated to be regulated by cdc42 (Sabharanjak et al., 2002), a small GTPase of the Rho family involved in regulating signalling cues including cell migration (Nobes & Hall, 1995)

Interleukin-2 receptor β is a receptor that is internalised in caveolin- and clathrin-independent manner and has been demonstrated to be regulated by RhoA and Rac1 unlike GPI-anchored proteins enriched early endosomal compartment (Lamaze et al., 2001). Arf6-dependant endocytosis is dynamin independent and endosomal compartment has been demonstrated to contain GPI-anchored protein

(Naslavsky et al., 2004). Depletion of membrane cholesterol using filipin has shown to block Arf6-dependant endocytosis suggesting that Arf6 endocytosis is cholesterol dependant (Naslavsky et al., 2004). Flotillin-mediated endocytosis is another clathrin-independent endocytosis and has been thought to be associated with caveolin-mediated endocytosis. Flotillin assembly induces plasma membrane invagination similar to caveolae implying that it is part of it. However, a study has demonstrated that flotillin is distinct from caveolae and not enriched within the caveolar structure (Frick et al., 2007; Glebov et al., 2006). Clathrin- and caveolin-independent mechanisms have been reported in the literature suggesting that such alternative mechanisms may play a role in signal transduction (Spoden et al., 2008). However, they are poorly characterised and required additional investigations to define them. For example, two studies have demonstrated an internalisation mechanism that was independent of dynamin, caveolin and clathrin (Damm et al., 2005). Hence, clathrin- and caveolin-independent entry of ligands to intracellular compartment could be beneficial to improve therapeutic drug delivery for treatment of cancer and other diseases.

1.4 Constitutive and ligand dependant endocytosis of integrin

Changes of ECM play a role in surface receptor expression and availability to exert a biological effect. Transferrin receptor, which is known to be regulated by clathrin-mediated endocytosis, is one of the early examples of surface receptors that can be endocytosed to be recycled back to the cell surface or degraded without any changes in ECM (Hopkins et al., 1985; Watts, 1985). Such a ligand-independent endocytic process is referred to as constitutive endocytosis applied to replenish material on the cell surface. Other receptors, on the other hand, require ligand binding and are sensitive to changes at ECM including PDGF (Sorkin et al., 1991). The internalisation of such receptors is induced after receptor engagement triggering rapid ligand-bound receptor uptake within 5-10 minutes (Hopkins et al., 1985; Stoscheck & Carpenter, 1984). This type of endocytosis is referred to as ligand-dependant endocytosis.

A study has been demonstrated that caveolae are not involved in constitutive endocytosis suggesting that caveolae are internalised upon ligand binding only (Thomsen et al., 2002). This was demonstrated by the internalisation of α5β1-integrin post ligand binding by caveolar endocytosis. However, some reports have demonstrated constitutive endocytosis of α5β1-integrin in the absence of fibronectin and fibronectin matrix providing more diversity for the mechanosensing receptor (Bass et al., 2011; Rocha et al., 2018; Shi & Sottile, 2008). Syndecan-4 engagement has been reported to initiate downstream signalling to regulate caveolar endocytosis of α5β1-integrin demonstrating ligand-dependant endocytosis as explained above. Moreover, extracellular engagement of integrin drives receptor clustering and recruits adaptors for clathrin-mediated endocytosis, including AP-2, Dab2 and Numb as

explained previously, demonstrating ligand-dependant endocytosis via clathrin-mediated endocytosis (Nishimura & Kaibuchi, 2007; Shemesh et al., 2005; Yu et al., 2011).

Taken together, integrin undergoes receptor uptake by both caveolin and clathrin endocytic pathways and can be internalised in a constitutive- and ligand-dependant manner. Although some of integrin-downstream effect has been developed based on syndecan-4 or ECM engagement with integrin, it is not clear how syndecan-4 is regulated, nor internalised exposing a gap that is critical to understand in order to appreciate the coordination between integrin and syndecan-4 during skin injury to achieve proper wound healing.

1.5 Recycling of internalised integrin vesicles

Following receptor endocytosis, the integrin-containing vesicle is fused with early endosome to be sorted and directed further. The majority of integrin vesicles are recycled to the plasma membrane, but some vesicles undergo lysosomal degradation as demonstrated in migrating cells where vesicles containing α5β1-integrin are transported to late endosome for lysosomal degradation (Lobert et al., 2010; Shi & Sottile, 2008). There are two small GTPases that contribute to integrin trafficking and recycling: Rab and Arf. Rab5 is the first mediator that directly interacts with integrin-containing vesicles at early endosome fusing plasma membrane with the newly pinched off vesicle (Arriagada et al., 2019; Christoforidis et al., 1999; Mendoza et al., 2013). Rab4 is another GTPase that is found in early endosome and can interact directly with Rab5 to recycle integrin vesicles back to the cell surface via a fast short loop in a Rab4-dependant manner, or traffic integrin vesicle via slow long loop to perinuclear recycling compartment before recycling back to the plasma membrane in Rab11 dependant manner (Caswell & Norman, 2006; Moreno-Layseca et al., 2019).

During cell migration, αVβ3-integrin is recycled to the plasma membrane via short loop bypassing perinuclear compartment. Whilst both α5β1- αVβ3-integrin can enter the longer loop via perinuclear compartment in unstimulated fibroblast, stimulation with platelet-derived growth factor can trigger αVβ3-integrin recycling via Rab4 short loop by exiting the endosome. Whereas α5β1 is recycled via Rab11 long loop. To exit the early endosome without entering Rab11 compartment, PKC-related kinase (PRKD1) phosphorylates Rab5 effector protein (rabaptin-5) forming a complex with Rab4 that drives αVβ3-integrin delivery to the leading edge of migrating fibroblast to maintain cell motility and αVβ3-integrin-dependant invasion in low fibronectin settings (Christoforides et al., 2012; Woods et al., 2004). Additional study has demonstrated fast short loop recycling of αVβ3-integrin via interaction of Rab4, Rab5 with the actin and myosin-binding protein supervillain (Fang et al., 2010). Genetic perturbation of PRKD1 or Rab4 effector protein (Rab IP4) that are associated with αVβ3 recycling has blocked αVβ3-

integrin recycling, reduced cell adhesion, spreading and increased α5β1 recycling. This suggests that fast short loop of αVβ3-integrin recycling does not directly interfere with cell motility by increasing recycling of αVβ3 to the leading edge, but rather antagonising the recycling of α5β1-integrin which affects directional migration of stimulated cells (Vukmirica et al., 2006; White et al., 2007).

α5β1 integrin passes first to Rab4 compartment before Rab11 of the perinuclear compartment. One of the Rab11 effector protein is Rab coupling protein, also known as Rab11 family of interactive proteins (Rab11-FIPs), which controls the recycling of α5β1 integrin via interacting with Rab11 and Arf6. The increase of α5β1 integrin can be demonstrated by inhibiting the adhesive function of αVβ3 using cilengitide, soluble cyclic RGD ligand, leading to Rab coupling protein-dependant recycling of α5β1 integrin to the cell surface. The association of the effector protein with integrin promotes random cell migration on 2D and directional migration on 3D matrices proposing a role of Rab coupling protein and Rab11 in regulating cell migration by controlling α5β1 recycling (Caswell et al., 2008).

Arf6 has been demonstrated to associate with integrin recycling from the perinuclear compartment of the long loop cycle back to the plasma membrane. Inactive β1-integrin is recycled via a short loop, similarly to αVβ3-integrin, in Arf6-positive protrusions at the plasma membrane in a Rab4 dependant mechanism, while active β1-integrin is recycled via Rab11 long loop. The active β1-integrin is thought to require more for time for ligand dissociation and has been observed in Rab7 late lysosomal compartment. However, a study has reported that active α5β1-integrin and bound ligand complex has been observed exiting late endosomal compartment and recycled back to the plasma membrane of the invading cancer cells via Rab25 (Arjonen et al., 2012; Dozynkiewicz et al., 2012; Lobert et al., 2010).

1.6. Regulation of endocytosis by syndecan-4

Switching integrin surface expression is regulated by the phosphorylation status of syndecan-4 and Arf6 activity. The phosphorylation of syndecan-4 inhibits Arf6 activity, explained earlier, which plays a role in stabilising focal adhesion and maintaining persistent directional migration of fibroblasts via increasing αVβ3 integrin recycling and inhibiting Arf-6 dependant recycling of α5β1 integrin (Morgan et al., 2013). Moreover, phosphorylation of syndecan-4 facilitates the binding of syntenin and PIP2 to syndecan-4 cytoplasmic domain inhibiting Arf6 activity to achieve the same outcome, degrading α5β1 integrin and recycling αVβ3 integrin. Interfering with PIP2 binding syntentin has been demonstrated to result in syndecan-4 and β1-integrin accumulation in the perinuclear compartment affecting cell spreading (Grootjans et al., 1997; Zimmermann et al., 2001; 2005). Figure 1.10 summarises integrin internalisation and recycling.

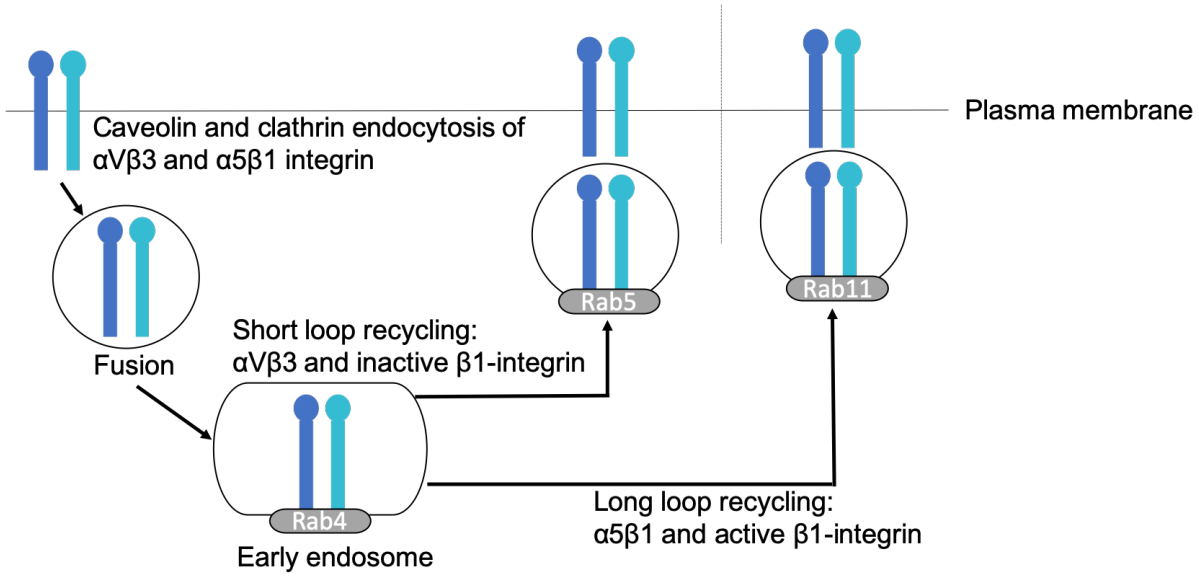


Figure 1.10: Summary of integrin internalisation and recycling. Both α5β1 and αVβ3 are internalised by caveolin and clathrin dependant mechanisms. Upon fusion of integrin containing vesicle with early endosome, αVβ3 and inactive β1 integrin are recycled back to the plasma membrane via Rab5 short recycling loop to stabilise focal adhesion and maintain directional migration. The long recycling loop of α5β1 and active β1 occurs via Rab11.

Sorting nexins are family of cytosolic and membrane-bound proteins with FERM-like domain. Sorting nexins contain phosphoinositide-binding motif and have been demonstrated to be involved in cellular tracking and retrieval of internalised transmembrane receptors from the lysosomal compartment. The sorting nexin 17 (SNX17) has been investigated in our research group and has been reported to colocalised almost completely with early endosomal compartments (EEA1/Rab4 positive compartment) containing internalised transmembrane receptor, not late endosome compartment. The role of SNX17 in regulating integrin recycling has been established in HeLa in which SNX17 has been associated with β1- and β5-integrin and rescue the internalised vesicle from lysosomal degradation compartment. The rescue of integrin has been demonstrated by binding of membrane distal NPXY motif of β-integrin with the FERM-like domain of SNX17 in the early endosomal compartment (EEA1/Rab4 positive) preventing entry into the degradation pathway mediating α5β1 integrin recycling (McNally et al., 2017; Steinberg et al., 2012).

Syndecan-4-dependant activation of PKCα following receptor engagement regulates multiple cellular events in a syndecan-4 dependant manner. Protein kinase C and casein kinase substrate in neurons protein 2 (PACSIN2) is a surface membrane sculpting F-BAR domain-containing protein that is regulated by PKCα and is tightly linked to caveolar endocytosis (Senju et al., 2015). PACSIN2 is essential for caveolae structure and has been reported to colocalised with caveolin-1 at caveolae (Senju et al., 2011). Hence, knockdown of PACSIN2 has resulted in abnormal plasma membrane shaped invagination

demonstrating the central role of PACSIN2 in inducing membrane tubulation and caveolae structure. PKCα is enriched at caveolae and phosphorylates PACSIN2 at serine 313 recruiting dynamin-2 at the cell surface to apply constriction and scission of the vesicle for intracellular trafficking (Senju et al., 2011; Smart et al., 1995). PKCα is activated by syndecan-4, but it is not known if PACSIN2 is linked to syndecan-4 endocytosis.

PKCα cooperates the actin-binding protein filamin which links the actin cytoskeleton to the plasma membrane. Binding of filamin C-terminus to β-integrin can localise filamin to focal adhesion through the interaction with focal adhesion associated proteins including vinculin and talin (Calderwood et al., 2001). The fact that PKCα is localised to focal adhesion has driven many investigations to study the involvement of PKCα with filamin. PKCα has been demonstrated to phosphorylate filamin which is directly linked to the caveolin endocytic mechanism (Ohta et al., 1999; Smart et al., 1995). Similar to PACSIN2, filamin stabilises caveolae at the plasma membrane by linking it to the actin cytoskeleton. PKCα phosphorylation of filamin leads to detachment and inward trafficking of caveolae (Muriel et al., 2011). Filamin can bind to integrin cytoplasmic domain and interact with PKCα but there is no evidence of filamin association with syndecan-4.

Syndecan-4 cytoplasmic domain contains a conserved region (C1) which is similar in all syndecans containing MKKK motif. This motive has been documented to play a role in syndecan clustering and endocytosis. Syndecan-1 has been documented to be internalised after inducing receptor clustering with a ligand (Chen & Williams, 2013). The internalisation of syndecan-1 has several key elements including MKKK motif, extracellular signal-regulated kinases (ERK; member of the mitogen activated protein kinase family involved in transmitting extracellular signals to intracellular compartment which is involved in many vital processes including angiogenesis and cell proliferation), α-tubulin, Src family kinase and cortactin which all have been reported in not only syndecan-4-mediated events but other syndecans as well (Cizmeci-Smith et al., 1997; Corti et al., 2013; Elfenbein & Simons, 2013; Morgan et al., 2013; Rauch et al., 2005). Syndecan-1 has been reported to internalised in two phases with each phase requiring a kinase and a cytoskeletal protein (Chen & Williams, 2013). When syndecan-1 is not engaged with a ligand α-tubulin is attached to the receptor suggesting its role in anchoring syndecan-1 to the plasma membrane. However, syndecan-1 receptor engagement, as in syndecan-4, triggers receptor clustering and rapid activation of ERK localising the receptor into lipid rafts. This interaction is MKKK-dependant as replacing the motive has abolished ERK phosphorylation. Phosphorylation of syndecan-1 transmembrane and cytoplasmic domain by Src family mediates receptor clustering and recruits cortactin, an intracellular protein linked to endocytosis and cell migration by modulating the

polymerisation and rearrangement of the actin cytoskeleton (Kinnunen et al., 1998), to promote endocytosis. Cortactin-Src-syndecans signalling pathway is well established in endocytosis and cell migration (Afratis et al., 2017) and inhibiting cortactin by small interference RNA (siRNA), receptor clustering or Src can block cell endocytosis and cell migration (Chen & Williams, 2013). Since syndecans share C1 region and all the mediators have been reported in syndecans-downstream signalling, it is possible that syndecan-4, as in syndecan-1, could be internalised in the same manner.

Arginine-rich cell-penetrating peptides (arginine-rich CPP) are molecules that can be used to deliver biologically active material including therapeutic drugs and siRNA to the intracellular compartment. These peptides have been utilised by many research groups to study endocytic mechanism demonstrating the internalisation of these molecules in a caveolin- (Fittipaldi et al., 2003), clathrin-dependant (Richard et al., 2005) and caveolin- and clathrin-independent manner (Nakase et al., 2004). HSPG has been demonstrated to internalised octa-arginine (R8), member of the arginine-rich CPPs (Richard et al. 2005). Syndecan-4 is one of the HSPG which have been demonstrated to regulate the internalisation of these peptides in a clathrin-dependant manner (Kawaguchi et al., 2016). The role of syndecan-4 in internalising these peptides have been investigated by syndecan-4 overexpression demonstrating an increase in internalised peptides while genetic knockdown of the receptor has resulted in decreased endocytic peptides suggesting that syndecan-4 is central for internalising R8. Inhibition of clathrin by using siRNA and pharmacological inhibitors have reduced endocytic R8 whereas caveolin and macropinocytosis inhibitors have no effect on internalised arginine-rich CCP concluding that R8 endocytosis is clathrin-mediated. Moreover, syndecan-4 has demonstrated colocalisation with R8 confirming the direct association of R8 and syndecan-4-mediated endocytosis (Kawaguchi et al., 2016). Collectively, there is a direct link between syndecan-4 and clathrin-mediated endocytosis which could contribute to receptor endocytosis.

Syndecan-4 ectodomain has been reported to bind R-spondin 3 (a member of secreted R-spondin family which modulate Wnt signalling) with high affinity promoting clathrin-mediated endocytosis of R-spondin 3 to induce non-canonical Wnt signalling (Ohkawara, Glinka & Niehrs 2011). R-spondins have a critical role during embryonic development and R-spondin 3 particularly has been demonstrated to be essential for the development of vasculogenesis and angiogenesis in mice and Xenopus (Aoki et al., 2007; Kazanskaya et al., 2008). Syndecan-4 receptor induces Wnt signalling via internalising the complex of syndecan-4 bound R-spondin 3 (Ohkawara, Glinka & Niehrs 2011). This has been demonstrated by inhibiting either syndecan-4 or R-spondin 3 using morpholinos directed against either one confirming the involvement of syndecan-4 in internalising R-spondin 3. Ohkawara research group

has demonstrated that R-spondin 3 is internalised in a clathrin-dependant manner by targeting AP-2 using morpholino and pharmacological inhibitors which blocked R-spondin 3 endocytosis. Indeed, depletion of caveolin using RNAi or pharmacological inhibitors has no effect on R-spondin 3 endocytosis confirming that clathrin is responsible for R-spondin 3 internalisation in a syndecan-4-dependant manner (Ohkawara et al., 2011).

Lastly, proteoglycan core protein synthesis is increased in cells overexpressing dynamin mutant, K44A, (Llorente et al., 2001). Those cells have increased PKC activity resulting from impaired clathrin-mediated endocytosis demonstrating that PKC regulates the synthesis of the proteoglycan core protein. The activity of PKC has been evaluated using BIM-I demonstrating that PKC activity has been increased in cells overexpressing dynamin mutant, which is correspondent to increased proteoglycan synthesis in cells with impaired clathrin-mediated endocytosis. Llorente research group has monitored the expression of syndecan-1, but not other syndecans, to report that cells overexpressing dynamin mutant have increased syndecan-1 expression compared to control (Llorente et al., 2001). It is plausible to link syndecan-4 expression to clathrin-mediated endocytosis since PKCα can bind syndecan-4 more specifically compared to syndecan-1 and monitor syndecan-4 expression when clathrin endocytic mechanism is impaired. To conclude, syndecan-4 has been reported to regulate endocytosis of many proteins and has been detected in endosomal compartment associating with both endocytic mediators, caveolin and clathrin, but the mechanism for regulating syndecan-4 itself is still unclear.

1.7. Summary

Syndecan-4 function as a sensor detecting minimal changes in the surrounding environment and regulates integrin mechanosensing function. Both receptors are found at focal contact recruiting mediators (including members of Rho GTPase family, PKCα, caveolin and clathrin) to control the dynamics of focal adhesion. Cells can spread on fibronectin following integrin engagement but cannot form focal adhesion as additional signalling is required by syndecan-4 engagement to form vinculin-containing focal adhesion demonstrating that cooperation of the two surface receptors is fundamental during wound healing (Bernfield et al., 1999).

Syndecan-4 regulates the dynamic of focal adhesion by controlling the surface expression of integrin and recycling. Syndecan-4 dependant switching of integrin surface expression via endocytosis and recycling of α5β1 and αVβ3 mediates the stability of focal adhesion as αVβ3 integrin stabilises the adhesion resulting in persistent and directional migration where α5β1 is responsible for weaker adhesion and random motility.

The cytoplasmic domain of syndecan-4 is unique in activating and localising PKCα to focal adhesion site providing specialised downstream signalling to Rho family GTPases regulating cell migration during wound healing. Defective cell migration is a hallmark observed in chronic wounds due to defective fibroblasts activation resulting in limited fibroblasts recruitment and differentiation, hence delay healing (Hinz, 2007). Syndecan-4-knockout fibroblasts fail to respond to fibronectin, while the knockout mice suffer delayed healing due to migration defect, establishing the importance of these molecules to the healing response (Bass et al., 2011; Echtermeyer et al., 2001). Thus, understanding fibroblast activation and receptor trafficking during wound healing are essential elements for the development of prospective healing therapeutics.

Syndecan receptor recycling is reported to occur in Rab11 and Arf6-dependant manner, pathways involved in integrin recycling. Interfering with the PDZ cytoplasmic-binding domain of syntenin can alter the binding of PIP2 to syndecan-2. Syntenin contains two PDZ domains: PDZ1 and PDZ2 where each domain can interact with PIP2 or syndecans. However, both are needed to form a complex where PDZ1 and PDZ2 bind PIP2 and syndecans, respectively regulating cell surface recycling and dynamics (Zimmermann et al., 2002). Interfering with syntenin cytoplasmic interaction can result in accumulation of both syndecan and β1 integrin at the perinuclear compartment demonstrating that syndecan is not only colocalised in the same vesicle for recycling but also regulates postinternalisation steps of integrin (Zimmermann et al., 2005).

In addition, syndecan-4 has been reported as a key regulator of carcinogenesis. Some studies have documented upregulation of syndecan-4 in breast cancer, hepatocellular carcinoma, mesothelioma (Roskams et al., 1998). The role of syndecan-4 in cancer is not fully understood and studies show contradictory outcomes. For example, in breast cancer, it has been reported that syndecan-4 is upregulated and associated with good prognosis in patients positive for oestrogen and progesterone receptors (Lendorf et al., 2011; Lim et al., 2015), while a different study reported that syndecan-4 is associated with poor prognosis showing significant syndecan-4 expression with high histological grade and negative oestrogen receptor (Baba et al., 2006). Furthermore, studies show that syndecan-4 can enhance or suppress tumour growth, invasion and progression according to tumour cells involved (Cavalheiro et al., 2017). Taken together, syndecan-4 plays a key role in cell migration, cell adhesion and cytoskeletal reorganisation, hence it is probable to contribute to tumour progression.

1.8 Project aims and objectives

Fibroblast activation during wound healing is critical to mediate cell signalling for cell migration and contraction. Syndecan-4 and integrin coordinate cell signalling by binding fibronectin from leaking blood vessel at the injury site. PKCα and Rho GTPases including Rac1 activate a wide range of mediators that drive cell polarisation, stress fibre rearrangement, and surface receptor redistribution that in turn lead to efficient cell migration to the site of injury. In chronic wounds, fibroblast activation is limited leading to a reduction in recruited and differentiated cells, hence the delay in healing. The role of syndecan-4 in wound healing has been established in the literature and the deletion of syndecan-4 results in a delay in healing. Some studies show how syndecan-4 regulates other receptors, like integrin, during skin injury, but the regulation of syndecan-4 itself has not been explored. Therefore, the focus of the project is testing syndecan-4 receptor trafficking and downstream signalling in order to help us understand its role in fibroblast activation and migration during wound healing to develop healing therapeutic strategies.

The aims of this project are as follow:

- To determine how syndecan-4 is endocytosed and how to trigger syndecan-4 endocytosis.
- To determine which mechanism mediates syndecan-4 endocytosis.
- To determine if syndecan-4 internalisation is ligand-regulated or constitutive.
- To determine if syndecan-4 regulates endocytosis of itself.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Stock and buffer solutions

All solutions were made up in distilled water unless otherwise specified

Buffer/solution	**Chemical composition**
10X Phosphate-buffered saline (PBS) pH 7.4	27mM KCl 1.4M NaCl 15mM KH_2PO_4 80mM Na_2HPO_4
10X Tris-buffered saline (TBS) pH 7.4 using HCl	100mM Tris-Base 140mM NaCl
TBS-Tween	1X TBS + 0.1% (v/v) Tween-20
10X Low molecular weight protein transfer buffer pH 8.0	192mM Glycine 25mM Tris-Base 10% Methanol
Western blot lysis buffer	10% Glycerol 20mM HEPES 140M NaCl 1% NP40 0.5% Sodium Deoxycholate 4mM EGTA 4mM EDTA 0.1% SDS 1X complete protease inhibitor (purchased from Roche #11697498001)
SDS-PAGE protein loading buffer	0.1M Tris-HCl, pH 6.8 15% Glycerol 2% SDS 0.01 Bromophenol Blue
Dulbecco PBS (with $MgCl_2$ and $CaCl_2$) (PBS^+)	Purchased from Sigma-Aldrich (#B8662)
Dulbecco PBS (PBS^-)	Purchased from Sigma-Aldrich (#B8537)
Bovine serum albumin (BSA)	Purchased from Thermo Fisher (#A9418)
H/0	Generated as described in (Sharma et al, 1999)

50K	Generated as described in (Sharma et al, 1999)
Biotin	B4501
Avidin	A9275

Table 2.1 Stock and buffer solutions.

2.1.2 Antibodies

Application: Western blot (WB), immunofluorescence (IF), Flow cytometry (FACS)

2.1.2.1 Primary antibodies

Target	Antibody source	Application	Supplier	Catalogue number
Syndecan-4	Human syndecan-4, goat polyclonal	IF (1:50 dilution)	Research and Diagnostic Systems	AF2918
	5G9, mouse monoclonal	IF and FC (1:50 dilution)	Santa Cruz	SC-12766
Actin	Atto 647N-Phalloidin	IF (1:200 dilution)	Sigma-Aldrich	65906
Dynamin	Anti-dynamin 2, goat polyclonal	WB (1:20 dilution)	Santa Cruz	SC-6400
PKCα	PKCα antibody, rabbit polyclonal	WB (1:100 dilution)	Cell Signalling	2056S
Early endosomal antigen 1 (EEA1)	14/EEA1, mouse monoclonal	IF (1:100 dilution)	BD Transduction Laboratories™	610457
GAPDH	14C10, rabbit monoclonal	WB (1:1000 dilution)	Cell Signalling	2118
Vinculin	hVIN-1, mouse monoclonal	WB, IF (1:1000 dilution)	Sigma-Aldrich	V9131
Caveolin-1	Anti-caveolin-1, rabbit polyclonal	WB, IF (1:1000 dilution)	BD-biosciences	610060
Clathrin heavy chain	Anti-clathrin, mouse monoclonal	WB (1:1000 dilution)	BD-biosciences	610499

Tubulin	Dm1A, mouse monoclonal	WB (1:1000 dilution)	Sigma-Aldrich	T9026

Table 2.2 Primary antibodies.

2.1.2.2 Secondary antibodies

Type	Supplier	Application	Catalogue number
Streptavidin	Thermo Fisher Scientific, Alexa Fluor 700 conjugate	WB (1:10000 dilution)	S21383
Secondary antibodies (WB)	Thermo Fisher Scientific, DyLight 680/800 conjugate anti-rabbit, anti-mouse	WB (1:10000 dilution)	Various
Secondary antibodies (FACS)	Serotech, FITC conjugate anti-mouse	FACS (1:200 dilution)	9B
Secondary antibodies (IF)	Jackson ImmunoResearch, Alexa Fluor-conjugated 594/647, anti-rabbit, anti-mouse	IF (1:200 dilution)	715-585-150 715-605-152

Table 2.3 Secondary antibodies.

2.1.3 Cell culture

2.1.3.1 Mammalian cells

Cell Type	Source
Wild type immortalised mouse embryonic fibroblasts (MEFs)	Generated by (Bass et al., 2007)
Syndecan-4 knockout MEFs	
Syndecan-4 rescue MEFs	
Syndecan-4 rescue mutant MEFs (Y188L)	
Caveolin wild type MEFs	Generated by (del Pozo et al., 2005)
Caveolin knockout MEFs	Generated by (del Pozo et al., 2005)
Human telomerase immortalised fibroblasts (TIFs)	In house
Primary human foreskin fibroblasts (HFFs)	In house
X22 mouse hybridoma B lymphocytes	Purchased from ATCC (catalogue number ATCC® CRL-2228™)

Table 2.4 Mammalian cell types used and sources.

2.1.3.2 Cell culture medium and reagents

Media/reagents	Suppliers/components	Catalogue number
MEFs media	Sigma-Aldrich, supplemented with 10% FBS (50 ml), 2mM L- glutamine (5 ml) and 20U/ml IFN-γ (11 µl) (Sigma-Aldrich #I4777). Caveolin knockout MEFs did not require IFN-γ	DMEM-5796
TIFs media	Sigma-Aldrich, supplemented with 15% (75 ml) FBS and 200mM L-glutamine (10 ml)	DMEM-6171
HFFs media	Sigma-Aldrich, supplemented with 10% FBS (50 ml) and 200mM L-glutamine (10 ml)	DMEM-6171
X22 media	ATCC, supplemented with 15% FBS (75 ml)	30-2002
Sterile PBS^-	Sigma-Aldrich	D8537
Foetal bovine serum (FBS)	Gibco by Life Technologies	10347-028
Trypsin-EDTA (0.05%)	Gibco by Life Technologies	25300-062
Freezing media	90% FBS, 10% Dimethylsulfoxide (DMSO) (Sigma-Aldrich #D2650)	N/A
Cell dissociation buffer	Invitrogen	C5914

Table 2.5 Cell culture media and reagents.

2.1.4 Pharmacological inhibitors, ligands and labels

Inhibitors	Application	Supplier	Catalogue number
Dynasore	Dynamin inhibition (20 µM)	Apexbio Technology	A1605
Tetradecyl trimethyl ammonium bromide (MiTMAB)	Dynamin inhibition (50 and 70 µM)	Sigma-Aldrich	324411
Bisindolylmaleimide-I (BIM-I)	PKC inhibition (100 and 200 nM)	Cayman chemical	13298
Biotinylated human syndecan-4 antibody	Syndecan-4 endocytic assay (200 µg/ml stock)	Research and Diagnostic Systems	BAF2918
EZ-link Sulfo NHS-LC Biotin (non-cleavable)	Syndecan-4 endocytic assay	Thermo Fisher	21335

Mix-n-Stain™ CF™ 488A Antibody Labeling Kit	Syndecan-4 colocalisation	Sigma-Aldrich	MX488AS100
Pierce™ Fab Micro Preparation Kit	Generating syndecan-4 fab fragment from IgG	Thermo Fisher	44685

Table 2.6 Pharmacological inhibitors, ligands and labels

2.1.5 Mammalian cell transfections

2.1.5.1 siRNA transfection reagents

Reagents	Suppliers
Opti-MEM® Reduced serum medium	Gibco by Life Technologies; catalogue number 31985070
DharmaFECT 2 Transfection reagent	Dharmacon; catalogue number T-2002-03
5X siRNA buffer	Dharmacon; catalogue number B-002000-UB-100

Table 2.7 siRNA transfection reagents

2.1.5.2 siRNA oligonucleotides

The following siRNA duplexes with ON TARGET modifications were purchased from Dharmacon (Thermo Fisher Scientific), and upon receipt all siRNAs were resuspended in 1 ml of sterile siRNA buffer following manufacturer's instructions. Stock solutions were prepared to 100 µM solutions and stored at -20^{0}C. Working concentrations (20 µM) were aliquoted as 200 µl and stored at -20^{o}C until use.

siRNA	Catalogue number	Target sequence 5'-3'
Human dynamin-2 (DNM2)	J-004007-05	GGCCCUACGUAGCAAACUA
Human caveolin (CAV1)	J-003467-07	GCAAAUACGUAGACUCGGA
Human caveolin (CAV1)	J-003467-09	GCAUCAACUUGCAGAAAGA
Human clathrin (CAV1)	J-004001-11	GCAGAAGAAUCAACGUUAU
Human clathrin (CLTC)	J-004001-13	CGUAAGAAGGCUCGAGAGU
Mouse clathrin (CLTC)	J-063954-05	GGAAAGCAAUCCAUACAGA
Mouse clathrin (CLTC)	J-063954-06	UCAGAAGAAUUGCUGCUUA
Mouse PKCα	J-040348-05	GAAGGGUUCUCGUAUGUCAUU
Mouse PKCα	J-040348-06	CAGCAAGUCGGGAAAUUUA

Table 2.8 siRNA oligonucleotides

2.1.6 Western blotting reagents

Specification	Supplier	Catalogue number
Mini Gel Tank	Invitrogen	A25977
Mini Blot Module	Invitrogen	B1000
NuPAGE® 4-12% Bis-Tris protein gels (12 and 15-well)	Thermo Fisher Scientific	NPO322, NPO323
20X Bolt™ MES SDS running buffer	Thermo Fisher Scientific	B000202
PageRuler™ Plus Prestained Protein Ladder	Thermo Fisher Scientific	26620
0.45 µm Nitrocellulose membrane	Bio-Rad	1620115
10X Casein blocking buffer	Sigma-Aldrich	B6429
Odyssey® Sa Infrared Imaging System	LI-COR, Biosciences	9260-01

Table 2.9 Western blotting reagents

2.2 Methods

2.2.1 Routine mammalian cell culture

Immortalised syndecan-4 wild type, rescue and Y188L were generated as described in (Bass et al., 2007). Briefly, syndecan-4 wild type and syndecan-null mice were crossed with immorto mouse carrying the simian virus 40 large T antigen (SV40) under the control of the temperature-sensitive H-2K^{b}-tsA58 promoter (Bass et al., 2007; Ishiguro et al., 2000). Primary fibroblasts were isolated from mice at 13.5 embryonic day (e13.5) carrying at least one copy of the transgene H-2K^{b}-tsA58. Immortalisation was achieved by passaging the cells approximately ten times at 33^{0}C in MEF media containing IFN-γ to allow expression of the large T antigen. Syndecan-4 wild type, rescue and Y188L were generated using retroviral transduction where human syndecan-4 wild type cDNA was cloned into pBabe Puro vector and transfected into the retroviral packaging cells AM-12. Syndecan-encoding virions were harvested to infect syndecan-null MEFs. The generated cells underwent two rounds of cell sorting in order to maintain a similar level of syndecan-4 expression, which was verified using FACS (Bass et al., 2007).

All cells were maintained in their corresponding media at 33°C (including syndecan-4 wild type, rescue, knockout and caveolin wild type and knockout MEFs) or 37°C (including TIFs and HFFs) incubator supplemented with 5% CO_2. X22 cells were not adherent and were maintained at 37°C to generate mouse clathrin antibody. Routinely, confluent fibroblasts were washed with 5 ml sterile PBS^{-} and incubated with 5 ml 0.05% trypsin for 5 minutes until cells detached from the culture surface. Detached

cells were centrifuged at 1500 rpm at room temperature for 3 minutes and cells were resuspended in 3 to 4 ml of appropriate media and split into culture flasks containing fresh culture media at 1:3 or 1:4 ratio every 3-4 days. However, X22 cells were spun at 1500 rpm at room temperature for 3 minutes and a third of the supernatant containing the antibody was transferred to a clean tube for storage at 4^{o}C. An equal volume of the transferred media was added to resuspend the cell pellet and cells were added to a new culture flask to produce more anti-clathrin heavy chain antibody (X22).

2.2.1.1 Freezing and defrosting cell stocks

For freezing cell stocks, cell pellets were resuspended in 90% FBS with 10% DMSO to be transferred into precooled cryovials. 1 ml of the resuspended pellet was transferred to cryovials at -20^{o}C freezer for 30 minutes then moved to -80^{0}C freezer for overnight storage before transferred to liquid nitrogen for prolonged storage. To resurrect cells from liquid nitrogen, 2 ml of fresh culture media was prewarmed before defrosting cryovials using 37^{o}C water bath. Defrosted cells were transferred to 15 ml falcon tube containing prewarmed media to be centrifuged at 1500 rpm for 3 minutes. The cell pellet was resuspended in 1 ml of media and transferred to a T25 flask containing fresh media. The culture media was changed the following day to remove any floating or dead cells.

2.2.2 Focal adhesion formation

2.2.2.1 Preparation of ligands, coverslips and ligand coating

Recombinant polypeptides of soluble syndecan-4-binding fragment (H/0) and soluble α5β1-integrins-binding fragment (50K) of fibronectin were prepared as described in (Sharma et al, 1999). H/0 stock concentration was determined using Bradford assay and 50 µg/ml was prepared using fresh culture media on the day of the experiment.

Each 13 mm glass coverslips were placed in 24-well plate and washed with 300 µl PBS^{-} prior coating with 250 µl of 1mM sulfo-3-maleimidobenzoyl- N-hydroxysulphosuccinimide ester (Sulfo-MBS) for 30 minutes at room temperature. Sulfo-MBS was washed 3 times with 300 µl PBS^{+} and washed coverslips were coated with 300 µl of 10 µg/ml 50K in PBS^{+} to be incubated at 4^{o}C on a rocker overnight. The next day, coverslips were washed 3 times with PBS- and blocked with 500 µl of 10 mg/ml heat-denatured BSA at room temperature for 30 minutes.

2.2.2.2 Cell preparation, spreading and adhesion complex formation

Cells planned for experiment were passaged two days before each experiment to reach 70-80% confluency on the day of the experiment. Where appropriate, cells were treated for 2 hours with 300 µl of 25 µg/ml cycloheximide to prevent de novo synthesis of ECM and cells were spread on 10 µg/ml

50K coated coverslips for 2 hours. Spread cells were then stimulated with 300 µl of 50 µg/ml H/0 in media or 300 µl of 10 µg/ml anti-syndecan-4 antibody in media (1:50 dilution) for 1 hour and fixed in 4% paraformaldehyde for 10 minutes at room temperature. Paraformaldehyde was washed 3 times using PBS^- and quenched with 300 µl of 0.1M glycine in PBS^- for 20 minutes at room temperature followed by washing with PBS^- and permeabilisation with 400 µl 0.5% Triton X-100 for 5 minutes at room temperature. Cells were then blocked overnight at 4^0C in 500 µl of 4% BSA. Primary antibodies (Table 2.2) were added for 1 hour at room temperature followed by washing with PBS^- to remove unbound antibodies before adding the secondary antibodies (Table 2.3). Coverslips were then washed 2 times with PBS^- followed by washing with distilled water and mounted using ProLong™ Gold Antifade mounting media with DAPI (Thermo Fisher #P36931) to be examined using Olympus BX51 fluorescence microscope.

Obtained images were analysed using ImageJ software to measure focal adhesion area. The total area of adhesion complex within cells was obtained after subtracting the background of selected images using rolling ball function size 15. Image threshold was adjusted for all conditions within each experiment using "Otsu" pre-set to measure focal adhesion area within cells with minimal background staining. The selected regions within cells were then used to measured focal adhesion area. The same microscope settings were used to capture all images within experiments.

2.2.3 Mammalian cell transfection using RNAi

Cells were allowed to grow in T-25 flask to reach 80-90% confluency before using RNA oligonucleotides to knockdown mediators in Table 2.8. A non-targeting siRNA was used in each transfection as control knockdown. For each knockdown experiment, 10 µl oligonucleotide (of 20 µM) and 5 µl DharmaFECT transfection reagent was diluted in 300 µl Opti-MEM and incubated for 5 minutes at room temperature. The diluted oligonucleotide was then transferred to the tube containing transfection reagent and incubated for 20 minutes at room temperature before transfer to T-25 flasks containing 2.5 ml of fresh culture media and allowed to grow overnight. Cells were passaged the next day and allowed to reach the same confluency in order to perform a second round of transfection 24 hours after splitting. After passaging the second round of transfection, cells were used for experiments and the efficiency of knockdown was tested using Western blotting.

Where appropriate, pharmacological agents were used in parallel to oligonucleotide experiments. For example, MiTMAB (50 and 70 µM) and dynasore (20 µM) were independently applied to 80-90%

confluent cells for 30 minutes to inhibit dynamin while BIM-I (100 and 200 nM) was used to inhibit PKCα activity.

2.2.4 Syndecan-4 internalisation assay

2.2.4.1 Labelling syndecan-4 ligands

Targeting syndecan-4 ectodomain was published previously by our group, hence syndecan-4 antibody (5G9) and heparin-binding H/0 were biotinylated using non-cleavable EZ-link Sulfo NHS-LC Biotin (Thermo Fisher) following manufacturers' instructions. Briefly, 100 µl of 1 mg/ml H/0 or 0.2mg/ml of 5G9 was incubated with the labelling agent in PBS^- for 30 minutes at 37^0C. The labelled material was transferred to dialysis tube to remove excess unbound biotin in a 2 L beaker containing PBS at 4°C overnight. The concentration of labelled material was determined using Pierce BCA Protein Assay Kit (Thermo Fisher #23225).

The human anti-syndecan-4 antibody (AF2918) was labelled for subcellular localisation of internalised syndecan-4 using Mix-n-Stain™ CF™ 488A Antibody Labelling Kit following manufacturer's instruction. The 10X reaction buffer in the labelling kit was diluted to 1X using the antibody and transferred to the vial containing the labelling dye. The content was mixed and vortex for a few seconds and incubated in the dark at room temperature for 30 minutes. The labelled antibody was diluted using the provided storage buffer and kept at 4°C until use.

2.2.4.2 Triggering syndecan-4 endocytosis

Two 6-well plates were coated with 10 µg/ml 50K in PBS^+ to be incubated at 4°C on a rocker overnight. The next day, wells were washed 3 times with PBS^- and blocked with 10mg/ml heat-denatured BSA at room temperature for 30 minutes. Cell density was adjusted to $3X10^5$ cell/well using appropriate media and plated cells were spread on 50K for 2 hours. Syndecan-4 ligands: biotinylated H/0 (2 µg/ml), biotinylated 5G9 (1:50 dilution) or pre-biotinylated syndecan-4 antibody BAF2918 (1:70 dilution) were diluted in media and 300 µl was added to each well. Each plate was either incubated on ice or at 37°C for 30 minutes. After 30 minutes, plates were transferred immediately on ice and washed with 2 ml precooled PBS^-. Free biotin in each well was quenched with 2 ml of precooled 50 µg/ml avidin and incubated on ice for 30 minutes. Plates were washed 3 times with 2 ml precooled PBS^- and the quenched avidin was re-quenched with 2 ml of precooled 50 µg/ml biotin for 30 minutes on ice. Wells were washed 3 times with 2 ml precooled PBS^- and cells were lysed with 200 µl lysis buffer (see Table 2.1) and scraped using a cell scraper to be collected in cooled 1.5 ml microcentrifuge tubes. Each tube was centrifuged for 2 minutes at 4°C and supernatant was stored at -20°C to be analysed using western blotting.

2.2.4.3 Western blotting

Samples were briefly spun down and 10-20 µl of the supernatant was transferred to a clean microcentrifuge tube containing an equal volume of SDS-PAGE protein loading buffer (see Table 2.1.1). Each sample was heated for 3-5 minutes at 85^0C before loading on NuPAGE® 4-12% Bis-Tris protein gels (12 or 15-well) with 1X Bolt™ MES SDS running buffer. PageRuler™ Plus Prestained Protein Ladder was used as a molecular weight standard. All samples were run at 200 volts for 22 minutes. Following SDS-PAGE separation, samples were transferred from the gel to 0.45 µm nitrocellulose membrane using mini blot module and the transfer was done at 30 volts for 90 minutes. Following protein transfer, the nitrocellulose membrane was blocked with 1X casein containing 1% TBS-Tween-20 for 60 minutes on a rocker at room temperature. The blocked membrane was incubated with appropriate primary antibody diluted (see Table 2.1.2.1) using the same blocking solution for 1 hour at room temperature or left overnight at 4^0C on a rocker. The membrane containing the primary antibody was washed for 30 minutes with 4 ml TBS-Tween-20 (4 times with 5 minutes/wash) and incubated with fluorophore-conjugated secondary antibodies (see Table 2.1.2.2) diluted in 1X casein containing 1% TBS-Tween-20 for 30 minutes at room temperature on a rocker away from light. The membrane was washed 5 times with 4 ml TBS-Tween and immunoblotted proteins were detected using Odyssey infrared imaging system (700 nm and 800 nm channels, 169 µm resolution). Band intensity was determined by digital-densitometric analysis using a free version of Image Studio™ Lite by LI-COR version 5.2.5. Band intensities were normalised to loading control and control condition at 30 within each experiment.

2.2.4.5 Subcellular localisation of internalised syndecan-4

Cells were prepared and spread on coated coverslips similar to 2.2.2.2. Spread cells were allowed to internalise the 488A fluorescently-labelled syndecan-4 (see 2.2.4.1) at 1:200 dilution of 125 µg/ml stock in prewarmed culture media for 10 and 30 minutes. Cells were fixed in 300 µl of 4% formaldehyde in the dark for 10 minutes at room temperature. Formaldehyde was washed 3 times using 500 µl PBS^- and quenched with 300 µl of 0.1M glycine in PBS^- for 20 minutes at room temperature and followed by washing with 500 µl PBS^- and permeabilisation with 300 µl of 0.5% Triton X-100 for 5 minutes at room temperature. Cells were then blocked overnight at 4^oC in 300 µl of 4% BSA. Anti-EEA1 and anti-caveolin-1 were used at 1:100 dilution of 0.25 mg/ml stocks while the clathrin antibody produced by X-22 cells was used as it is. 300 µl of primary antibodies were applied and incubated for 1 hour at room temperature and followed by washing steps with PBS^- to remove unbound antibodies. Secondary antibodies (see Table 2.3) were used at 1:500 dilution of 0.5mg/ml stocks and applied to appropriate coverslips for incubation in the dark at room temperature for 30 minutes. Coverslips were then washed 2 times with PBS^- followed by washing with distilled water and mounted using ProLong™ Gold Antifade

mounting media with DAPI (Thermo Fisher #P36931) to be examined using Nikon A1 Confocal Laser Microscope. The same microscope settings were applied to capture and analyse all the images.

Obtained images were analysed using ImageJ software to measure vesicle intensity and colocalisation with internalised syndecan-4. The background of the obtained image was subtracted from syndecan-4 stained images using rolling ball function size 15. Image threshold was adjusted for all images using a pre-set threshold to measure the intensity of syndecan-4 (MaxEntropy), caveolin and clathrin (Moments) within cells with minimal background staining. Pearson's and Manders' M1 coefficients were used to demonstrate syndecan-4 association with both endocytic mediators using JACoP plugin which enables visualisation of colocalisation by generating pixel intensity scatterplots (Bolte & Cordelières, 2006). The selected regions within cells were then used to measured vesicles intensity and association with syndecan-4.

2.2.5 Flow cytometry and fluorescently activated cell sorting (FACS)

A confluent T75 flask was washed twice with PBS^- followed by two washes with 7 ml cell dissociation buffer. Cells were incubated with cell dissociation buffer for 10 minutes at 37^{o}C to detach from culture surface. Cells were collected and centrifuged in precooled tubes 1500 rpm for 5 minutes at 4^{0}C. Each pellet was resuspended in 150 µl DMEM media supplemented with 1% FBS. Cells were stained with 50 µl of syndecan-4 antibody (5G9 1:100 dilution of 0.2 mg/ml stock) diluted in PBS^- containing 0.1% sodium azide for 60 minutes on ice. Cells stained with primary antibody were washed twice with 150 µl PBS^- supplemented with 1% FBS and centrifuged for 5 minutes at 1500 rpm at 4^{o}C. The pellet was stained with 50 µl of secondary fluorophore-tagged antibody (9B 1:200 dilution of 0.01 mg/ml stock) diluted in PBS^+ supplemented with 10% FBS in the dark for 30 minutes on ice. Cells were washed, resuspended in 400 µl PBS^+ and fixed with 2% paraformaldehyde. Samples were sent to the Flow Cytometry core facility at the University of Sheffield Medical School for sorting and analysis.

2.2.6 Enzymatic preparation and labelling of syndecan-4 fab fragment

Human syndecan-4 antibody (AF2918) was enzymatically digested using Pierce Fab Micro Preparation Kit (44685) to produce syndecan-4 fab fragment following the manufacturer's instructions. Briefly, the antibody was digested by papain generating two fragments: fragment of crystallisation (Fc) and 2 fragments antigen binding (Fab). Fab fragments were purified using the provided spin column while undigested antibody and Fc fragment were eluted in a separate tube. 500 µl of the generated fab fragment was biotinylated similar to (2.2.4.1) and tested using western blotting a day before the experiment. The biotinylated fab fragment was stored at 4^{o}C until use.

2.2.6.1 Endocytosis of labelled fab fragment

Cells were plated and spread on two 50K-coated plates (as described in 2.2.4.2) and the 300 µl of biotinylated fab fragment (diluted in media as 10 µg/ml) was applied to cells to be incubated on ice or at 37^{o}C for 30 minutes. The two plates containing biotinylated fab fragment were transferred immediately on ice and washed twice with 3 ml of precooled PBS^{-} and free biotin was quenched using 0.2 mg/ml avidin as described previously. An extra well was evaluated for fab surface staining (not quenched with avidin) to distinguish internalised fab from surface staining. Following two washes with PBS^{-}, free unbound avidin was quenched with biotin and lysed to be analysed using Western blotting.

2.2.7 Statistical analysis

All error bars represented standard error of the mean (SEM). Data were statistically analysed using a free version of GraphPad Prism 8 software. Comparisons were made using Student's *t*-test, where appropriate, to compare between two samples assuming unequal variance. One-way Analysis of Variance (ANOVA) was applied to compare the mean of more than two samples within the same experiment with Tukey's multiple comparisons test for post hoc comparisons. P value of less than 0.05 was considered significant.

Chapter 3: Syndecan-4 antibody targets syndecan-4 and mimics downstream signalling

Focal adhesions are anchorage points where integrin and signalling macromolecules cluster, linking ECM and cytoskeleton. Engagement of clustered integrin with fibronectin recruits cytoskeletal proteins to develop into focal adhesion. Syndecan-4 is a fibronectin co-receptor and is an integrin regulator which can independently mediate syndecan-4 downstream signalling and regulate focal adhesions by recruiting proteins involved in regulating integrin trafficking. Both integrin and syndecan-4 are equally needed for focal adhesions development to allow transmission of contractile force between the intracellular actin cytoskeleton and ECM (Bass et al., 2007, 2011; Greene et al., 2003). The recruitment of proteins during receptors engagement can be used to monitor the formation of focal adhesion. Vinculin is an example of recruited cytoskeletal proteins that link ECM-bound integrin to actin cytoskeleton and plays a key role in focal adhesion maturation (Morgan et al., 2013), thus can be used as a marker for focal adhesion. However, the cells used in this chapter do not exclusively express syndecan-4, but express other syndecans including syndecan-1 and syndecan-2 (Bass et al., 2007), to which heparin-binding growth factors could bind and mediate downstream signalling. Hence, biotinylated syndecan-4 antibody was used to target the receptor more specifically and compare the outcomes to syndecan-4-binding ligand H/0.

3.1 Syndecan-4 antibody activates fibroblasts to form focal adhesion

Syndecan-4 receptor engagement with fibronectin is known to trigger receptor activation and focal adhesion formation. To determine the functionality of syndecan-4 receptor, internalisation and capability of the ligand to engage the receptor in order to mediate syndecan-4-mediated signalling, syndecan-4 receptor was engaged using the native soluble syndecan-binding fragment of fibronectin comprising type III repeats 12–15 (H/0) (Sharma et al., 1999) or syndecan-4 polyclonal antibody directed against the ectodomain. However, native ligands are not specific and could bind other targets including other syndecans involved in receptor activation, hence the antibody demonstrates more specific downstream effect. Syndecan-4 receptor was engaged using H/0 or syndecan-4 antibody for 60 minutes followed by staining for vinculin as an adhesion marker (Morgan et al., 2013). The syndecan-4 receptor of human telomerase immortalised fibroblasts (Tif) was engaged with H/0 to stimulate the ectodomain, while syndecan-4 antibody was applied to syndecan-4 rescue cells and syndecan-4 knockout mouse embryonic fibroblasts (MEFs).

Stimulating Tif cells with H/0 triggered a significant increase in focal adhesion as shown by vinculin staining within 60 minutes when compared to unstimulated cells (Figure 3.1).

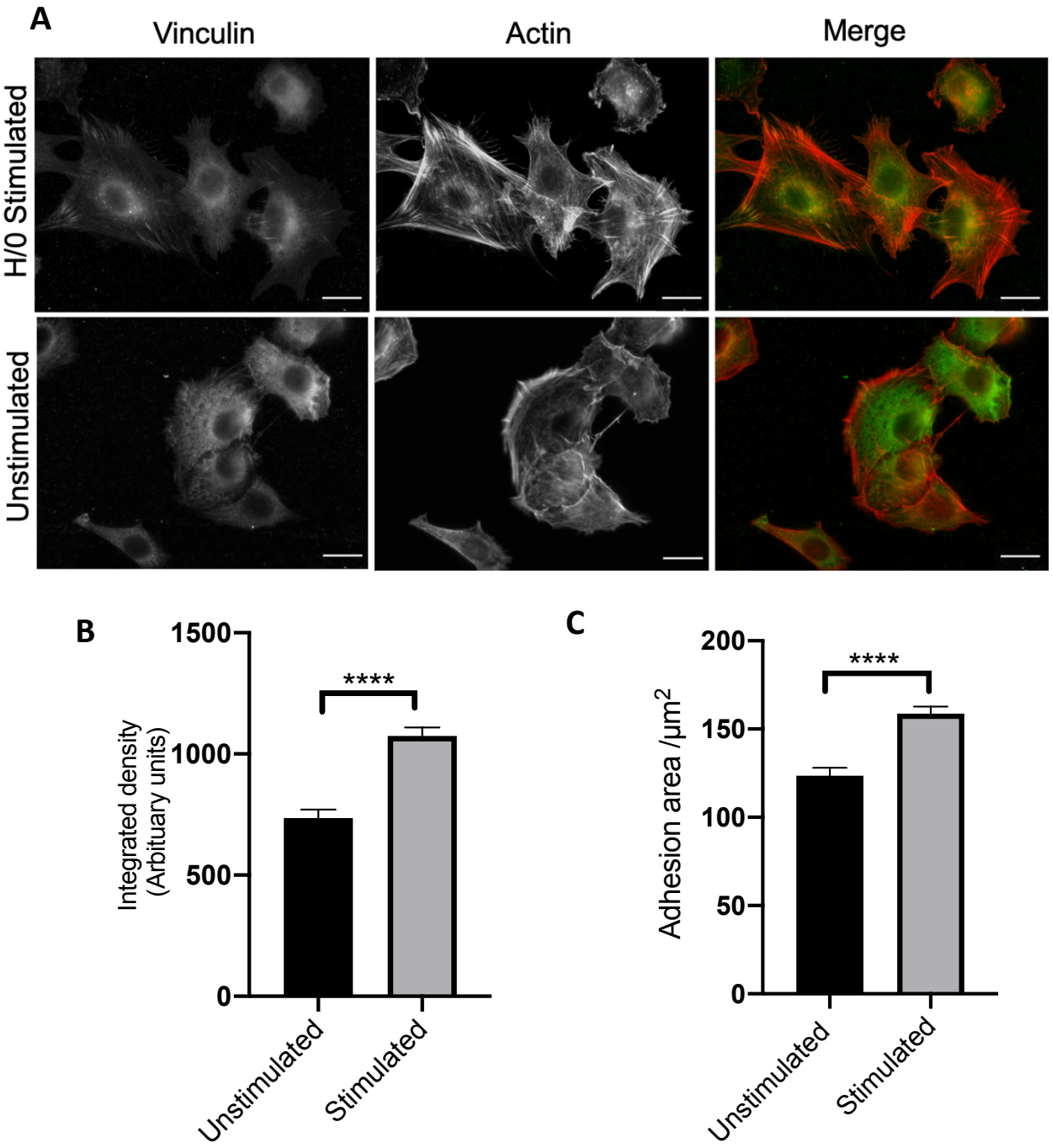


Figure 3.1: H/0 engagement drives focal adhesion complex formation in Tif cells. Tif cells were spread on 10 µg/ml 50K for 2 hours before stimulation with or without 50 µg/ml of H/0 for 60 minutes. Cells were then fixed with 4% PFA and stained for focal adhesion marker, vinculin (green), to measure focal adhesion formation. Fluorescently-conjugated phalloidin (red) was used as a counterstain for actin cytoskeleton. H/0 engagement was capable of triggering focal adhesion formation and significantly induced vinculin-containing adhesions when compared to unstimulated cells. **(A)** Representative immunofluorescence images of Tif cells stained for vinculin and actin with or without H/0 engagement. **(B)** Quantification of vinculin-containing adhesions from A of stimulated and unstimulated cells by recording the area of fluorescence intensity above an empirically determined threshold after rolling ball background subtraction. **(C)** Quantification of focal adhesion area from A comparing both conditions (n=80-100 cells). Scale bar=20 µm. Error bars represent SEM. Statistical significance was calculated by Student's t-test, (**** $p \leq 0.0001$).

Syndecan-4 rescue MEFs are stable syndecan-4 knockout MEFs rescued by expressing the full length of human syndecan-4 to discriminate if focal adhesion is mediated specifically via syndecan-4 receptor by comparing the outcomes with syndecan-4 knockout MEFs where syndecan-4 protein is obliterated. Syndecan-4 rescue MEFs stimulated with the antibody showed a significant increase in focal adhesion marker and area when compared to unstimulated syndecan-4 rescue MEFs (Figure 3.2). Antibody-stimulated syndecan-4 knockout MEFs failed to engage the antibody nor develop focal adhesion demonstrating that formation of focal adhesion is mediated specifically via syndecan-4 receptor. These outcomes agree with previous findings and demonstrate that clustering syndecan-4 ectodomain using the antibody can be used as a ligand mimetic in these experiments (Morgan et al., 2007).

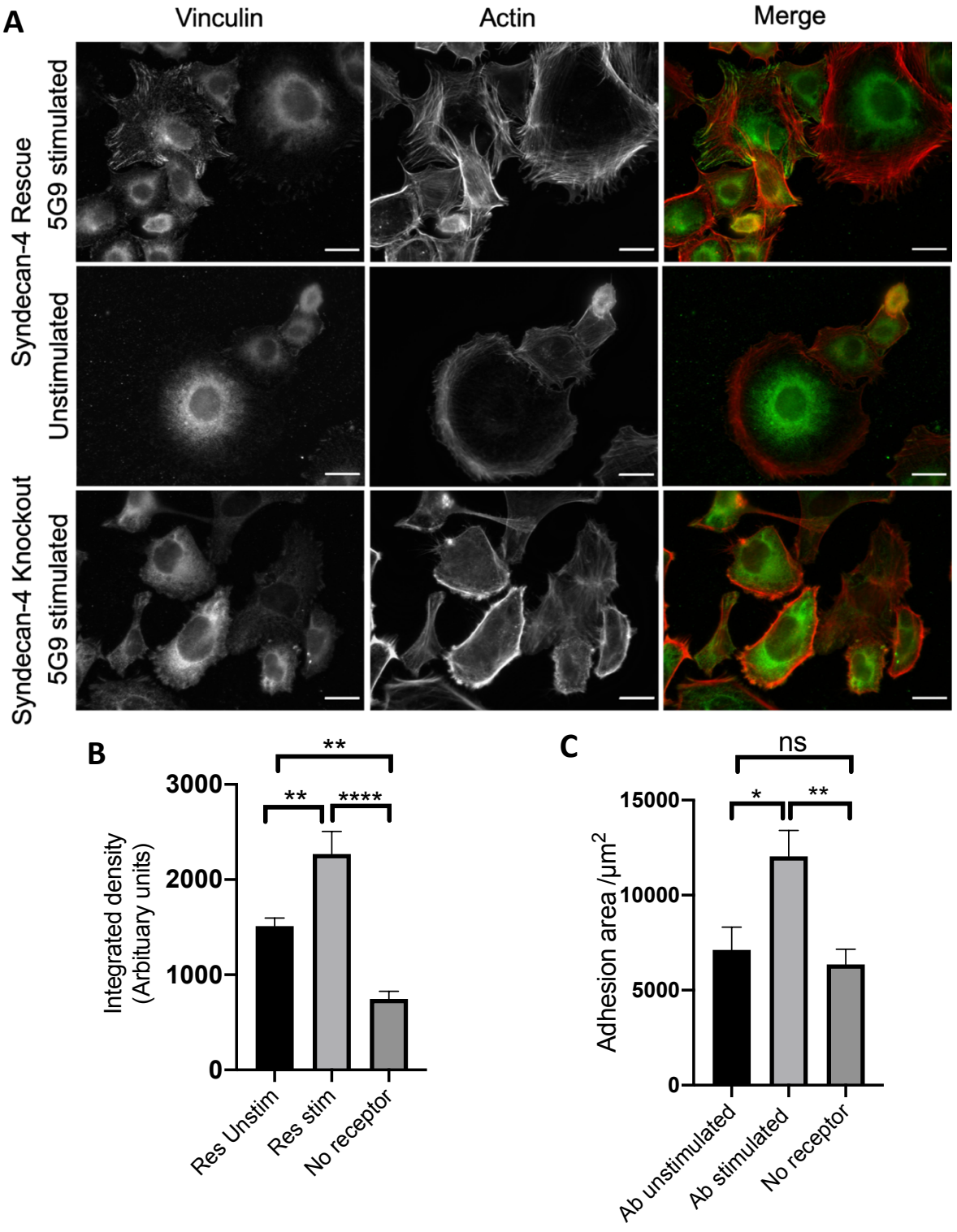


Figure 3.2: Syndecan-4 antibody engagement drives focal adhesion complex formation. Rescue cells and syndecan-4 knockout cells were spread on 10 µg/ml 50K for 2 hours before stimulation with 10 µg/ml anti-syndecan-4 antibody for 60 minutes. Cells were fixed with 4% PFA and stained for focal adhesion marker, vinculin (green), to measure focal adhesion formation. Fluorescently-conjugated phalloidin (red) was used as a counterstain for actin cytoskeleton. antibody engagement was capable of triggering focal adhesion formation and significantly induced vinculin-containing vesicles in antibody-stimulated cells when compared to unstimulated cells. **(A)** Representative immunofluorescence images of rescue cells or syndecan-4 knockout cells stained for vinculin and actin with or without antibody engagement. **(B)** Quantification of vinculin-containing vesicles from A by recording the area of fluorescence intensity above an empirically determined threshold after rolling ball background subtraction. **(C)** Quantification of focal adhesion area from A comparing both conditions (n=50-700 cells). Scale bar=20 µm. Error bars represent SEM. Statistical significance was calculated using One-way ANOVA with Tukey's post hoc test. (* $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$).

3.2 Tracking syndecan-4 antibody in MEFs

The aim was to detect the endocytosis of syndecan-4 by measuring the uptake of native ligand H/0 and antibody. First, H/0 was biotinylated following manufacturer's instructions and excess unbound biotin was removed by membrane dialysis to be used at two internalisation conditions: on ice (0) and at 37°C (30) for 30 minutes in syndecan-4 wild type (WT) and syndecan-4 knockout MEFs. Biotinylated H/0 was the only protein detected using western blot following membrane dialysis suggesting successful biotinylation of the protein at the different concentrations obtained (Figure 3.3 panel A). However, biotinylated H/0 was undetectable in both cells used (Figure 3.3 panel B) suggesting that biotinylated H/0 probably needed further purification steps including desalting column or washed off during the experiment. Moreover, cell detachment was consistently observed after the 30 minutes of endocytosis even when switching to a different buffer, thus ligand purification was recommended.

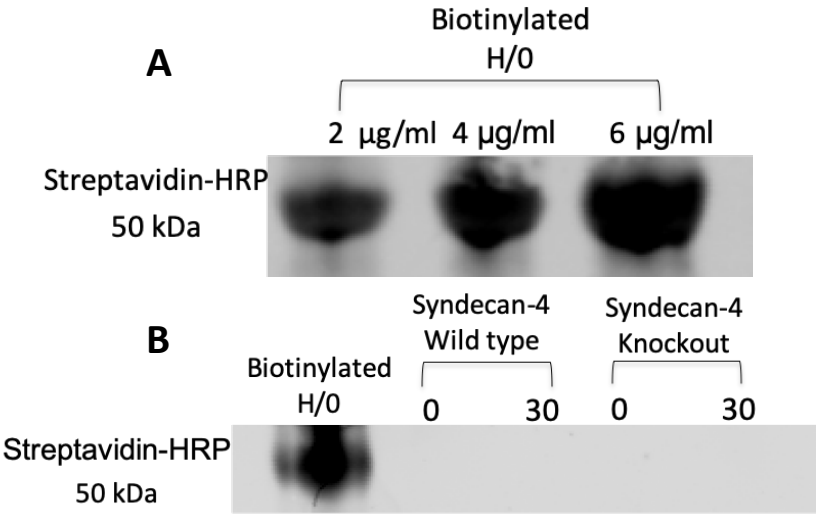


Figure 3.3: Syndecan-4 native ligand is biotinylated but not internalised in MEFs. 2, 4 and 6 µg/ml of biotinylated H/0 stocks were purified using membrane dialysis and detected by western blotting the day before experiment using HRP-conjugated streptavidin antibody indicating successful biotinylation. Anti-streptavidin antibody showed that 2 µg/ml of biotinylated H/0 was not internalised by neither syndecan-4 wild type nor knockout cells within 30 minutes. **(A)** Representative western blot confirming the biotinylation of different stocks of H/0 at different concentrations (2, 4 and 6 µg/ml) using HRP-conjugated streptavidin. **(B)** Representative western blot of cell lysate demonstrating undetectable H/0 endocytosis in syndecan-4 wild type and knockout MEFs by anti-streptavidin antibody.

Next, the pre-biotinylated anti-syndecan-4 antibody was used to establish syndecan-4 endocytosis assay by clustering syndecan-4 ectodomain and comparing internalised receptor-bond antibody during the endocytic periods in syndecan-4 wild type and knockout by western blotting using HRP-conjugated streptavidin antibody. Following syndecan-4 engagement with the antibody, internalised syndecan-4 antibody was significantly induced within 30 minutes in the wild type cells when comparing the endocytic conditions 0 to 30 (Figure 3.4). On the other hand, syndecan-4 knockout cells failed to internalise syndecan-4 antibody within 30 minutes due to deletion of syndecan-4 in those cells validating this method to be applied to detect syndecan-4 receptor internalisation. Consequently, syndecan-4 antibody uptake can be used specifically to detect receptor endocytosis.

3.3 Syndecan-4 endocytosis is dynamin-dependant

Cellular endocytic pathways including caveolin, clathrin and micropinocytosis are all potential mechanisms that may regulate syndecan-4 receptor uptake and signalling. Dynamin-2 is known to mediate caveolin and clathrin endocytic pathways scissoring nascent vesicles (Damke et al., 1994; Henley et al., 1998). To determine the role of dynamin-2 in syndecan-4 internalisation, siRNA was used to knockdown dynamin, and dynamin inhibitors, myristyl trimethyl ammonium bromide (MiTMAB) (70 µM) and dynasore (20 µM), were applied for 30 minutes prior to syndecan-4 endocytosis experiment. These pharmacological agents are widely used in endocytosis studies to prevent interaction of phospholipid and dynamin assembly at the cell membrane by competitive inhibition, as in MiTMAB, or non-competitively, as in dynasore, preventing any dynamin-mediated endocytosis (Hill et al., 2004; Joshi et al., 2010). Evaluation of syndecan-4 endocytosis was carried out in Tif cells and 30 minutes endocytic period was fixed for the two internalisation conditions: on ice (0) and at 37^{o}C (30).

Syndecan-4 endocytosis in control knockdown almost doubled when comparing HRP-streptavidin antibody staining of the two conditions within 30 minutes (Figure 3.5 Panel A). While RNAi-mediated knockdown of dynamin-2 showed significant attenuation in antibody uptake within 30 minutes when

comparing the two conditions and when comparing dynamin knockdown 30 to control knockdown 30 (Figure 3.5 Panel A). Although the efficiency of dynamin-2 knockdown was up to 90% (Figure 3.5 B) and the reduction in syndecan-4 uptake was statistically significant, it did not block it. It is possible that due to incomplete knockdown of dynamin-2, the internalisation of syndecan-4 was not abolished (Figure 3.5 Panel C). This experiment demonstrates that syndecan-4 internalisation is dynamin-dependant.

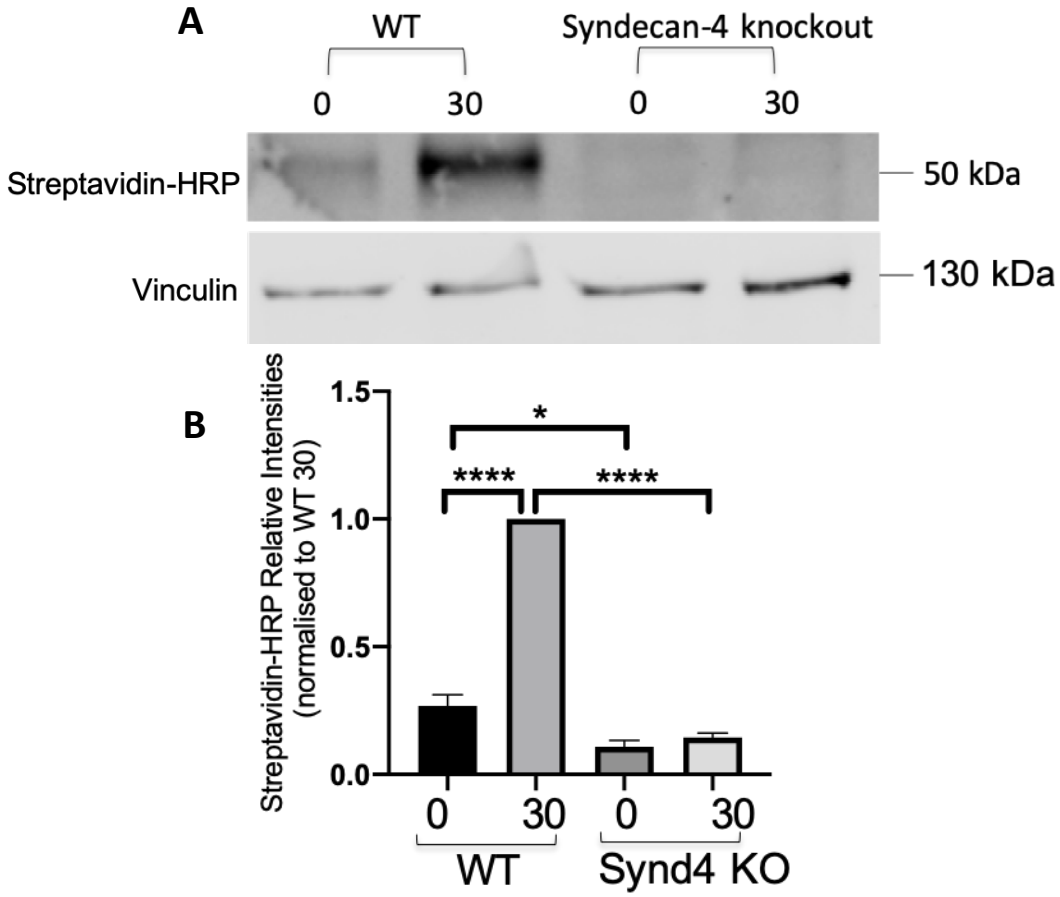


Figure 3.4: Validation of biotinylated syndecan-4 antibody uptake in syndecan-4 wild type and syndecan-4 knockout MEFs. Syndecan-4 uptake was measured in syndecan-4 wild type and syndecan-4 knockout cells using 10 μg/ml biotinylated syndecan-4 antibody and allow 30 minutes of internalisation on ice (0) or 37°C (30) followed by lysing the cells. Cell lysates containing internalised syndecan-4 were analysed by western blotting to compare biotinylated syndecan-4 in each condition using HRP-conjugated streptavidin antibody. Syndecan-4 endocytosis was significantly induced in syndecan-4 wild type within 30 minutes, whereas syndecan-4 knockout cells fail to internalise the antibody. **(A)** Representative western blot showing cell lysate of syndecan-4 wild type with a notable increase in syndecan-4 antibody uptake reflected by induced anti-streptavidin antibody staining between the two conditions 0 and 30, while syndecan-4 knockout cells showed no syndecan-4 endocytosis. Vinculin was used as loading control. **(B)** Graph summarising the quantification of anti-streptavidin antibody demonstrating internalised syndecan-4 antibody in syndecan-4 wild type and syndecan-4 knockout cells for 30 minutes at 0 (on ice) and 30 (37°C). Data were normalised to loading control (vinculin) and wild type 30. (n=6 from independent experiment; WT= wild type, Synd4 KO=syndecan-4 knockout). Error bars represent SEM. Statistical significance was calculated using One-way ANOVA with Tukey's post hoc test.

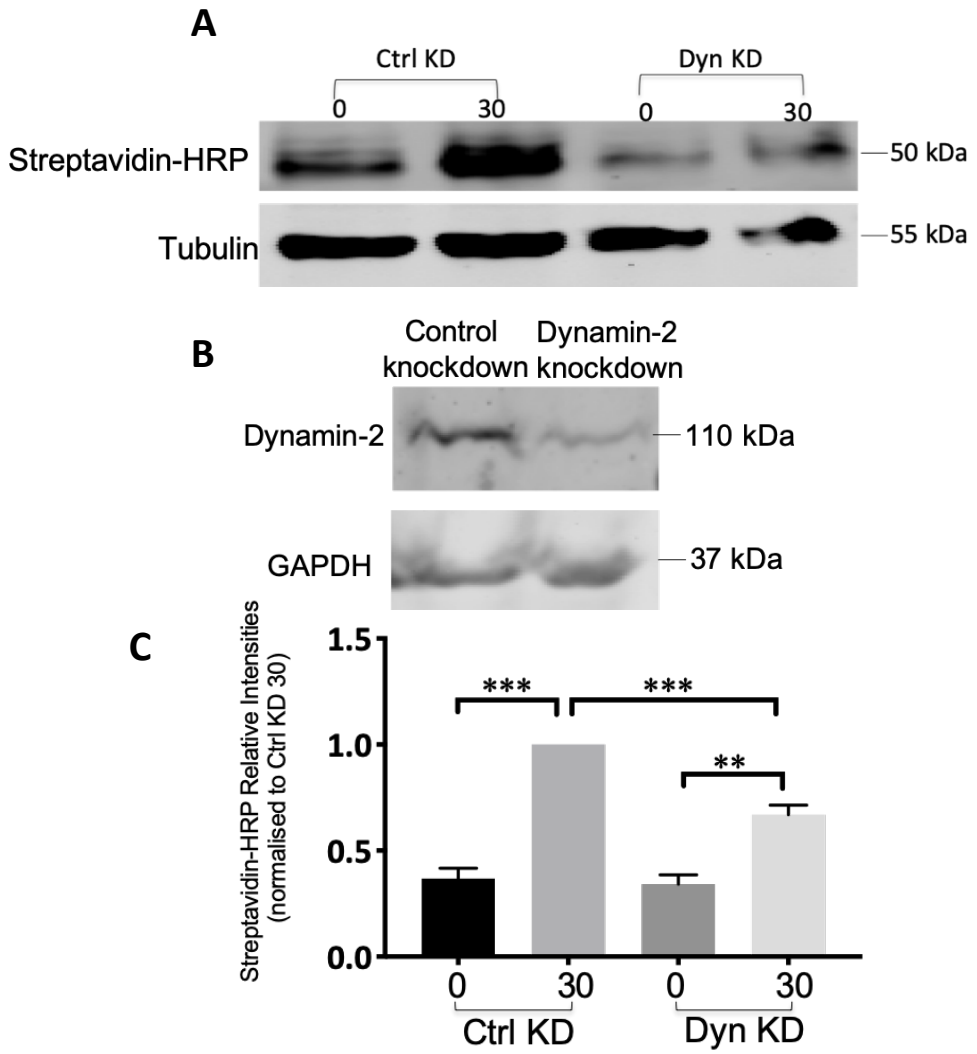


Figure 3.5: Syndecan-4 internalisation in Tif cells is dynamin dependant. Syndecan-4 uptake was measured in control knockdown cells and dynamin-2 knockdown cells using 10 μg/ml biotinylated syndecan-4 antibody and allow 30 minutes of internalisation on ice (0) or 37°C (30) followed by lysing the cells and compare cell lysates by western blot using HRP-conjugated streptavidin antibody. Syndecan-4 uptake was reduced, but not blocked, in dynamin-2 knockdown cells when compared to dynamin-2 knockdown 0 or control knockdown within 30 minutes. **(A)** Representative western blot showing cell lysate of Tif cells with a notable difference in syndecan-4 antibody uptake as reflected by anti-streptavidin antibody staining when comparing the two conditions (0 and 30) of control knockdown or dynamin-2 knockdown. Similarly, antibody uptake was reduced in dynamin-2 knockdown 30 when compared to control knockdown 30. Tubulin was used as loading control. **(B)** Western blot lysate showing dynamin-2 level in control knockdown and dynamin-2 knockdown cells demonstrating 90% reduction in dynamin expression. GAPDH was used as loading control. **(C)** Graph summarising the quantification of anti-streptavidin antibody demonstrating internalised syndecan-4 antibody in control knockdown and dynamin-2 knockdown for 30 minutes at 0 (on ice) and 30 (37°C). Data were normalised to loading control (tubulin) and control knockdown 30. (n=4 from independent experiment; Ctrl= control, Dyn-2=dynamin-2). Error bars represent SEM. Statistical significance was calculated using One-way ANOVA with Tukey's post hoc test.

The use of MiTMAB and dynasore has the advantage of prompt inhibition of dynamin compared to siRNA approach. MiTMAB-untreated cells showed 4 folds higher syndecan-4 uptake when comparing the two conditions (0 and 30), while treatment of cells with MiTMAB resulted in a notable reduction of receptor internalisation at the concentration gradient used (50 and 70 μM) when comparing

MiTMAB 30 to control 30 (Figure 3.6). In parallel, the level of syndecan-4 internalisation reflected by anti-streptavidin antibody in dynasore-untreated cells (DMSO control) was 2.5 folds higher between control 0 and control 30, whereas dynasore-treated cells showed statistically lower syndecan-4 internalisation when compared to untreated cells 30 within the internalisation period (Figure 3.7). Taken together, pharmacological inhibitors demonstrate that dynamin-2 mediate syndecan-4 endocytosis.

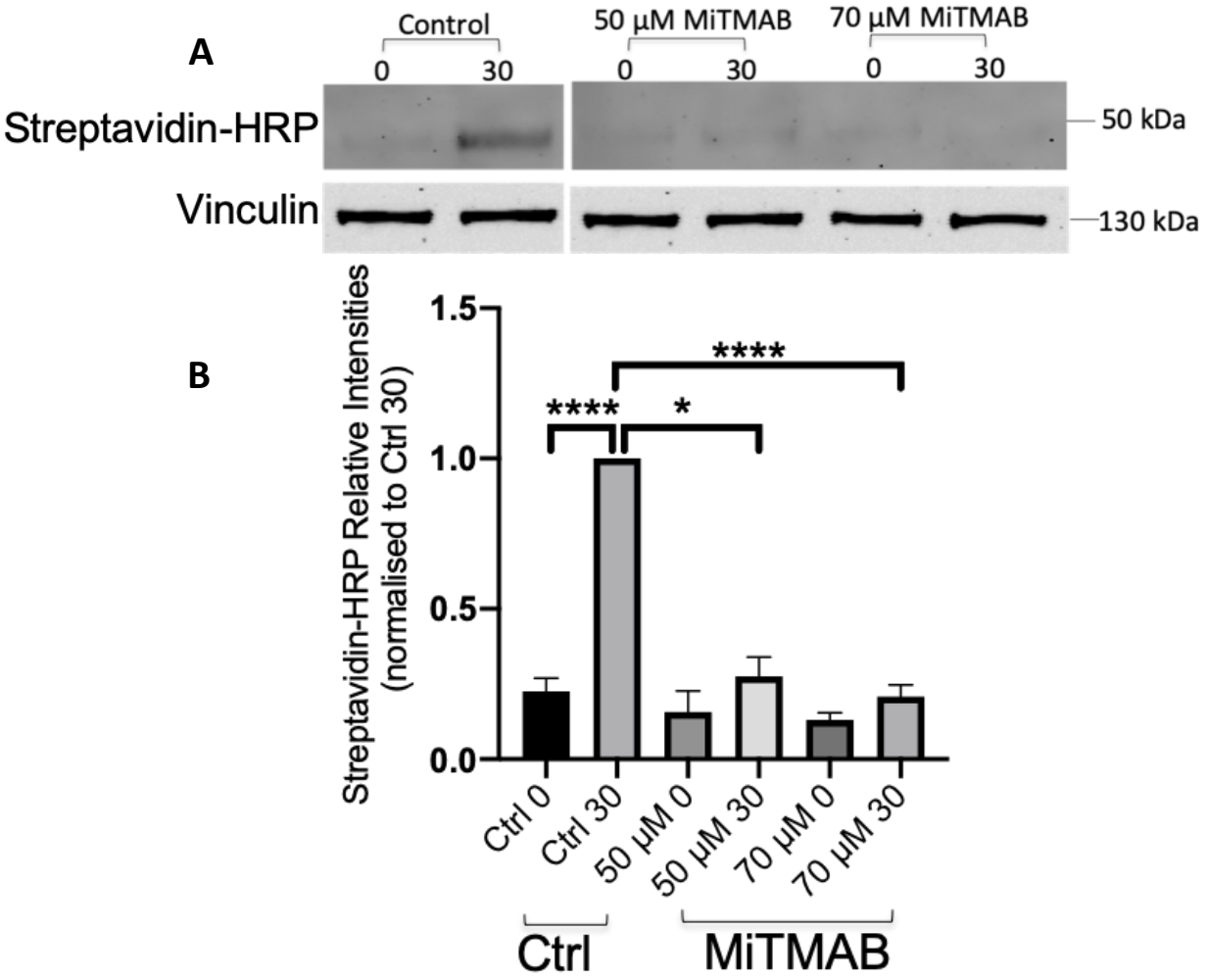


Figure 3.6: MiTMAB treatment reduces dynamin-mediated syndecan-4 endocytosis in Tif cells. Tif cells were treated with 50 µM and 70 µM MiTMAB for 30 minutes after spreading on 10 µg/ml 50K to inhibit dynamin interaction with phospholipid at the cell surface. Then biotinylated syndecan-4 uptake was measured in treated and untreated cells using HRP-conjugated streptavidin antibody. 10 µg/ml biotinylated syndecan-4 antibody was added and allowed to internalise for 30 minutes on ice (0) or 37°C (30). Cells were harvested after 30 minutes and cell lysates were compared using western blot. MiTMAB treatment remarkably reduced syndecan-4 uptake at both concentrations used when compared to untreated cells. (**A)** Representative western blot of cell lysates showing a reduction in streptavidin staining indicating lower syndecan-4 uptake in treated cells when compared to control. Vinculin was used as loading control. **(B)** Graph summarising the quantification of anti-streptavidin antibody demonstrating internalised syndecan-4 in control untreated and MiTMAB-treated cells for 30 minutes at 0 (on ice) and 30 (37°C). Data were normalised to loading control (vinculin) and control untreated 30. (n=11 from independent experiment; Ctrl= control). Error bars represent SEM. Statistical significance was calculated using One-way ANOVA with Tukey's post hoc test.

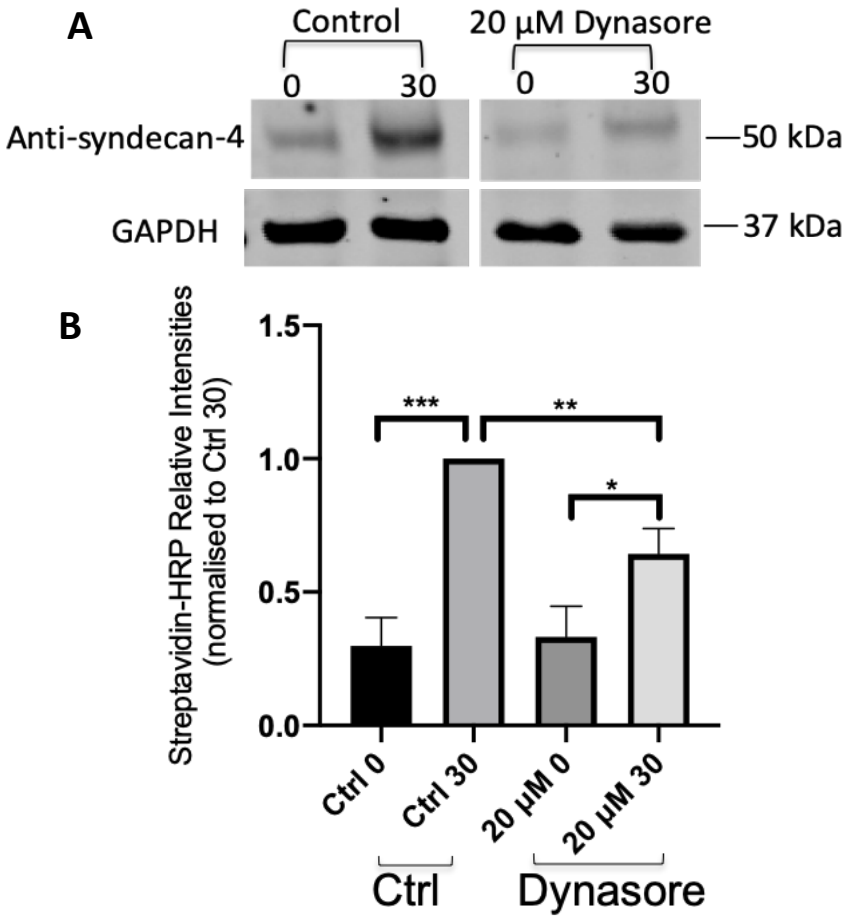


Figure 3.7: Dynasore treatment reduces dynamin-mediated syndecan-4 endocytosis in Tif cells. Tif cells were treated with 20 µM dynasore for 30 minutes after spreading on 10 µg/ml 50K to inhibit dynamin GTPase activity. Then syndecan-4 uptake was measured in treated and untreated cells using 10 µg/ml biotinylated syndecan-4 antibody and allow 30 minutes of internalisation on ice (0) or 37°C (30) followed by lysing of cells and comparing cell lysates using western blot. Dynasore treatment reduced syndecan-4 uptake when comparing the two conditions in treated cells to untreated DMSO control cell. (**A)** Representative western blot showing cell lysate of Tif treated and untreated cells with a notable reduction in HRP-conjugated streptavidin antibody reflecting lower syndecan-4 uptake in dynasore-treated cells compared to control untreated cells. GAPDH was used as loading control. **(B)** Graph summarising the quantification of anti-streptavidin antibody indicating internalised syndecan-4 antibody in DMSO control untreated and dynasore-treated cells for 30 minutes at 0 (on ice) and 30 (37°C). Data were normalised to loading control and control untreated cells 30. (n=4 from independent experiment; Ctrl= control). Error bars represent SEM. Statistical significance was calculated using One-way ANOVA with Tukey's post hoc test.

Data in this chapter demonstrated that syndecan-4 antibody is capable of activating syndecan-4 downstream signalling to form focal adhesion complex mimicking the natural ligand H/0 (Figure 3.1-3.2). The antibody approach targeted the receptor more specifically compared to H/0, as fibroblasts used express other syndecans. The endocytic mechanism for syndecan-4 upon receptor engagement with the antibody is proposed to be dynamin-mediated as shown in Figures 3.4-3.7 and further investigation is required to delineate the internalisation mechanism.

Multiple concentrations of biotinylated H/0 were used to determine the cause of unsuccessful internalisation of H/0. Biotinylated H/0 might require additional purification to improve ligand binding and retrieval. It could be possible that H/0 was degraded or washed away during the experimental procedures. Cell detachment was consistently observed during quenching of free biotin with avidin even when switching to different buffers, which led to switching to biotinylated antibody approach. Another challenge was maintaining the cells after the second round of dynamin-2 knockdown as some experiments where abandon due to infection which occurred frequently after the second round of transfection. Aseptic technique was improved, and separate incubator was used to overcome infections during transfection of cells with siRNA.

Chapter 4: Syndecan-4 endocytosis is blocked when caveolin and clathrin are perturbed

There are a number of different dynamin-dependant endocytic pathway including caveolin- and clathrin-mediated endocytosis. Data in the previous chapter demonstrated that dynamin mediated syndecan-4 antibody internalisation, hence the role of caveolin and clathrin in syndecan-4 endocytosis will be tested in this chapter. To determine the role of each endocytic pathway, siRNA was used to knockdown caveolin and clathrin independently and together in Tifs, and syndecan-4 uptake was evaluated using the same approach in previous chapter.

4.1 syndecan-4 is internalised in a caveolin-dependant manner

Caveolin-1 was reported to be an essential component of the caveolae structure, and without it the structure was undetectable in endothelial and epithelial cells of caveolin-1 knockout mice (Drab et al., 2001; Fra et al., 1995; Razani et al., 2001). To determine caveolin involvement in syndecan-4 internalisation, two different siRNA oligonucleotides were used to knockdown caveolin-1 in Tifs and syndecan-4 endocytosis was evaluated using pre-biotinylated anti-syndecan-4. Following 30 minutes of antibody uptake, cells were lysed and analysed using western blotting.

Syndecan-4 uptake was not blocked, but it was partially attenuated in caveolin knockdown cells when comparing the two conditions 0 and 30, and when compared to control knockdown in both siRNA oligonucleotides used (Figure 4.1 and 4.2). Caveolin expression was reduced to 70% by both oligonucleotides used confirming the same outcomes. This experiment demonstrates that syndecan-4 can be internalised in a caveolin-dependant manner.

4.2 Syndecan-4 is internalised in a clathrin-dependant manner

Similar to caveolin knockdown, clathrin was knocked down using two different oligonucleotides to knockdown clathrin in Tifs, and syndecan-4 endocytosis was evaluated using the same approach.

Like in caveolin knockdown, internalised syndecan-4 was significantly reduced at 30 minutes in clathrin knockdown compared to control knockdown cells for both oligonucleotides used (Figure 4.3 and 4.4). These experiments demonstrate that clathrin also contributes to syndecan-4 endocytosis.

Data here showed that both caveolin and clathrin contribute to syndecan-4 uptake and knockdown of either affected endocytosis. The internalised syndecan-4 upon caveolin-1 or clathrin knockdown could be due to other mechanisms. Therefore, the hypothesis built was that knockdown of both (double

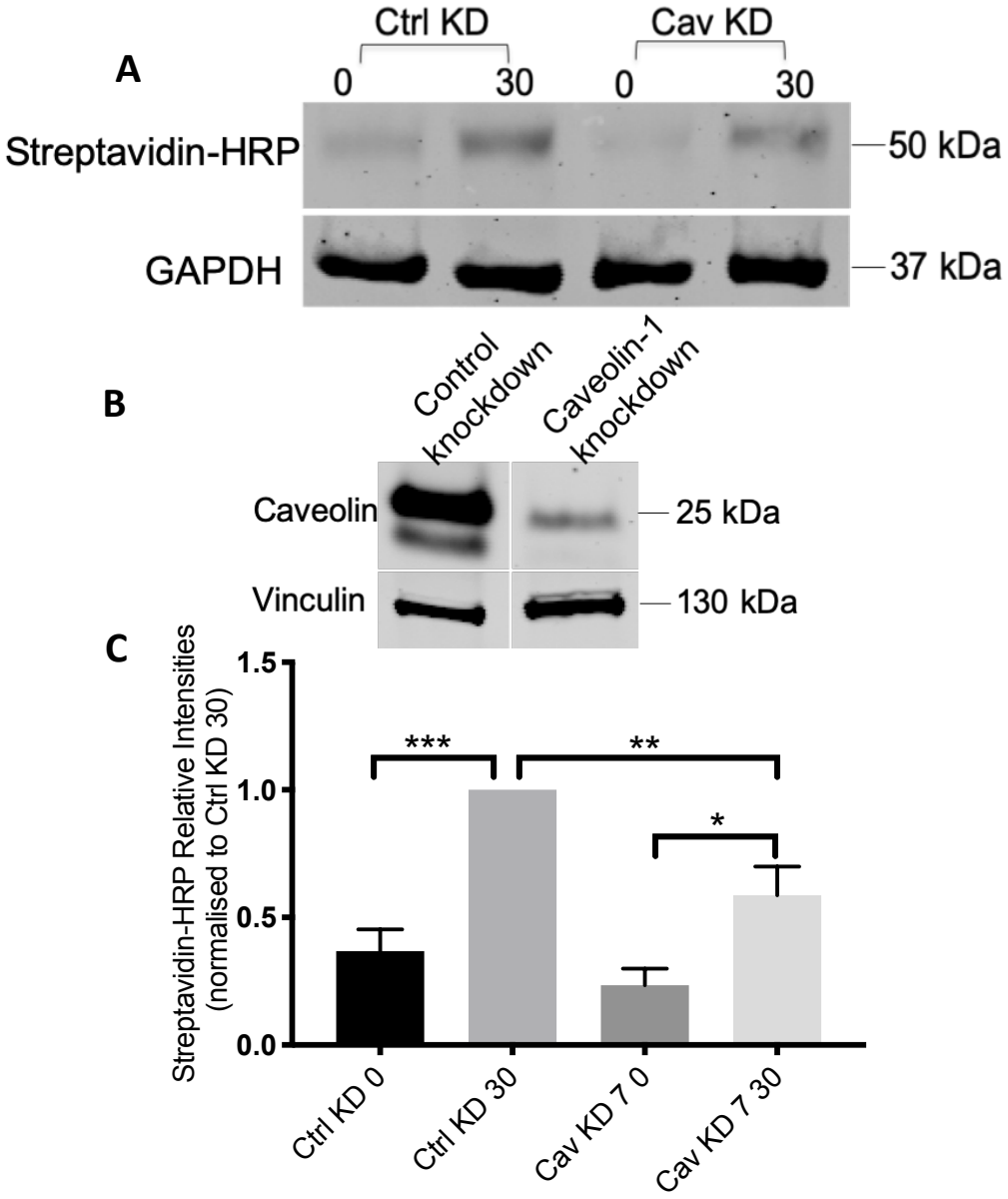


Figure 4.1: Syndecan-4 internalisation in caveolin-1 knockdown Tifs (using oligonucleotide 7) is caveolin dependant. Syndecan-4 uptake was measured in control knockdown cells and caveolin-1 knockdown cells (oligonucleotide 7) using 10 µg/ml biotinylated syndecan-4 antibody and allow 30 minutes of internalisation on ice (0) or 37°C (30) followed by lysing the cells. Cell lysates containing internalised syndecan-4 were analysed by western blotting to compare biotinylated syndecan-4 in each condition using HRP-conjugated streptavidin antibody. Syndecan-4 uptake was significantly reduced in caveolin-1 knockdown cells in both conditions (0 and 30) and when compared to control knockdown 30. **(A)** Representative western blot showing cell lysate of Tif cells with a notable difference in syndecan-4 uptake reflected by reduced anti-streptavidin antibody when comparing caveolin-1 knockdown 30 control knockdown 30. GAPDH was used as loading control. **(B)** Western blot lysate showing caveolin-1 levels in control knockdown and caveolin-1 knockdown cells (oligonucleotide 7) with 70% reduction in caveolin expression. Vinculin was used as loading control. **(C)** Graph summarising the quantification of anti-streptavidin antibody demonstrating internalised syndecan-4 antibody in control knockdown and caveolin-1 knockdown for 30 minutes at 0 (on ice) and 30 (37°C). GAPDH was used as loading control. Data were normalised to loading control (GAPDH) and control knockdown 30. (n=5 from independent experiment; Ctrl= control, KD= knockdown, cav= caveolin, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). Error bars represent SEM. Statistical significance was calculated using One-way ANOVA with Tukey's post hoc test.

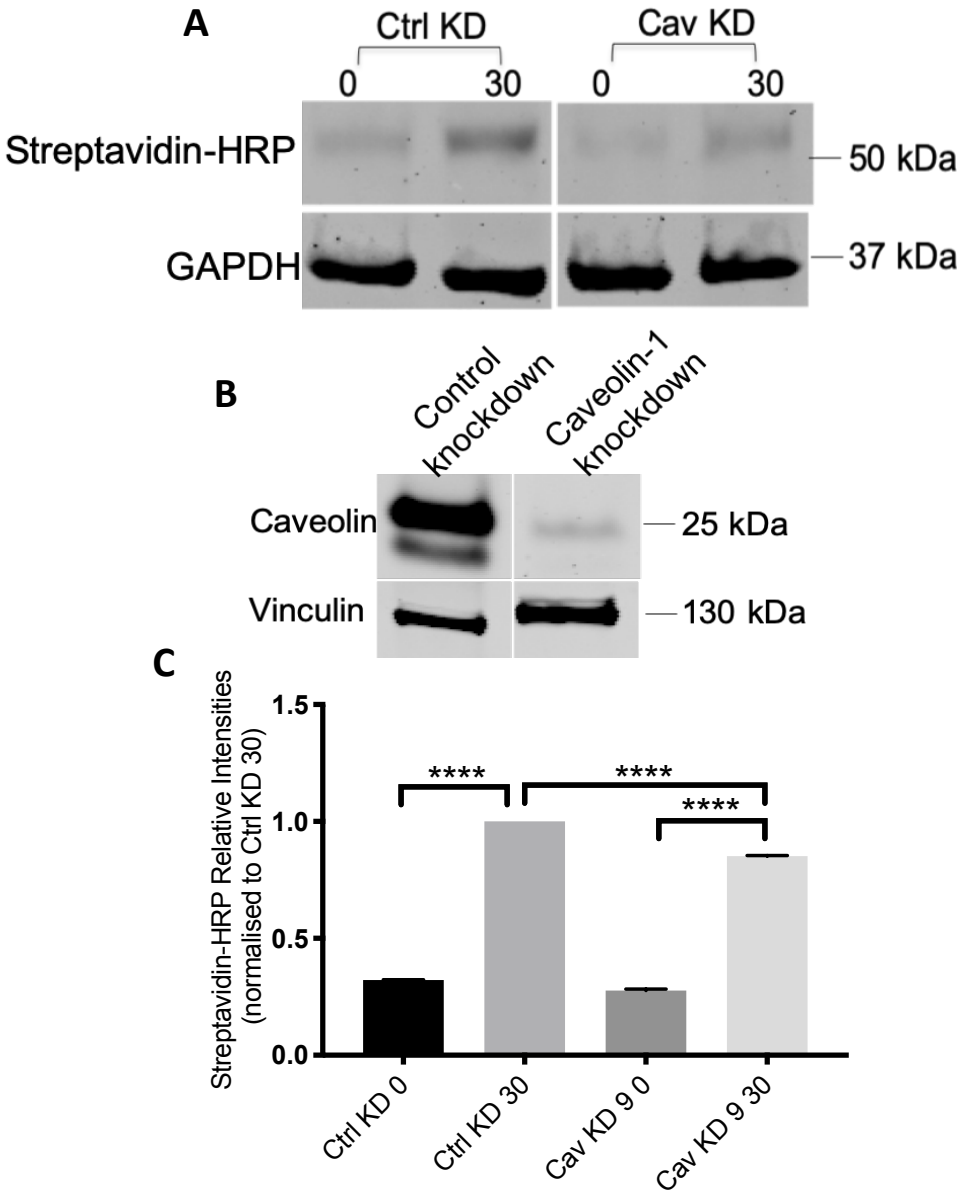


Figure 4.2: Syndecan-4 internalisation in caveolin-1 knockdown Tifs (using oligonucleotide 9) is caveolin dependant. Syndecan-4 uptake was measured in control knockdown cells and caveolin-1 knockdown cells (oligonucleotide 7) using 10 µg/ml biotinylated syndecan-4 antibody and allow 30 minutes of internalisation on ice (0) or 37°C (30) followed by lysing the cells. Cell lysates containing internalised syndecan-4 were analysed by western blotting to compare biotinylated syndecan-4 in each condition using HRP-conjugated streptavidin antibody. Syndecan-4 uptake was significantly reduced in caveolin-1 knockdown cells in both conditions (0 and 30) and when compared to control knockdown 30. Syndecan-4 uptake was reduced in caveolin-1 knockdown cells when comparing caveolin-1 knockdown 30 to control knockdown 30. **(A)** Representative western blot showing cell lysate of Tif cells with a notable difference in syndecan-4 uptake indicated by reduced anti-streptavidin antibody when comparing caveolin-1 knockdown 30 control knockdown 30. GAPDH was used as loading control. **(B)** Western blot lysate showing caveolin-1 levels in control knockdown and caveolin-1 knockdown cells (oligonucleotide 9) with 90% reduction in caveolin expression. Vinculin was used as loading control. **(C)** Graph summarising the quantification of anti-streptavidin antibody demonstrating internalised syndecan-4 in control knockdown and caveolin-1 knockdown for 30 minutes at 0 (on ice) and 30 (37°C). GAPDH was used as loading control. Data were normalised to loading control (GAPDH) and control knockdown 30. (n=4 from independent experiment; Ctrl= control, KD= knockdown, cav= caveolin, **** $p \leq 0.0001$). Error bars represent SEM. Statistical significance was calculated using One-way ANOVA with Tukey's post hoc test.

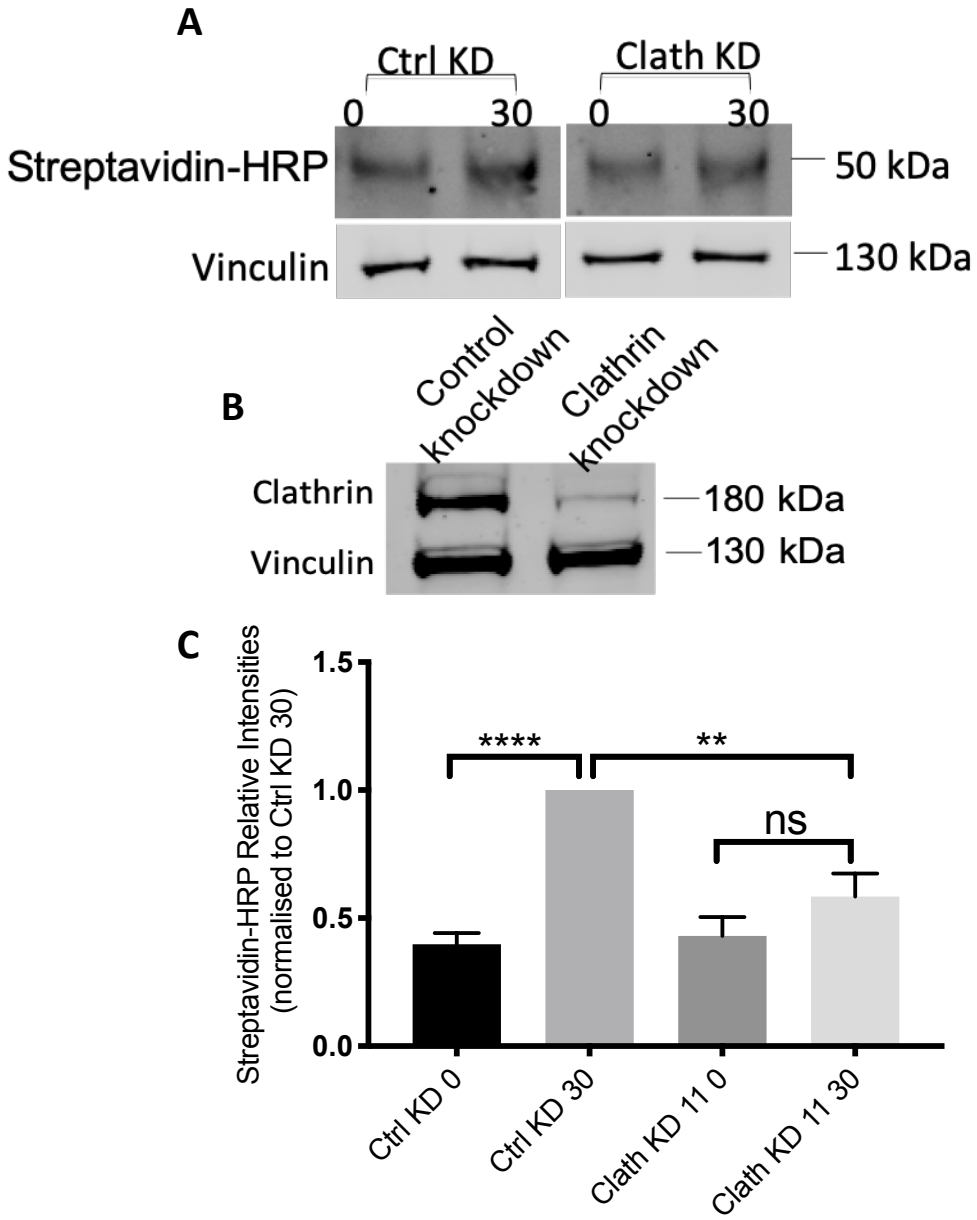


Figure 4.3: Syndecan-4 internalisation in clathrin knockdown Tifs (using oligonucleotide 11) is clathrin dependant. Syndecan-4 uptake was measured in control knockdown cells and clathrin knockdown cells (oligonucleotide 11) using 10 µg/ml biotinylated syndecan-4 antibody and allow 30 minutes of internalisation on ice (0) or 37°C (30) followed by lysing the cells. Cell lysates containing internalised syndecan-4 were analysed by western blotting to compare biotinylated syndecan-4 in each condition using HRP-conjugated streptavidin antibody. Syndecan-4 uptake was significantly reduced in clathrin knockdown cells when compared to control knockdown within 30 minutes. **(A)** Representative western blot showing cell lysate of Tif cells with a difference in syndecan-4 uptake indicated by reduced anti-streptavidin antibody when comparing clathrin knockdown 30 to control knockdown 30. Vinculin was used as loading control. **(B)** Western blot lysate showing clathrin expression in control knockdown and clathrin knockdown cells (oligonucleotide 11) with 90% reduction in clathrin expression. Vinculin was used as loading control. **(C)** Graph summarising the quantification of anti-streptavidin antibody demonstrating internalised syndecan-4 in control knockdown and clathrin knockdown for 30 minutes at 0 (on ice) and 30 (37°C). Vinculin was used as loading control. Data were normalised to loading control (vinculin) and control knockdown 30. (n=4 from independent experiment; Ctrl= control, KD= knockdown, clath= clathrin, ns=not significant, ** $p \leq 0.01$, **** $p \leq 0.0001$). Error bars represent SEM. Statistical significance was calculated using One-way ANOVA with Tukey's post hoc test.

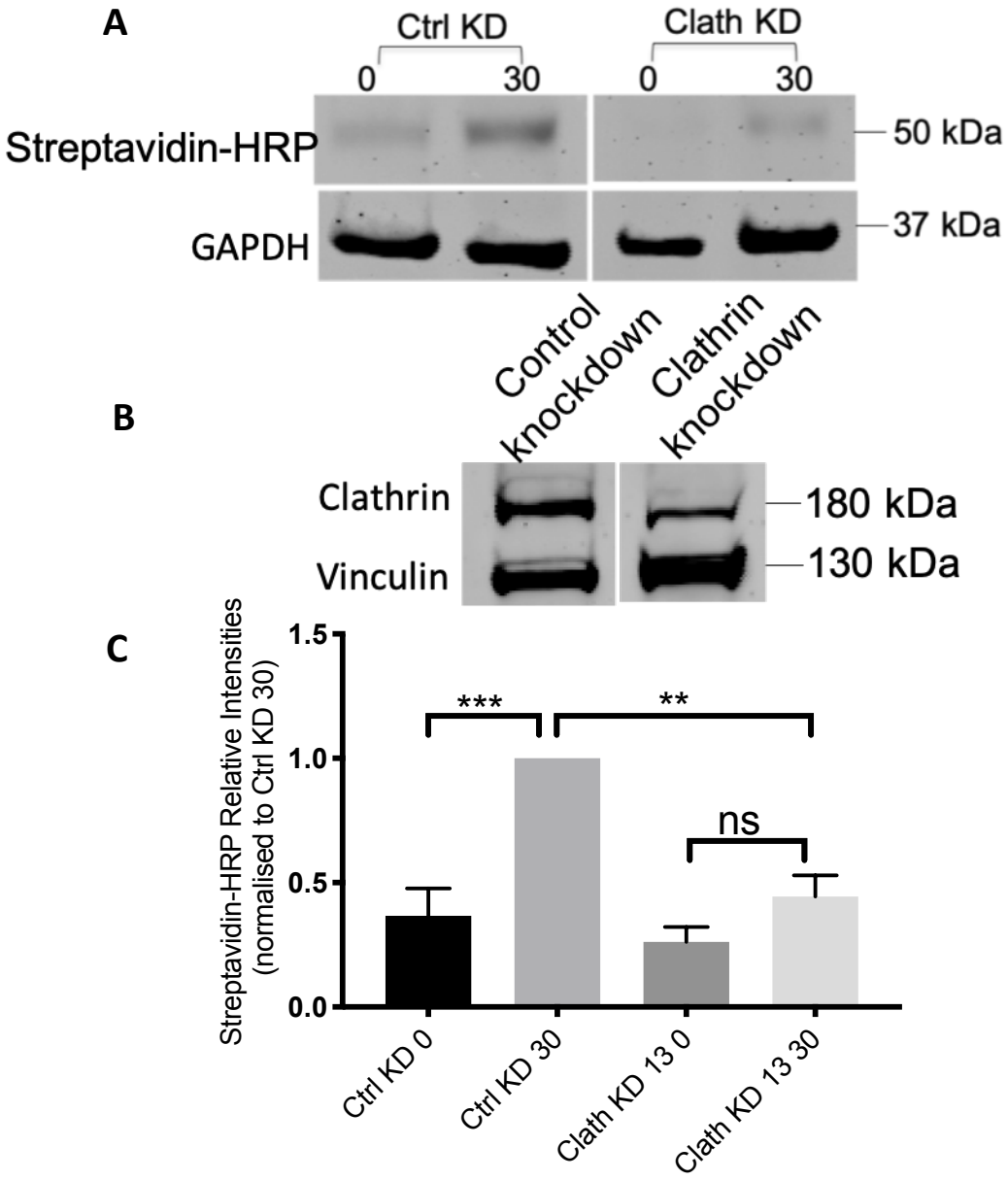


Figure 4.4: Syndecan-4 internalisation in clathrin knockdown Tifs (using oligonucleotide 13) is clathrin dependant. Syndecan-4 uptake was measured in control knockdown cells and clathrin knockdown cells (oligonucleotide 13) using 10 µg/ml biotinylated syndecan-4 antibody and allow 30 minutes of internalisation on ice (0) or 37°C (30) followed by lysing the cells. Cell lysates containing internalised syndecan-4 were analysed by western blotting to compare biotinylated syndecan-4 in each condition using HRP-conjugated streptavidin antibody. Syndecan-4 uptake was significantly reduced in clathrin knockdown cells when compared to control knockdown within 30 minutes. **(A)** Representative western blot showing cell lysate of Tif cells with a difference in syndecan-4 uptake indicated by significant reduction of anti-streptavidin antibody when comparing clathrin knockdown 30 to control knockdown 30. GAPDH was used as loading control. **(B)** Western blot lysate showing clathrin expression in control knockdown and clathrin knockdown cells (oligonucleotide 11) with 80% reduction in clathrin expression. Vinculin was used as loading control. **(C)** Graph summarising the quantification of anti-streptavidin antibody demonstrating internalised syndecan-4 in control knockdown and clathrin knockdown for 30 minutes at 0 (on ice) and 30 (37°C). GAPDH was used as loading control. Data were normalised to loading control (GAPDH) and control knockdown 30. (n=5 from independent experiment; Ctrl= control, KD= knockdown, clath= clathrin, ns=not significant, ** $p \leq 0.01$, *** $p \leq 0.001$). Error bars represent SEM. Statistical significance was calculated using One-way ANOVA with Tukey's post hoc test.

knockdown of caveolin-1 using oligonucleotide 7 and clathrin using oligonucleotide 13) would block syndecan-4 endocytosis. However, double knockdown of caveolin and clathrin in Tifs resulted in attenuated, but not block receptor uptake when comparing control knockdown 30 to double knockdown 30 (Figure 4.5). This could be due to incomplete knockdown of both mediators; thus, further investigation will be carried out to evaluate the hypothesis.

4.3 Caveolin is not solely responsible for syndecan-4 internalisation

Clathrin and caveolin demonstrated contribution to syndecan-4 endocytosis, hence incomplete knockdown of caveolin and/or clathrin might contribute to residual uptake of syndecan-4 seen in Figure 4.5. Therefore, the use of caveolin-1 knockout MEFs could simplify the interpretation of endocytic mediators involved in syndecan-4 uptake. Caveolin wild type and caveolin-1 knockout MEFs were used to measure caveolin-independent endocytosis contributing to syndecan-4 endocytosis. The hypothesis was built on caveolin and clathrin contribution to syndecan-4 endocytosis proposing that knockdown of clathrin and caveolin could block syndecan-4 endocytosis, as the knockdown of each has an effect and clathrin demonstrated compensation upon caveolin knockdown meaning that both are normally involved. The hypothesis was tested using siRNA (two different oligonucleotides) to knockdown clathrin in caveolin-1 knockout cells and syndecan-4 internalisation was carried as previously.

As expected, syndecan-4 internalisation was significantly reduced in caveolin-1 knockout cells compared to caveolin wild type MEFs (Figure 4.6). To test whether residual uptake was mediated by clathrin compensation, clathrin was knockdown in caveolin-1 knockout MEFs and syndecan-4 internalisation was performed using pre-biotinylated antibody testing the later hypothesis of clathrin contribution in receptor uptake.

Clathrin knockdown cells (using two different oligonucleotides 5 and 6) in caveolin-1 knockout MEFs showed further reduction in syndecan-4 internalisation when compared to control knockdown to the extent that significant syndecan-4 endocytosis was no longer detected (Figure 4.7 and 4.8). These experiments demonstrate that, like caveolin, clathrin contribute to syndecan-4 internalisation.

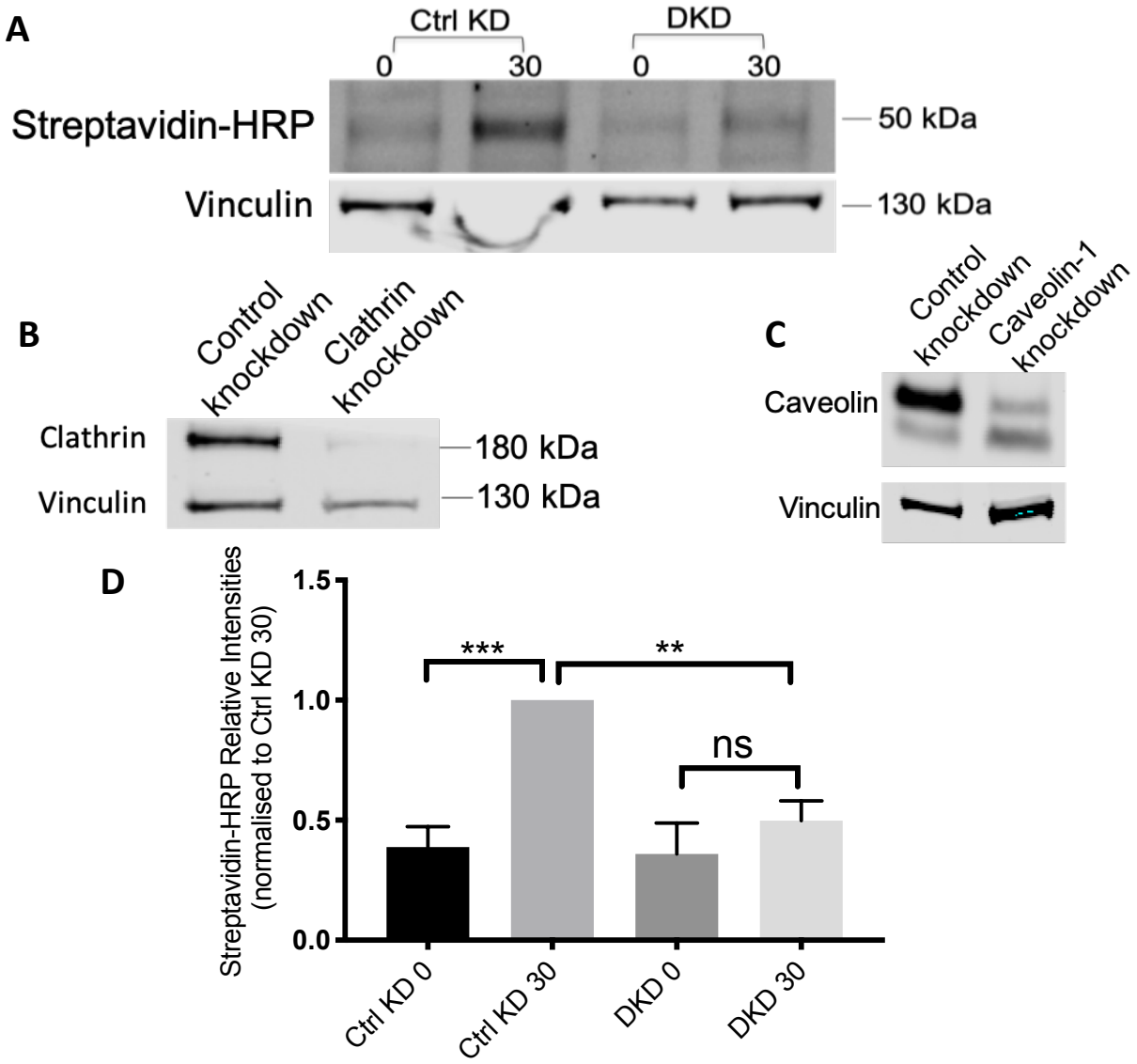


Figure 4.5: Syndecan-4 internalisation in double knockdown Tifs is attenuated, but not blocked. Syndecan-4 uptake was measured in control knockdown cells and double knockdown of both caveolin-1 (oligonucleotide 7) and clathrin (oligonucleotide 13) using 10 µg/ml biotinylated syndecan-4 antibody and allow 30 minutes of internalisation on ice (0) or 37°C (30) followed by lysing the cells. Cell lysates containing internalised syndecan-4 were analysed by western blotting to compare biotinylated syndecan-4 in each condition using HRP-conjugated streptavidin antibody. Syndecan-4 uptake was reduced in the double knockdown when compared to control knockdown within 30 minutes. **(A)** Representative western blot showing cell lysate of Tif cells with reduced syndecan-4 uptake indicated by significant reduction of anti-streptavidin antibody when comparing double knockdown 30 to control knockdown 30. Vinculin was used as loading control. **(B)** Western blot lysate showing clathrin expression in control knockdown and clathrin knockdown cells with 90% reduction in clathrin expression. **(C)** Western blot lysate showing caveolin expression in control knockdown and caveolin-1 knockdown cells with 80% reduction in clathrin expression. **(D)** Graph summarising the quantification of internalised anti-streptavidin antibody demonstrating internalised syndecan-4 antibody in control knockdown and double knockdown for 30 minutes at 0 (on ice) and 30 (37°C). Vinculin was used as loading control. Data were normalised to loading control (vinculin) and control knockdown 30. (n=5 from independent experiment; Ctrl= control, KD= knockdown, DKD= clathrin and caveolin knockdown, ns=not significant, ** $p \leq 0.01$, *** $p \leq 0.001$). Error bars represent SEM. Statistical significance was calculated using One-way ANOVA with Tukey's post hoc test.

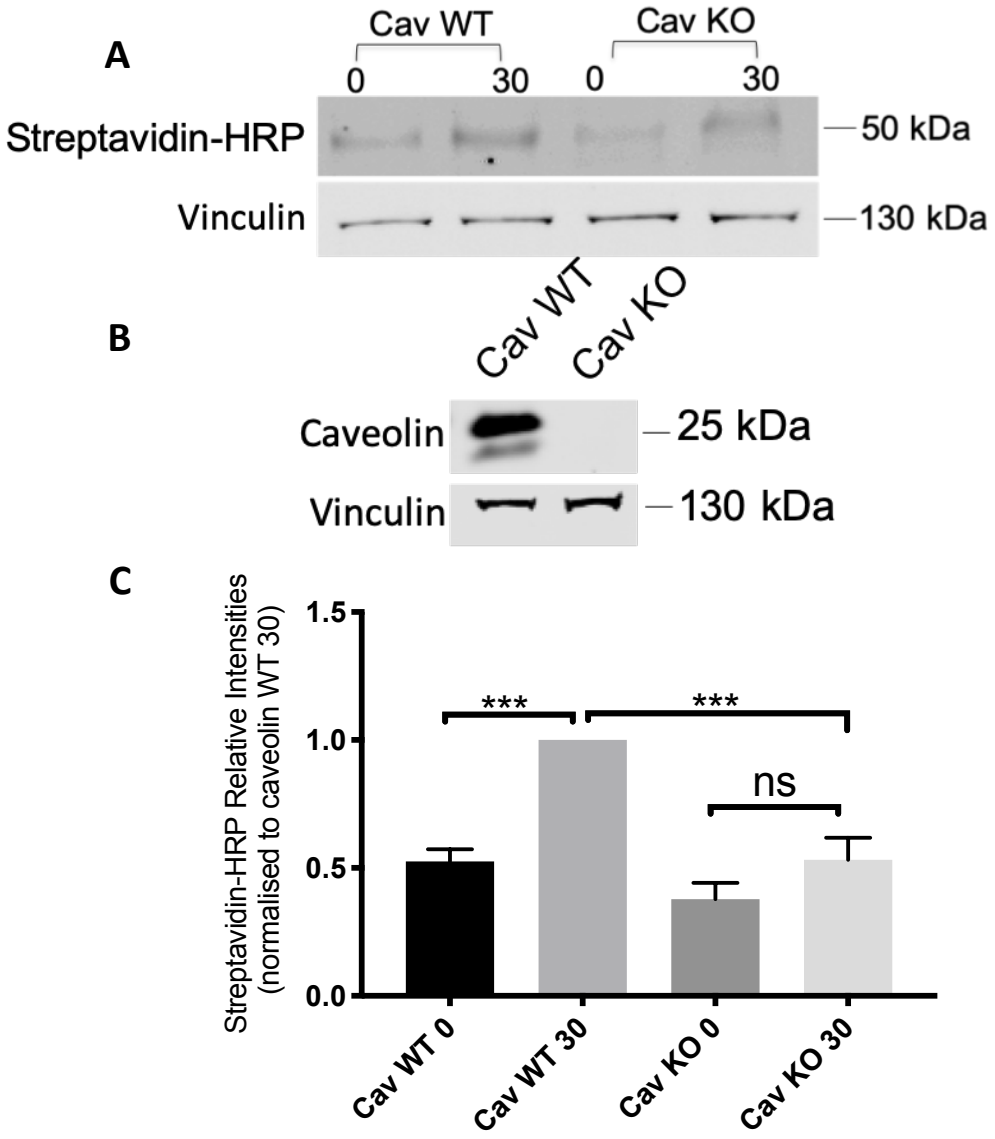


Figure 4.6: Caveolin alone is not responsible for syndecan-4 internalisation. Syndecan-4 uptake was measured in caveolin wild type and caveolin-1 knockout MEFs using 10 µg/ml biotinylated syndecan-4 antibody and allow 30 minutes of internalisation on ice (0) or 37°C (30) followed by lysing the cells. Cell lysates containing internalised syndecan-4 were analysed by western blotting to compare biotinylated syndecan-4 in each condition using HRP-conjugated streptavidin antibody. Syndecan-4 uptake was attenuated in caveolin-1 knockout MEFs when compared to caveolin wild type within 30 minutes. **(A)** Representative western blot showing cell lysate of caveolin wild type and knockout cells with a significant reduction in syndecan-4 antibody uptake reflected by reduced anti-streptavidin antibody staining when comparing caveolin knockout to wild type MEFs. **(B)** Western blot lysate showing the expression of caveolin in caveolin wild type and knockout. **(C)** Graph summarising the quantification anti-streptavidin antibody demonstrating internalised syndecan-4 antibody in caveolin wild type and knockout for 30 minutes at 0 (on ice) and 30 (37°C). Vinculin was used as loading control. Data was normalised to loading control (vinculin) and caveolin wild type 30. (n=6 from independent experiment; Cav= caveolin, WT= wild type, KO=knockout, ns=not significant, *** $p \leq 0.001$). Error bars represent SEM. Statistical significance was calculated using One-way ANOVA with Tukey's post hoc test.

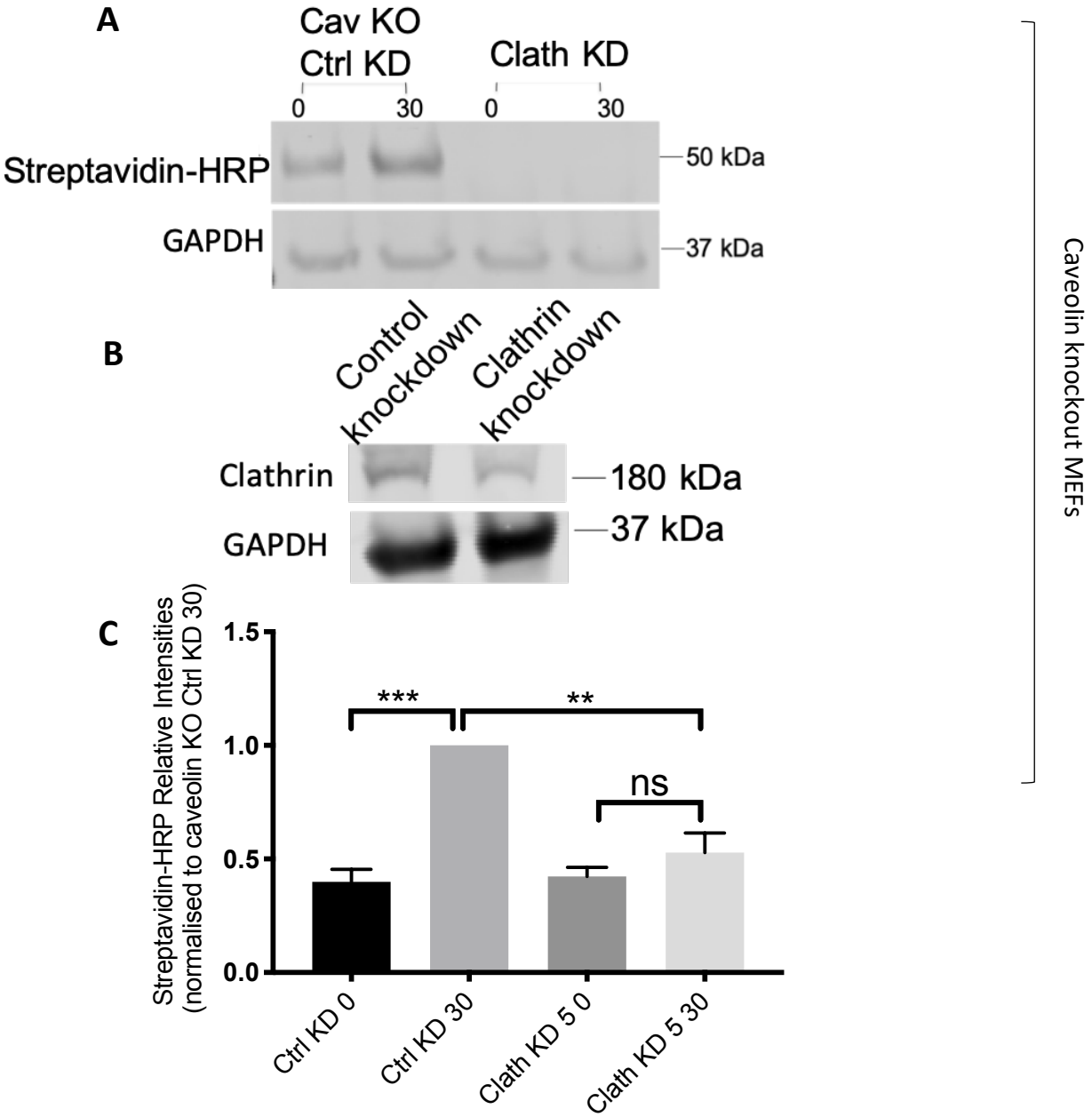


Figure 4.7: Syndecan-4 internalisation in clathrin knockdown caveolin-1 knockout MEFs (using oligonucleotide 5) is clathrin dependant. Syndecan-4 uptake was measured in control knockdown (caveolin knockout MEFs) and clathrin knockdown cells (oligonucleotide 5) using 10 µg/ml biotinylated syndecan-4 antibody and allow 30 minutes of internalisation on ice (0) or 37°C (30) followed by lysing the cells. Cell lysates containing internalised syndecan-4 were analysed by western blotting to compare biotinylated syndecan-4 in each condition using HRP-conjugated streptavidin antibody. Syndecan-4 uptake was significantly reduced to an undetectable level in clathrin knockdown cells when compared to control knockdown within 30 minutes. **(A)** Representative western blot showing cell lysate of clathrin knockdown in caveolin-1 knockout MEFs cells with no syndecan-4 uptake as reflected by anti-streptavidin antibody staining compared to control caveolin-1 knockout MEFs within 30 minutes. **(B)** Western blot lysate showing clathrin expression in control knockdown and clathrin knockdown cells (oligonucleotide 5) in caveolin-1 knockout MEFs with 80% reduction in clathrin expression. **(C)** Graph summarising the quantification of anti-streptavidin antibody demonstrating internalised syndecan-4 antibody in control knockdown and clathrin knockdown for 30 minutes at 0 (on ice) and 30 (37°C). GAPDH was used as loading control. Data was normalised to loading control (GAPDH) and control knockdown 30 of caveolin-1 knockout cells. (n=7 from independent experiment; Ctrl= control, KD= knockdown, clath= clathrin, ns=not significant, ** $p \leq 0.01$, *** $p \leq 0.001$). Error bars represent SEM. Statistical significance was calculated using One-way ANOVA with Tukey's post hoc test.

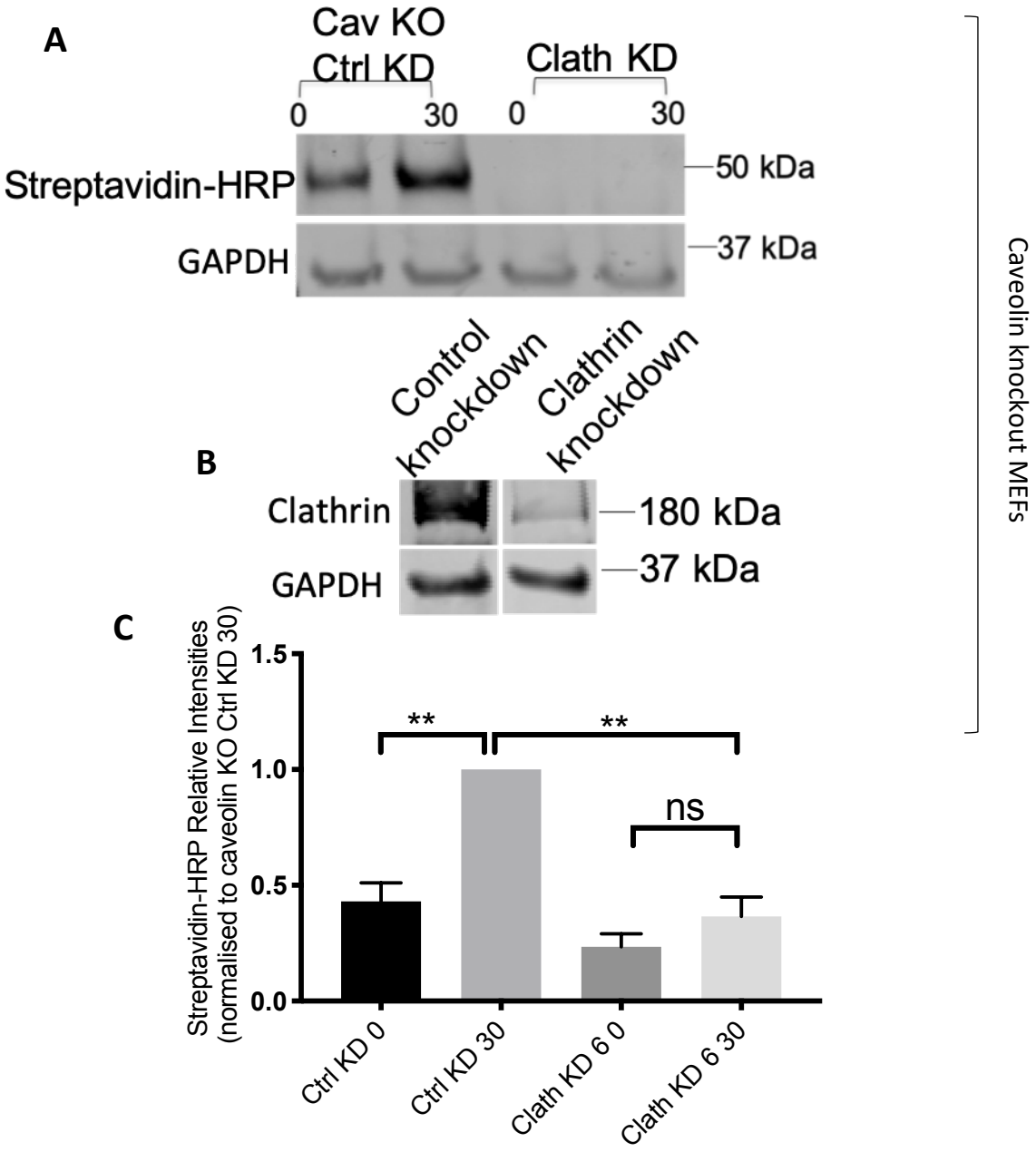


Figure 4.8: Syndecan-4 internalisation in clathrin knockdown caveolin-1 knockout MEFs (using oligonucleotide 6) is clathrin dependant. Syndecan-4 uptake was measured in control knockdown (caveolin knockout MEFs) and clathrin knockdown cells (oligonucleotide 6) using 10 µg/ml biotinylated syndecan-4 antibody and allow 30 minutes of internalisation on ice (0) or 37°C (30) followed by lysing the cells. Cell lysates containing internalised syndecan-4 were analysed by western blotting to compare biotinylated syndecan-4 in each condition using HRP-conjugated streptavidin antibody. Syndecan-4 uptake was significantly reduced to an undetectable level in clathrin knockdown cells when compared to control knockdown within 30 minutes. **(A)** Representative western blot showing cell lysate of clathrin knockdown in caveolin-1 knockout MEFs cells with no syndecan-4 uptake as reflected by anti-streptavidin antibody staining compared to control caveolin-1 knockout MEFs within 30 minutes. **(B)** Western blot lysate showing clathrin expression in control knockdown and clathrin knockdown cells (oligonucleotide 6) with 90% reduction in clathrin expression. **(C)** Graph summarising the quantification of anti-streptavidin antibody demonstrating internalised syndecan-4 antibody in control knockdown and clathrin knockdown for 30 minutes at 0 (on ice) and 30 (37°C). GAPDH was used as loading control. Data was normalised to loading control (GAPDH) and control knockdown 30 of caveolin-1 knockout cells. (n=6 from independent experiment; Ctrl= control, KD= knockdown, clath= clathrin, ns=not significant, ** $p \leq 0.01$). Error bars represent SEM. Statistical significance was calculated using One-way ANOVA with Tukey's post hoc test.

In conclusion, data in this chapter confirm that syndecan-4 endocytosis is mediated by both caveolin and clathrin endocytic mechanisms proposing both candidates as regulators for syndecan-4 receptor uptake. The data is supported by reduced and undetectable syndecan-4 in MEFs when caveolin-1 and clathrin were perturbed (Figure 4.5, 4.7 and 4.8). These data show that like integrin, syndecan-4 utilises both endocytic pathways reinforcing a hypothesis of possible co-trafficking of syndecan-4 and integrin, which needs further investigation.

One of the challenges was maintaining and supressing clathrin in the fast proliferative caveolin-1 knockout MEFs. However, this was expected as some studies demonstrated that caveolin had anti-proliferative effect in tumours. Caveolin-1 downregulation in tumours often leads to increase cell proliferation and lower apoptosis (Cerezo et al., 2009; Torres et al., 2006). Therefore, caveolin-1 knockout MEFs were maintained according to need and passaged at low ratios. Any unused MEFs were frozen immediately.

Chapter 5: Microscopic analysis of the subcellular localisation and internalisation of syndecan-4

Confocal microscopic analysis of subcellular localisation of syndecan-4 post endocytosis can provide a better understanding of internalised vesicles trafficking by colocalisation analysis of fixed time points of syndecan-4 following receptor stimulation. Data in previous chapter indicate that syndecan-4 uptake, as integrin, is mediated by caveolin and clathrin. Hence, it is not known if internalised syndecan-4 vesicles can demonstrate association with caveolin or clathrin. To determine the role of caveolin and clathrin vesicle in syndecan-4 trafficking, fluorescently labelled syndecan-4 antibody was added to media and allowed to internalise in MEFs for 30 minutes before cell fixation and staining for caveolin and clathrin for colocalisation analysis using ImageJ. Colocalisation analysis was done using JACoP plugin, a widely used tool in correlation studies, which enables measurement of both Pearson's and Mander's coefficients as well as visualisation of colocalisation by generating pixel intensity scatterplots (Bolte & Cordelières, 2006). Pearson's and Mander's coefficients are mathematically based on signal intensities but differ in how colocalisation is measured. Pearson's coefficient is based on intensity distribution between channels and deviation from a set threshold, while Mander's is based on the total intensity of fluorophores that coincide with each other (Adler & Parmryd, 2010).

5.1 Localisation analysis of syndecan-4 endocytosis in MEFs

In the biochemical approach, the syndecan-4 receptor was clustered using a biotinylated antibody to mediate receptor internalisation. The uptake of fluorescently labelled antibody in cells would determine if the antibody uptake was an accurate reporter of syndecan-4 internalisation. The hypothesis was that syndecan-4 knockout MEFs would be unable to cluster the receptor in order to internalise the antibody due to deletion of syndecan-4 protein, hence it would be critical to investigate if syndecan-4 antibody could be taken up in a receptor-independent endocytosis. The labelled antibody was compared in both syndecan-4 rescue and knockout MEFs for 10 or 30 minutes to test the hypothesis and distinguish antibody endocytic mechanism. The uptake of syndecan-4 green signal for both conditions was obtained by confocal microscopy and analysed using ImageJ.

Cytoplasmic staining of syndecan-4 antibody was present in syndecan-4 rescue MEFs showing significant increase within 30 minutes when comparing the two endocytic points (Figure 5.1 A), while syndecan-4 knockout MEFs failed to internalise the antibody, matching biochemical data observed in Chapter 3. Syndecan-4 knockout MEFs was used as a negative control to evaluate this assay (Figure 5.1B).

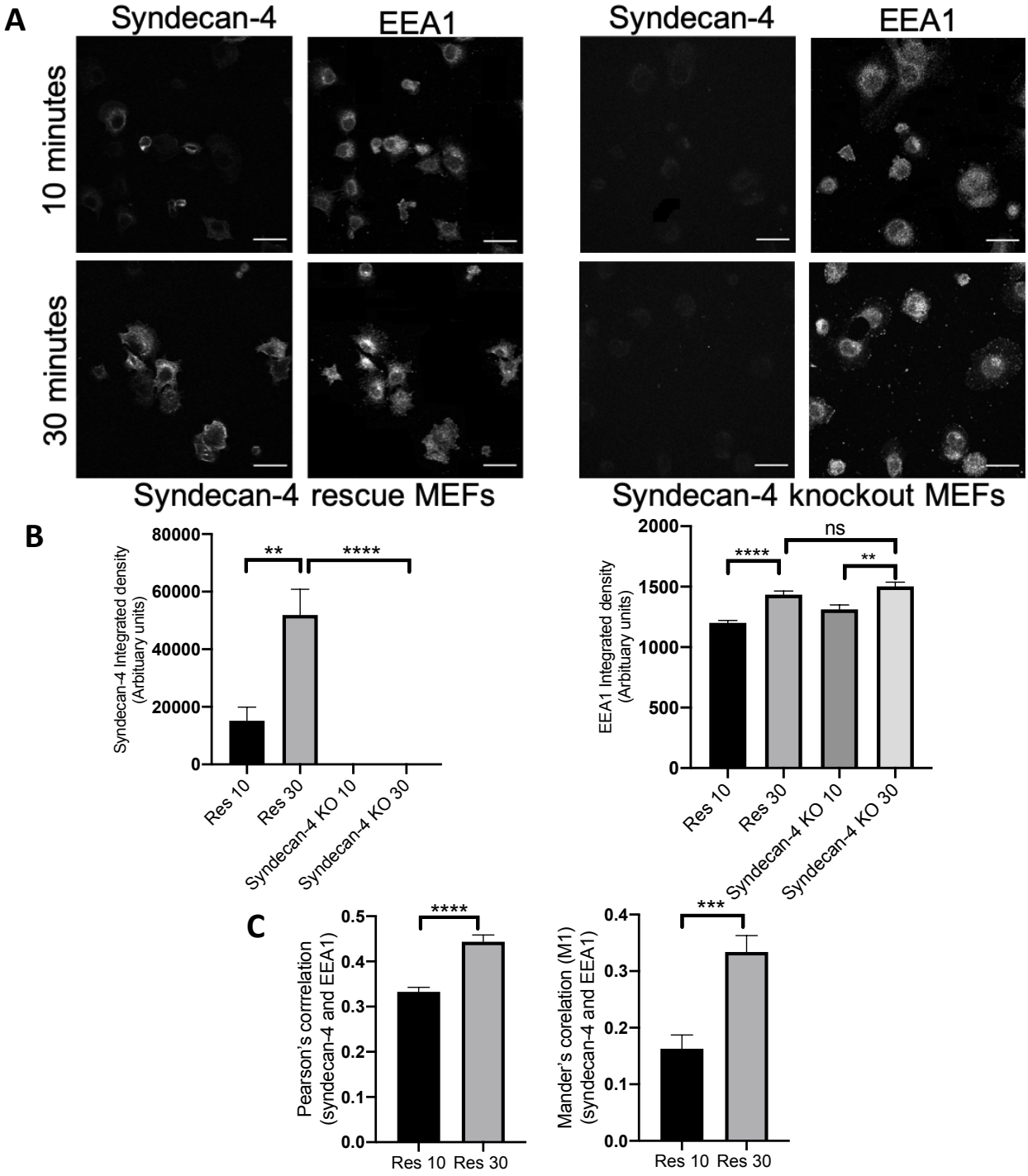


Figure 5.1: Syndecan-4 vesicles are internalised in syndecan-4 rescue MEFs via endosomal route and show colocalisation with EEA1. Syndecan-4 rescue and knockout MEFs were spread on 10 µg/ml 50K for 2 hours before adding 10 µg/ml fluorescent syndecan-4 antibody. Two internalisation time points (10 or 30 minutes) were used at room temperature before fixing and staining for EEA1. Cytoplasmic staining of syndecan-4 was increased within 30 minutes in syndecan-4 rescue MEFs, while EEA1 was similar in both MEFs. **(A)** Immunofluorescence images representing a significant difference in syndecan-4 antibody uptake after 10 and 30 minutes with, while EEA1 showed no clear difference within 30 minutes. **(B)** Quantification of syndecan-4 integrated density by recording the area of fluorescence intensity above an empirically determined threshold after rolling ball background subtraction. Syndecan-4 rescue demonstrated a significant increase in antibody uptake within 30 minutes, whereas knockout MEFs showed no antibody uptake. The quantification of EEA1 integrated density showed a difference within 30 minutes in both cell types when comparing the two time points (n=70-100 cells). **(C)** Colocalisation analysis of syndecan-4 and EEA1 using Pearson's and Mander's M1 correlation coefficients in ImageJ by JACoP plugin (n=11). Both analyses show significant colocalisation of syndecan-4 with EEA1 in syndecan-4 rescue MEFs within 30 minutes. Scale bar=20 µm. Error bars represent SEM. Statistical significance was calculated using One-way ANOVA with Tukey's post hoc test, and Student's t-test. (Res=syndecan-4 rescue MEFs, KO=knockout, ns=not significant, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

5.2 Syndecan-4 is internalised and trafficked via early endosomal route

Early endosomal antigen 1 (EEA1) is a Rab5 effector protein that regulates endocytic pathways by docking of incoming vesicles before fusion with early endosome (Simonsen et al., 1998). To determine if syndecan-4 is internalised and trafficked via endosomal route, syndecan-4 rescue and knockout MEFs were used. Following feeding MEFs with syndecan-4 antibody, fixed cells were stained for early endosomal marker EEA1. Obtained images from confocal microscopy were analysed for correlation using ImageJ.

The level of EEA1 was slightly increased within 30 minutes in both MEFs used showing a similar level when comparing both cell types at 30 minutes (Figure 5.1 B). However, this could be due to the arrangement of EEA1 vesicles in examined images and does not necessarily reflect the level of EEA1 in both cell types used. To determine the localisation of internalised syndecan-4 antibody in EEA1 vesicles, JACoP plugin was used to calculate Pearson's and Mander's M1 (the proportion of syndecan-4 in EEA1) correlation coefficients. The values of syndecan-4 knockout MEFs were not used, as it generated random numbers despite the absence of syndecan-4 antibody signal.

At 10 minutes and 30 minutes post syndecan-4 antibody feeding, syndecan-4 positive vesicles were found to be colocalised with EEA1 (Figure 5.1 C). The colocalisation of syndecan-4 and EEA1 was significantly increased within 30 minutes when comparing the two endocytic points. This data suggests that syndecan-4 may be trafficked via early endosomal route.

5.3. Internalised syndecan-4 vesicles colocalise with caveolin and clathrin

Caveolin and clathrin in the previous chapter demonstrated their roles as mediators of syndecan-4 endocytosis, hence testing internalised antibody for colocalisation with caveolin and clathrin was done in human foreskin fibroblasts (HFFs). To determine syndecan-4 colocalisation with caveolin and clathrin, the labelled syndecan-4 antibody was added to HFFs and antibody was allowed to be internalised for 10 or 30 minutes and fixed cells were stained for caveolin and clathrin using fluorescent antibodies to be examined by confocal microscope. Obtained images were analysed using ImageJ and correlation analysis was done using JACoP plugin.

Cytoplasmic staining of syndecan-4 positive vesicles was present in within 10 minutes post antibody engagement and was increased within 30 minutes (Figure 5.2 A). Caveolin vesicles showed a slightly higher signal at 10 minutes compared to 30 minutes post antibody engagement, which reflected the

arrangement of syndecan-4 vesicles, demonstrating syndecan-4 to be colocalised better with caveolin at 10 minutes compared to 30 minutes post antibody feeding (Figure 5.2 B).

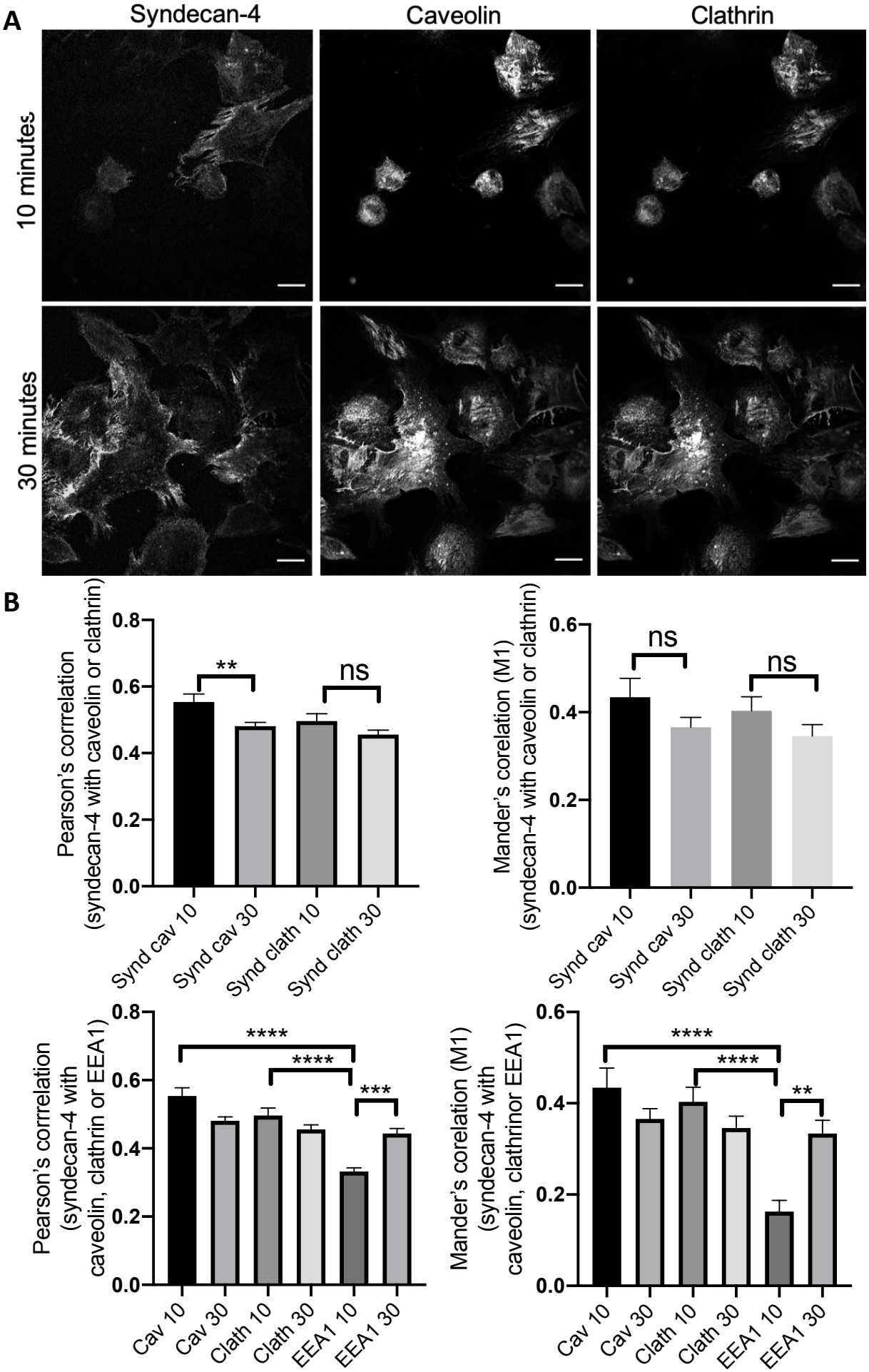
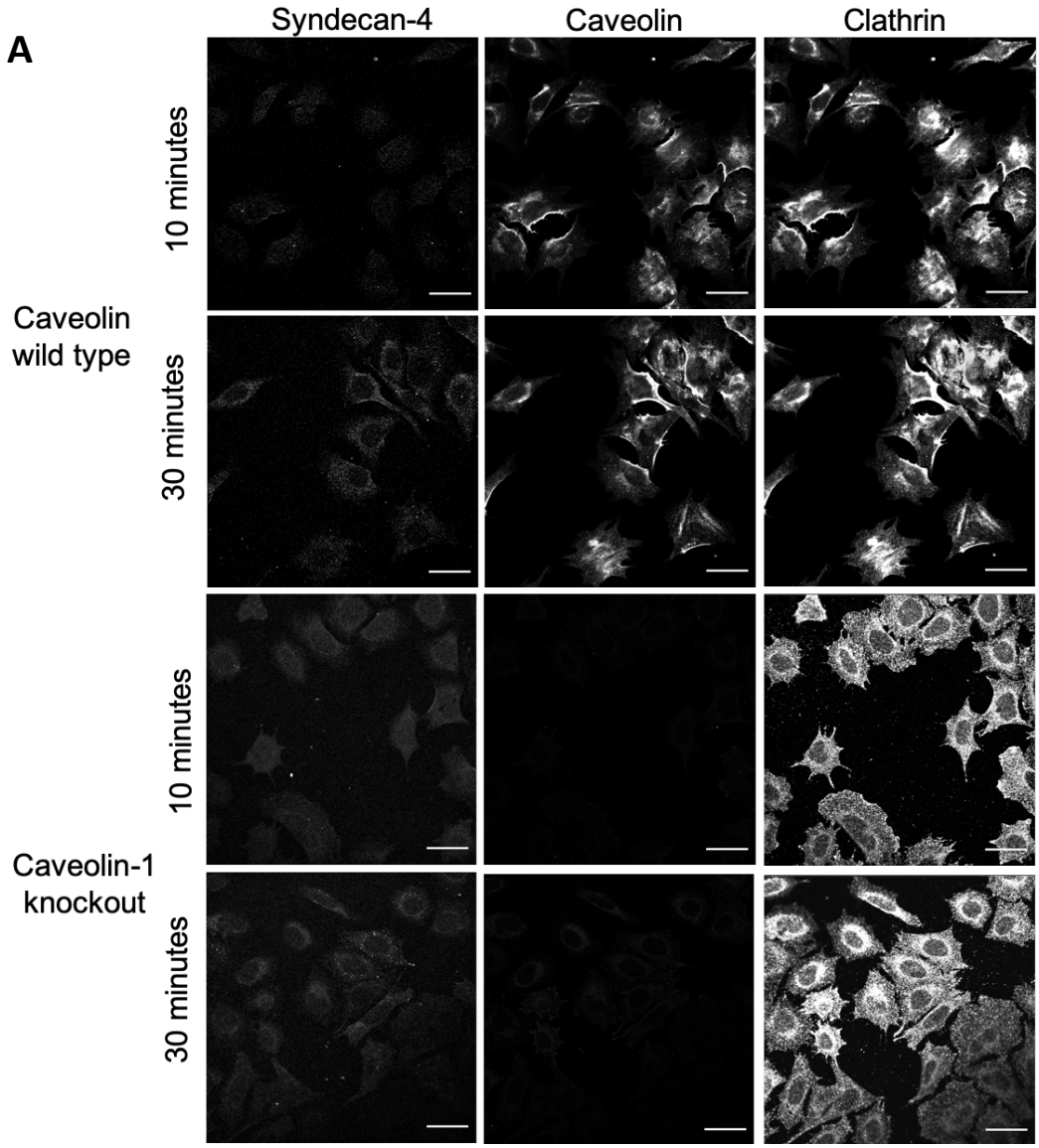
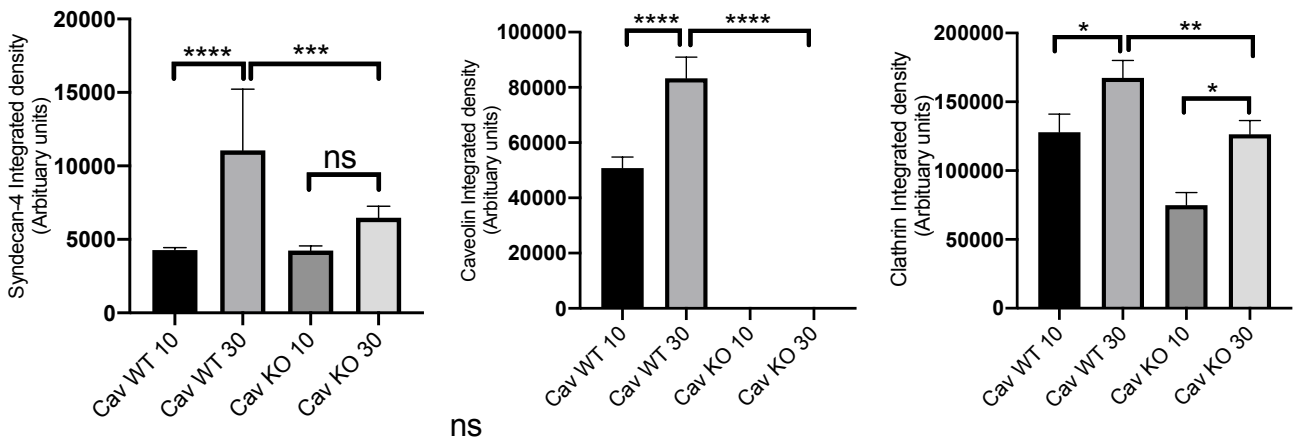


Figure 5.2: Syndecan-4 vesicles are internalised in HFFs and show some colocalisation with caveolin. HFFs were spread on 10 µg/ml 50K for 2 hours before adding 10 µg/ml fluorescent syndecan-4 antibody. Two internalisation time points (10 or 30 minutes) were used at room temperature before fixing and staining for caveolin and clathrin. Cytoplasmic staining of syndecan-4 was increased within 30 minutes, while caveolin and clathrin levels were almost the same. **(A)** Immunofluorescence images representing a significant difference in syndecan-4 antibody uptake after 10 and 30 minutes with, while caveolin and clathrin levels showed no difference within 30 minutes. **(B)** Colocalisation analysis of syndecan-4 and caveolin or clathrin was done after recording the area of fluorescence intensity above an empirically determined threshold after rolling ball background subtraction. Pearson's and Mander's M1 correlation coefficients were measured in ImageJ using JACoP plugin (n=12). Both analyses show similar colocalisation of syndecan-4 with caveolin and clathrin in HFFs within 30 minutes. **(C)** Syndecan-4 shows better association with caveolin and clathrin at 10 minutes when compared to EEA1 demonstrating that internalised syndecan-4 pass through caveolin or clathrin before fusing with early endosomal marker EEA1. Scale bar=20 µm. Error bars represent SEM. Statistical significance was calculated using One-way ANOVA with Tukey's post hoc test. (Synd=syndecan-4, cav=caveolin, clath=clathrin, ns=not significant, ** $p \leq 0.01$*** $p \leq 0.001$**** $p \leq 0.0001$).

Clathrin vesicles demonstrated association with syndecan-4 that was similar to caveolin, in which 10 minutes showed better colocalisation with syndecan-4 compared to 30 minutes when comparing the two time points. Moreover, when comparing the association of internalised syndecan-4 antibody at 10 minutes with EEA1, caveolin and clathrin vesicles, data suggested that syndecan-4 pass through either caveolin or clathrin compartment prior progressing to early endosome (Figure 5.2 C). This hypothesis is plausible as caveolin and clathrin are mediators for syndecan-4 endocytosis. This could explain the lower signal of EEA1 at 10 minutes as most of internalised syndecan-4 was either in caveolin or clathrin compartment.

Syndecan-4 association with clathrin and caveolin was tested further in caveolin wild type and knockout MEFs to test if syndecan-4 internalisation was mediated by clathrin compensation mechanism. Cytoplasmic staining of syndecan-4 positive vesicles was observed in both caveolin wild type and caveolin knockout MEFs within 10 minutes post syndecan-4 engagement (Figure 5.3 A). The level of internalised syndecan-4 was significantly increased within 30 minutes when comparing the two time points in caveolin wild type MEFs. While caveolin knockout MEFs showed minimal insignificant syndecan-4 antibody uptake when comparing 10 and 30 minutes (Figure 5.3 B). Caveolin signal was undetectable in caveolin knockout cells due to absence of caveolin-1 expression in these MEFs, while caveolin wild type showed significant increase within 30 minutes due to caveolin-mediated endocytosis of syndecan-4 resulted from antibody clustering of the syndecan-4 receptor. Clathrin signal showed a significant increase in caveolin wild type MEFs when comparing 10 to 30 minutes of antibody stimulation. Although caveolin knockout MEFs showed an increase in clathrin signal between the two


A
Syndecan-4
Caveolin
Clathrin
10 minutes
30 minutes
Caveolin wild type
Caveolin-1 knockout


B
Syndecan-4 Integrated density (Arbituary units)
Caveolin Integrated density (Arbituary units)
Clathrin Integrated density (Arbituary units)
Cav WT 10
Cav WT 30
Cav KO 10
Cav KO 30
****
***
ns
*
**

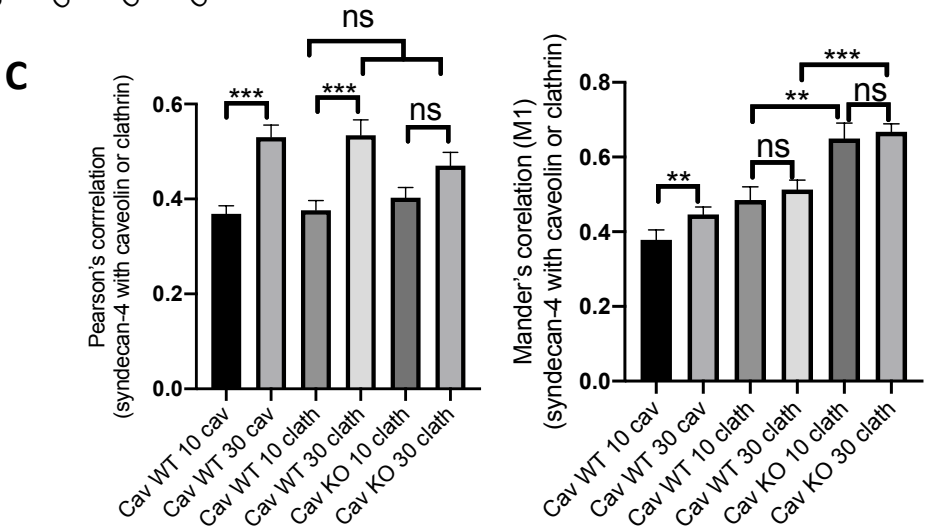

C
Pearson's correlation (syndecan-4 with caveolin or clathrin)
Mander's corelation (M1) (syndecan-4 with caveolin or clathrin)
Cav WT 10 cav
Cav WT 30 cav
Cav WT 10 clath
Cav WT 30 clath
Cav KO 10 clath
Cav KO 30 clath
***
**
ns

Figure 5.3: Syndecan-4 vesicles show colocalisation with caveolin and clathrin. Caveolin wild type and knockout MEFs were spread on 10 μg/ml 50K for 2 hours before adding 10 μg/ml fluorescent syndecan-4 antibody. Two internalisation time points (10 or 30 minutes) were used at room temperature before fixing and staining for caveolin and clathrin. Cytoplasmic staining of syndecan-4, caveolin and clathrin was increased in caveolin wild type and knockout MEFs within 30 minutes. However, clathrin level was lower in caveolin-1 knockout MEFs compared to wild type. **(A)** Immunofluorescence images representing a significant increase in syndecan-4, caveolin and clathrin within 30 minutes in the MEFs used (n=100-140 cells). **(B)** Quantification of syndecan-4, caveolin and clathrin integrated density was done after recording the area of fluorescence intensity above an empirically determined threshold after rolling ball background subtraction showing significant changes in both MEFs used within 30 minutes. **(C)** Colocalisation analysis of syndecan-4 with caveolin or clathrin using Pearson's and Mander's M1 correlation coefficients measured in ImageJ using JACoP plugin (n=10). Syndecan-4 showed colocalisation with caveolin and clathrin in wild type within 30 minutes. Clathrin showed some association with syndecan-4 within 30 minutes in caveolin knockout cells when compared to wild type suggesting weak clathrin compensation in knockout cells. Scale bar=20 μm. Error bars represent SEM. Statistical significance was calculated using one-way ANOVA with Tukey's post hoc test. (cav=caveolin, WT= wild type, KO=knockout, clath=clathrin, ns=not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

time points, it was lower compared to caveolin wild type. The decrease of clathrin signal in caveolin knockout could be technically related to image resolution as the pre-set threshold might have affected the generated value of clathrin signal. The cytoplasmic staining of clathrin in caveolin knockout MEFs could be due to clathrin redistribution, a hypothesis that needs further investigation.

To determine syndecan-4 colocalisation in both MEFs, JACoP plugin was applied to acquired confocal images and Pearson's and Mander's M1 (proportion of syndecan-4 in either compartment) correlation coefficients were calculated using ImageJ. After stimulating caveolin wild type with the syndecan-4 antibody for 30 minutes, syndecan-4 positive vesicles showed some association with caveolin (Figure 5.3 C). However, caveolin knockout cells showed no caveolin signal, hence no analysis was needed. Clathrin vesicles in caveolin wild type showed colocalisation as well with syndecan-4 in caveolin wild type within 30 minutes post antibody feeding. Caveolin knockout MEFs showed an association of clathrin vesicles with syndecan-4 within 30 minutes post antibody engagement when comparing the proportion of syndecan-4 in clathrin vesicles. Data from these experiments suggest that syndecan-4 colocalise with caveolin and clathrin vesicles and demonstrate clathrin compensation in caveolin knockout MEFs.

In conclusion, data in this chapter demonstrate that syndecan-4 might pass through either caveolin or clathrin compartment first before fusing with endosomal compartment confirming the role of caveolin and clathrin as mediators for syndecan-4 endocytosis. Syndecan-4 shows colocalisation with early endosomal marker EEA1 but further refinement is needed to test syndecan-4 colocalisation with

caveolin and clathrin as image quality was a limiting factor in the technique used. Clathrin showed some compensation in syndecan-4 internalisation matching the biochemical data in the previous chapter. However, it is recommended to investigate this further as image quality could be improved using digitalised image reconstruction techniques.

Calculating integrated density and colocalisation for this chapter was challenging as image quality affected data analysis, thus image reconstructing techniques including stochastic optical reconstruction microscopy imaging (STORM) might be a better alternative for enhanced quality images with better resolution compared to images acquired using confocal microscopy. The florescence signal generated from the caveolin channel (cy3) might have crossed to the clathrin channel (cy5) affecting image analysis, hence further optimisation is needed. Labelling syndecan-4 antibody using blue colour could be useful to simplify image analysis. Also switching to a different secondary antibody could be beneficial to overcome fluorescence cross over between channels. The antibody generated from X22 cells might require further purification as the it contained cell debris which was observed in microscopic images.

Chapter 6: Syndecan-4 internalisation requires syndecan-4 signalling via PKCα

Syndecan-4 engagement with fibronectin leads to PKCα activation, which is one of the early events that take place upon receptor engagement, in addition to Rac1 activation, RhoA suppression and RhoG activation regulating cell polarity and migration via forming membrane protrusion and caveolin-dependant endocytosis of integrin, respectively. The later events of receptor engagement are to stabilise focal adhesion and is achieved through RhoA reactivation by releasing it from the sequestering RhoGDI and Arf6 activation which regulates integrin recycling (Figure 1.4). The cytoplasmic V region of syndecan-4 (but not other syndecans) can bind the catalytic domain of PKCα directly, hence the hypothesis is that PKCα knockdown or inhibition could block syndecan-4 signalling downstream to RhoG, Rac1 and RhoA during cell migration and subsequent downstream events involved in cell migration (Bass et al., 2011; Brooks et al., 2012; Oh et al., 1997b). Although the relationship between PKCα and syndecan-4 was reported previously (Brooks et al., 2012; Koo et al., 2006), the role of PKCα in syndecan-4 endocytosis and its relationship with the cytoplasmic domain in syndecan-4 signalling during receptor activation is still unclear.

6.1 PKC-binding motif and PKCα are required for syndecan-4 endocytosis

To determine the role of PKC-binding motif of syndecan-4 cytoplasmic domain in receptor signalling and endocytosis, syndecan-4 rescue and PKC-binding mutant (Y188L) MEFs were utilised to compare biotinylated syndecan-4 antibody uptake by western blot using HRP-conjugated streptavidin antibody. In parallel, the effect of PKCα on syndecan-4 internalisation was investigated using PKCα inhibitor, bisindolylmaleimide I (BIM-I) and siRNA to knockdown PKCα**.**

Y188L demonstrated a notable reduction in syndecan-4 uptake when compared to the syndecan-4 rescue (Figure 6.1), which demonstrated significant uptake within 30 minutes of internalisation similar to earlier experiments. Although PKC-binding mutant showed residual uptake of syndecan-4 within 30 minutes, Y188L at 30 was three times lower than syndecan-4 rescue. Syndecan-4 surface expression of syndecan-4 rescue and Y188L was compared using flow cytometry demonstrating similar expression of syndecan-4 in both cell types tested (Figure 6.1 C). Data from 6.1 demonstrated that PKC-binding motif is required for receptor internalisation.

To determine the role PKCα in syndecan-4 endocytosis, BIM-1 was utilised to inhibit PKCα in MEFs. BIM-1 had the advantage of fast PKCα inhibition compared to PKCα knockdown, hence cells were treated for 30 minutes with 100 and 200 nM BIM-1 prior to biotinylated antibody uptake. BIM-I-treated

MEFs showed a significant reduction in syndecan-4 internalisation within 30 minutes compared to DMSO- control (Figure 6.2).

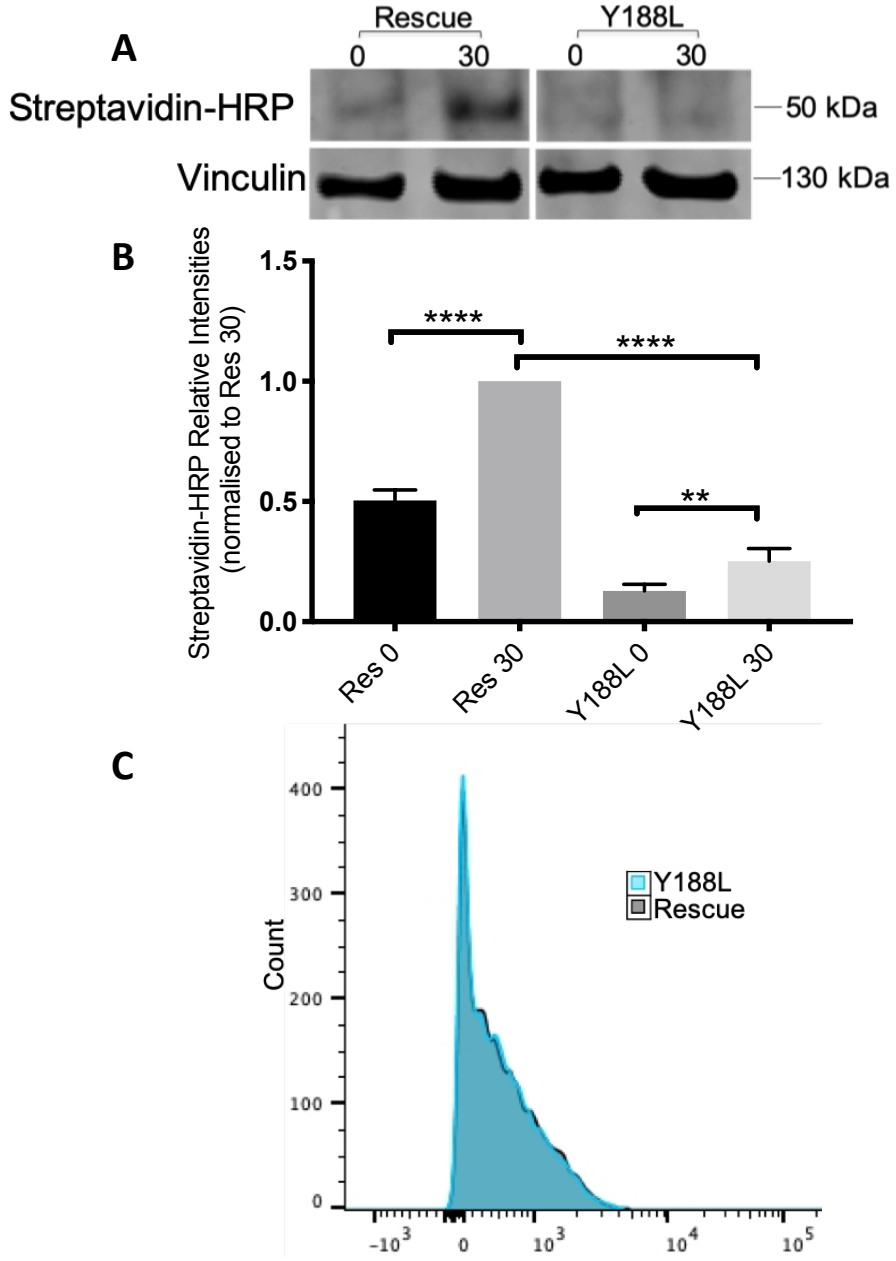


Figure 6.1: PKCα and PKC-binding motif is required for syndecan-4 internalisation and signalling. Syndecan-4 uptake was measured in syndecan-4 rescue and Y188L MEFs using using 10 µg/ml biotinylated syndecan-4 antibody and allow 30 minutes of internalisation on ice (0) or 37°C (30) followed by lysing the cells. Cell lysates containing internalised syndecan-4 were analysed by western blotting to compare biotinylated syndecan-4 in each condition using HRP-conjugated streptavidin antibody. Syndecan-4 uptake was blocked in Y188L within 30 minutes. Although Y188L showed residual receptor internalisation, it was 3 folds lower than syndecan-4 rescue. **(A)** Representative western blot showing cell lysate of syndecan-4 rescue and Y188L cells demonstrating undetectable antibody uptake in Y188L within 30. **(B)** Graph summarising the quantification of internalised anti-streptavidin antibody demonstrating significantly lower antibody uptake when comparing syndecan-4 recue 30 to Y188L 30. (n=14 from independent experiment. Vinculin was used as loading control. Data were normalised to loading control (vinculin) and syndecan-4 rescue 30. **(C)** Flow cytometry data showing similar expression of syndecan-4 in syndecan-4 rescue and Y188L. (Res= syndecan-4 rescue, ** $p \leq 0.01$, **** $p \leq 0.0001$). Error bars represent SEM. Statistical significance was calculated using one-way ANOVA with Tukey's post hoc test.

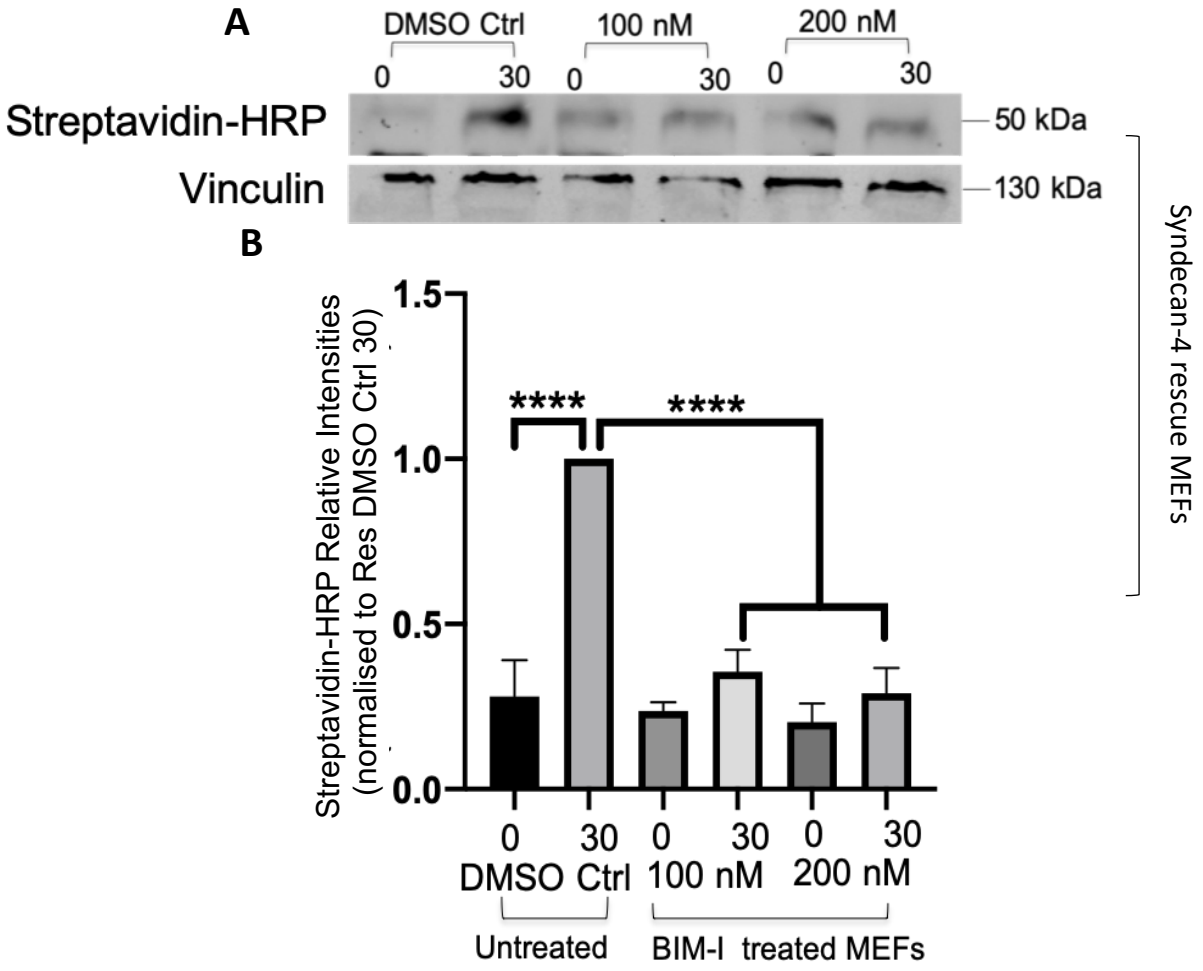


Figure 6.2: BIM-I treatment reduces syndecan-4 endocytosis in treated syndecan-4 rescue MEFs. Syndecan-4 rescue cells were treated with 100 or 200 nM BIM-I for 30 minutes after spreading on10 µg/ml 50K to inhibit PKCα activity. Then syndecan-4 uptake was measured in treated and untreated cells using 10 µg/ml biotinylated syndecan-4 antibody and allow 30 minutes of internalisation on ice (0) or 37°C (30) followed by lysing the cells. Cell lysates containing internalised syndecan-4 were analysed by western blotting to compare biotinylated syndecan-4 in each condition using HRP-conjugated streptavidin antibody. BIM-I treatment at 100 and 200 nM reduced syndecan-4 uptake in treated cells when compared to DMSO control within 30 minutes. **(A)** Representative western blot showing cell lysate of syndecan-4 rescue MEFs treated and untreated cells showing a difference in syndecan-4 antibody uptake reflected by reduced anti-streptavidin antibody staining when comparing the two concentration of BIM-I-treated cells at 30 to DMSO control 30. **(B)** Graph summarising the quantification of anti-streptavidin antibody demonstrating internalised syndecan-4 antibody in DMSO control untreated and BIM-I-treated cells for 30 minutes at 0 (on ice) and 30 (37°C). vinculin was used as loading control. Data were normalised to loading control (vinculin) and syndecan-4 rescue DMSO control 30. (n=7 from independent experiment; Ctrl= control, **** $p \leq 0.0001$). Error bars represent SEM. Statistical significance was calculated using one-way ANOVA with Tukey's post hoc test.

Although reports showed a wide range of concentrations to inhibit PKCα activity, (starting from 10 nM (Więdłocha et al. 2005) to 10 µM (Im et al., 2007)), both concentrations used were based on previous investigation testing PKCα downstream effect upon syndecan-4 receptor engagement with H/0 (Bass et al., 2007). Data obtained demonstrated that PKCα was required for syndecan-4 internalisation

matching previous reports. To eliminate possible off-target effect and specificity of the pharmacological agent BIM-I, PKCα knockdown was performed.

PKCα knockdown to less than 10% (Figure 6.3 B and 6.4 B) in syndecan-4 rescue MEFs showed attenuated syndecan-4 antibody uptake compared to control knockdown within 30 minutes in both oligonucleotides used (Figure 6.3 C and 6.4 C) matching the data of BIM-I-treated cells and confirming the key role of PKCα in syndecan-4 receptor uptake.

The role of PKCα in caveolin-dependant endocytosis was covered before in the lab (Bass et al., 2011) and data in Figure 6.1-6.4 showed evidence of syndecan-4 dependant signalling to PKCα, but PKCα involvement in clathrin dependant endocytosis of syndecan-4 has not been investigated. Hence, PKCα was suppressed in caveolin-1 knockout MEFs using BIM-I and siRNA followed by biotinylated syndecan-4 antibody uptake testing.

First, BIM-I was used to determine the role of PKCα in mediating clathrin-dependant endocytosis of syndecan-4 in caveolin-1 knockout cells. BIM-I pre-treatment for 30 minutes demonstrated lower syndecan-4 antibody uptake in treated cells when compared to DMSO-control (Figure 6.5). Caveolin-1 knockout MEFs treated with 100 and 200 nM BIM-I showed a significant reduction in receptor endocytosis by almost one and two folds, respectively when compared to DMSO-control at 30. BIM-I treatment data of caveolin-1 knockout demonstrated that PKCα was involved in mediating syndecan-4 endocytosis via clathrin-dependent endocytosis. Although BIM-I treatment in syndecan-4 rescue and caveolin-1 knockout MEFs showed that PKCα mediated syndecan-4 internalisation via caveolin and clathrin endocytosis, clathrin showed less sensitivity at 100 nM (compared to 200 nM) which could be due to BIM-I titration resulting in incomplete inhibition in both MEFs used.

Second, to eliminate possible off-target effect and specificity of BIM-I inhibition, PKCα was suppressed using siRNA and biotinylated antibody uptake was measured. PKCα knockdown to less than 30% and 20% (using oligonucleotide 5 Figure 6.6 B and 6 Figure 6.7 B, respectively) demonstrated ablation of syndecan-4 antibody uptake compared to control knockdown within 30 minutes in both oligonucleotides used (Figure 6.6 C and 6.7 C). Collectively, these experiments demonstrate the following: PKCα knockdown or substitution of PKCα-binding motif of syndecan-4 blocks syndecan-4 endocytosis, establishing the role of PKCα in syndecan-4 endocytosis. And that PKCα activation by syndecan-4 receptor engagement is essential for caveolin- and clathrin-mediated endocytosis.

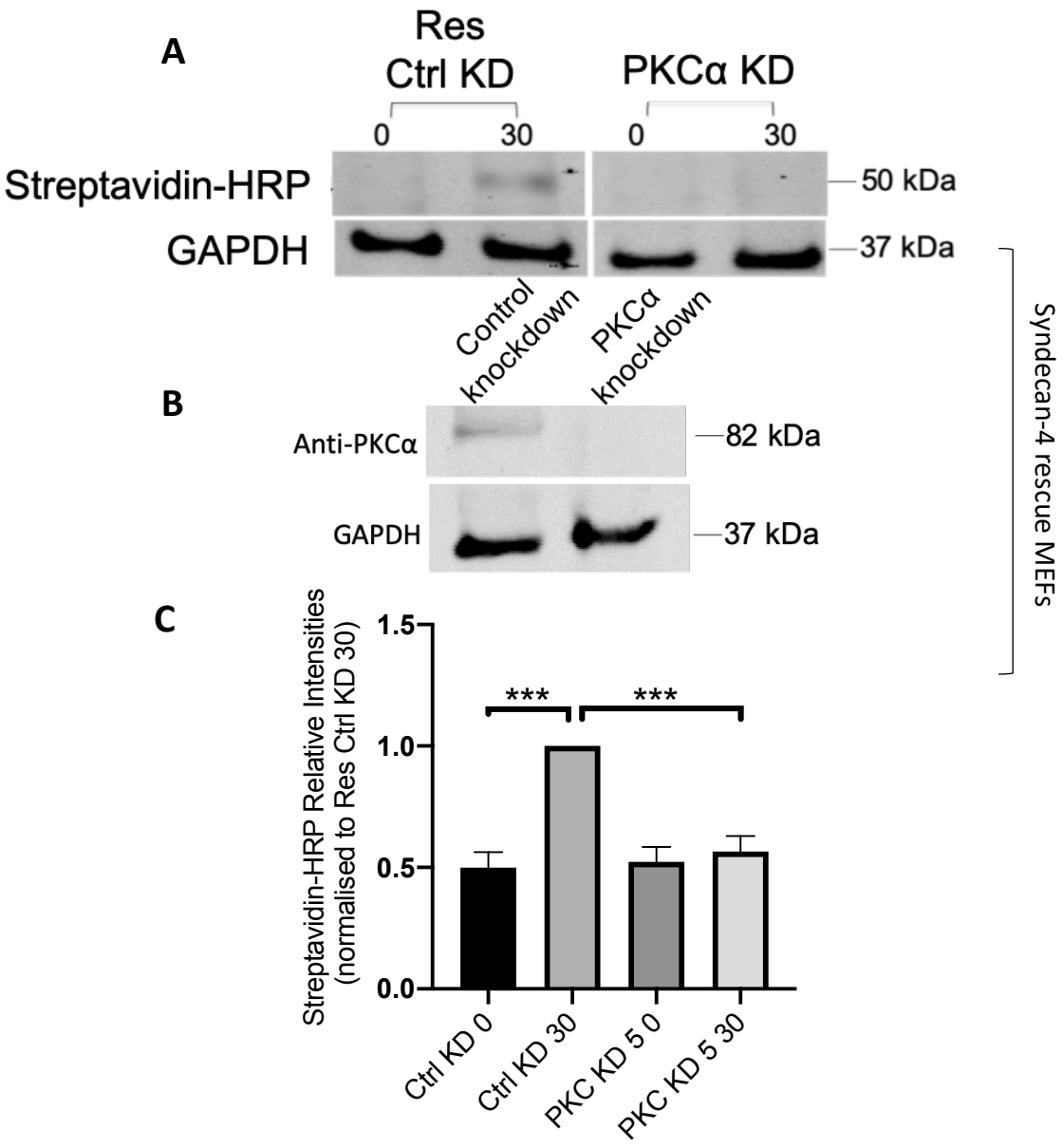


Figure 6.3: PKCα knockdown (using oligonucleotide 5) in syndecan-4 rescue MEFs blocks syndecan-4 internalisation. Syndecan-4 uptake was measured in control knockdown cells and PKCα knockdown (using oligonucleotide 5) using 10 μg/ml biotinylated syndecan-4 antibody and allow 30 minutes of internalisation on ice (0) or 37°C (30) followed by lysing the cells. Cell lysates containing internalised syndecan-4 were analysed by western blotting to compare biotinylated syndecan-4 in each condition using HRP-conjugated streptavidin antibody. Syndecan-4 uptake was blocked in PKCα knockdown 30 when compared to control knockdown 30. **(A)** Representative western blot showing cell lysate of control knockdown cells with showing a difference in syndecan-4 antibody uptake reflected by blocked anti-streptavidin antibody staining in PKCα knockdown when compared to control knockdown. **(B)** Western blot lysate showing PKCα levels in control and PKCα knockdown cells (oligonucleotide 5) showing 10% PKCα expression. **(C)** Graph summarising the quantification of internalised anti-streptavidin antibody demonstrating syndecan-4 antibody in control knockdown and PKCα knockdown for 30 minutes at 0 (on ice) and 30 (37°C). GAPDH was used as loading control. Data were normalised to loading control (GAPDH) and syndecan-4 rescue control knockdown 30. (n=4 from independent experiment; Ctrl= control, KD= knockdown, *** $p \leq 0.001$). Error bars represent SEM. Statistical significance was calculated using One-way ANOVA with Tukey's post hoc test.

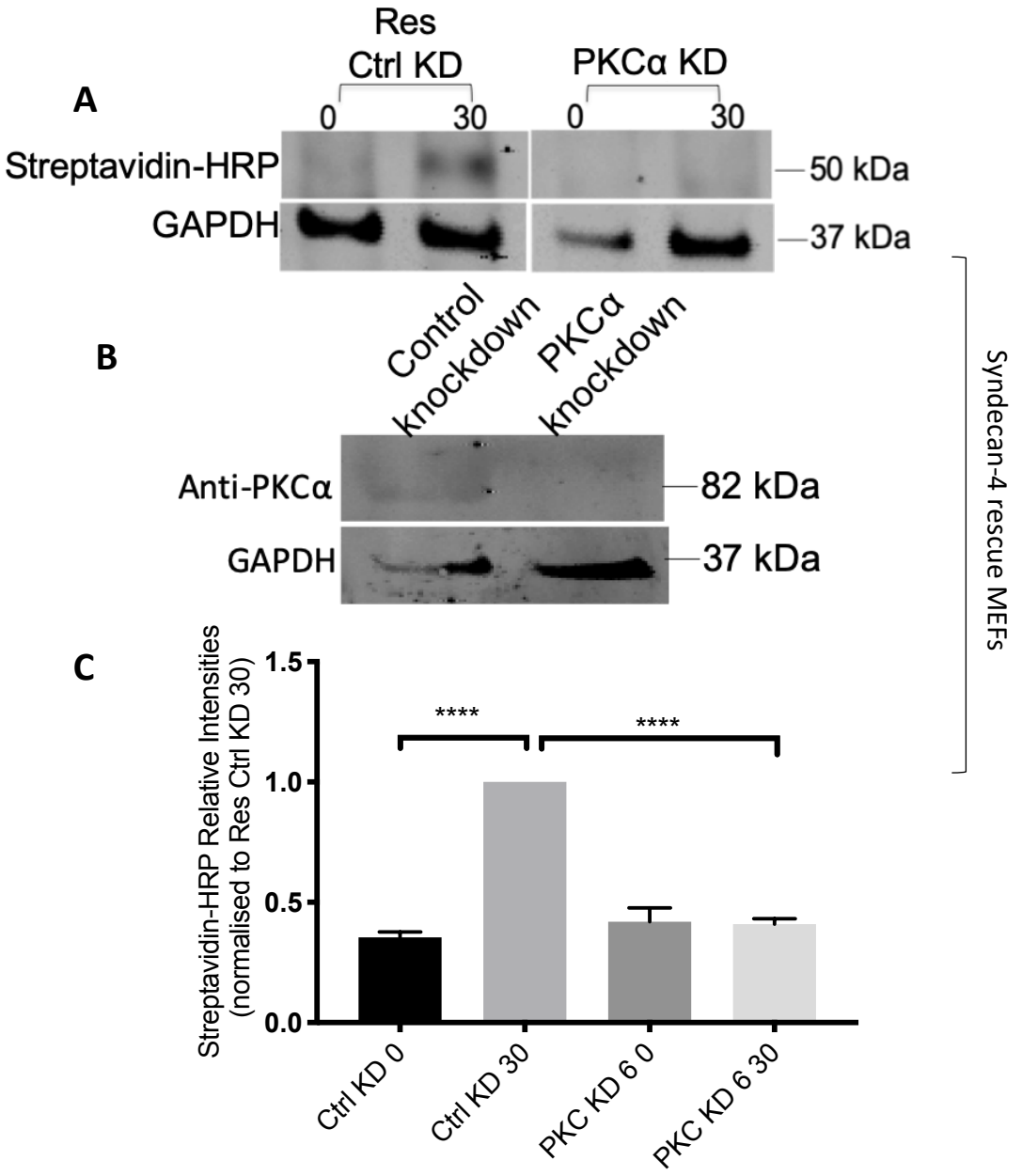


Figure 6.4: PKCα knockdown (using oligonucleotide 6) in syndecan-4 rescue MEFs blocks syndecan-4 internalisation. Syndecan-4 uptake was measured in control knockdown cells and PKCα knockdown (using oligonucleotide 6) using 10 μg/ml biotinylated syndecan-4 antibody and allow 30 minutes of internalisation on ice (0) or 37°C (30) followed by lysing the cells. Cell lysates containing internalised syndecan-4 were analysed by western blotting to compare biotinylated syndecan-4 in each condition using HRP-conjugated streptavidin antibody. Syndecan-4 uptake was blocked in PKCα knockdown 30 MEFs when compared to control knockdown 30. **(A)** Representative western blot showing cell lysate of control knockdown cells with showing a difference in syndecan-4 antibody uptake reflected by blocked anti-streptavidin antibody staining in PKCα knockdown when compared to control knockdown. **(B)** Western blot lysate showing PKCα levels in control and PKCα knockdown cells (oligonucleotide 6) showing 10% PKCα expression. **(C)** Graph summarising the quantification of internalised anti-streptavidin antibody demonstrating syndecan-4 antibody in control knockdown and PKCα knockdown for 30 minutes at 0 (on ice) and 30 (37°C). GAPDH was used as loading control. Data were normalised to loading control (GAPDH) and syndecan-4 rescue control knockdown 30. (n=5 from independent experiment; Ctrl= control, KD= knockdown, **** $p \leq 0.0001$). Error bars represent SEM. Statistical significance was calculated using One-way ANOVA with Tukey's post hoc test.

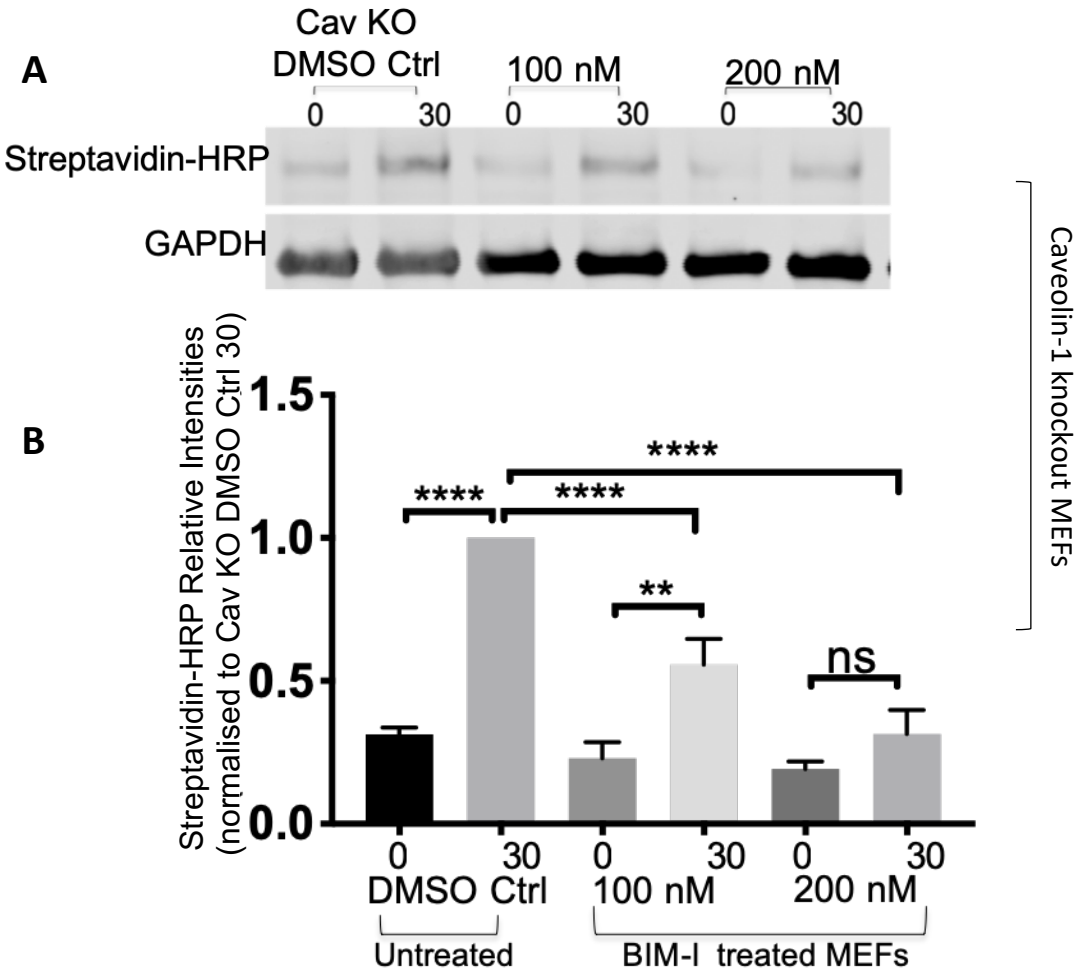


Figure 6.5: BIM-I treatment reduces syndecan-4 endocytosis in treated caveolin-1 knockout MEFs. Caveolin-1 knockout MEFs were treated with 100 or 200 nM BIM-I for 30 minutes after spreading on 10 µg/ml 50K to inhibit PKCα activity. Then syndecan-4 uptake was measured in treated and untreated cells using 10 µg/ml biotinylated syndecan-4 antibody and allow 30 minutes of internalisation on ice (0) or 37°C (30) followed by lysing of cells. Cell lysates containing internalised syndecan-4 were analysed by western blotting to compare biotinylated syndecan-4 in each condition using HRP-conjugated streptavidin antibody. BIM-I treatment at 100 and 200 nM reduced syndecan-4 uptake in treated cells when compared to DMSO control within 30 minutes. **(A)** Representative western blot showing cell lysate of caveolin-1 knockout MEFs treated and untreated cells showing a difference in syndecan-4 antibody uptake reflected by reduced anti-streptavidin antibody staining when comparing the two concentration of BIM-I-treated cells at 30 to DMSO control 30. **(B)** Graph summarising the quantification of anti-streptavidin antibody demonstrating internalised syndecan-4 antibody in DMSO control untreated and BIM-I-treated cells for 30 minutes at 0 (on ice) and 30 (37°C). GAPDH was used as loading control. Data were normalised to loading control (GAPDH) and caveolin-1 knockout DMSO control 30. (n=6 from independent experiment; Ctrl= control, ns=not significant, ** $p \leq 0.01$, **** $p \leq 0.0001$). Error bars represent SEM. Statistical significance was calculated using One-way ANOVA with Tukey's post hoc test.

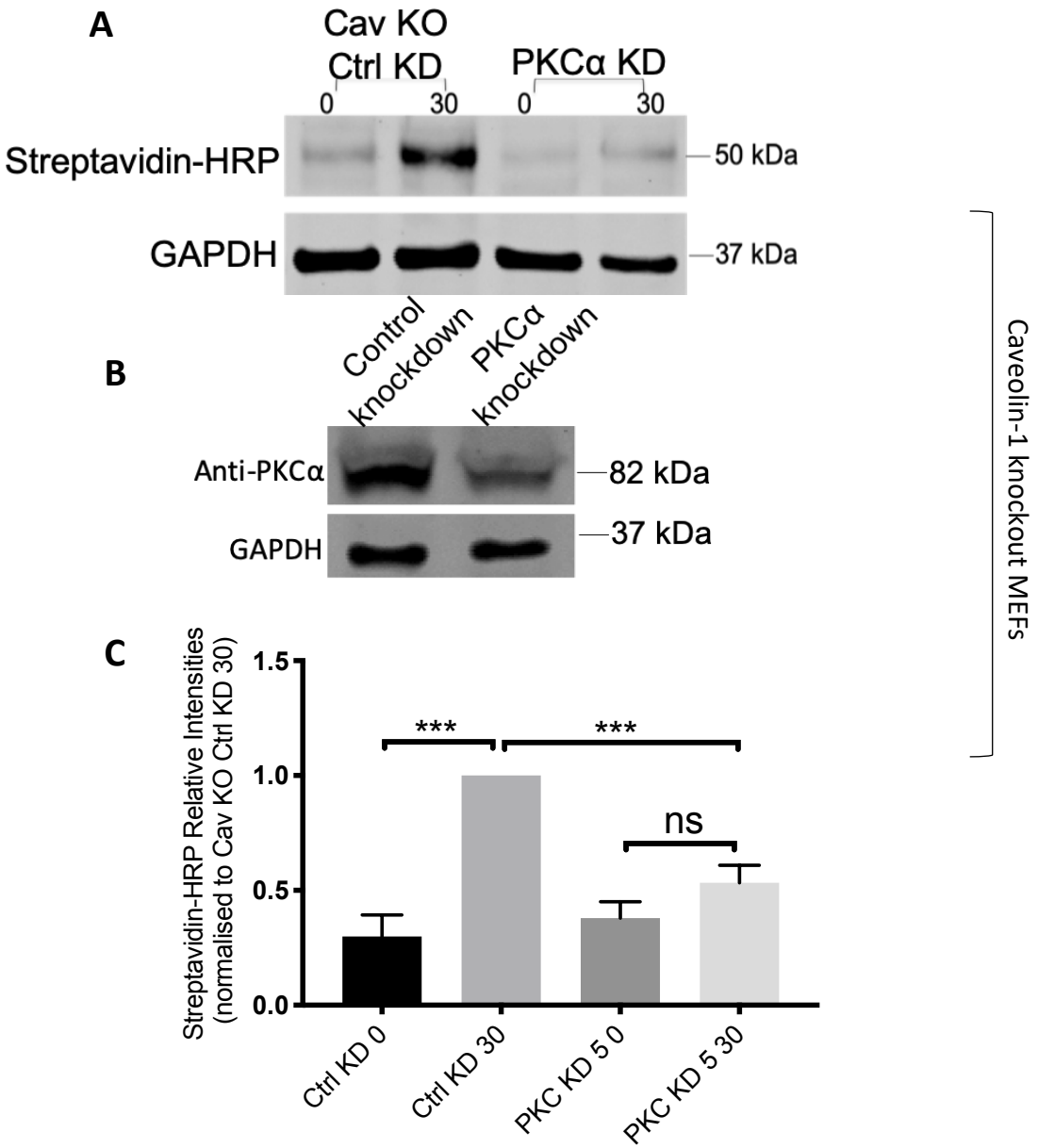


Figure 6.6: PKCα knockdown (using oligonucleotide 5) in caveolin-1 knockout MEFs blocks syndecan-4 internalisation. Syndecan-4 uptake was measured in control knockdown cells and PKCα knockdown (using oligonucleotide 5) using 10 μg/ml biotinylated syndecan-4 antibody and allow 30 minutes of internalisation on ice (0) or 37°C (30) followed by lysing the cells. Cell lysates containing internalised syndecan-4 were analysed by western blotting to compare biotinylated syndecan-4 in each condition using HRP-conjugated streptavidin antibody. Syndecan-4 uptake was blocked in PKCα knockdown 30 MEFs when compared to control knockdown 30 **(A)** Representative western blot showing cell lysate of control knockdown cells showing a difference in syndecan-4 antibody uptake reflected by reduced anti-streptavidin antibody staining in PKCα knockdown when compared to control knockdown. **(B)** Western blot lysate showing PKCα levels in control and PKCα knockdown cells (oligonucleotide 5) showing 30% PKCα expression. **(C)** Graph summarising the quantification of internalised anti-streptavidin antibody demonstrating syndecan-4 antibody in control knockdown and PKCα knockdown for 30 minutes at 0 (on ice) and 30 (37°C). GAPDH was used as loading control. Data were normalised to loading control (GAPDH) and caveolin-1 knockout control knockdown 30. (n=7 from independent experiment; Ctrl= control, KD= knockdown, *** $p \leq 0.001$). Error bars represent SEM. Statistical significance was calculated using One-way ANOVA with Tukey's post hoc test.

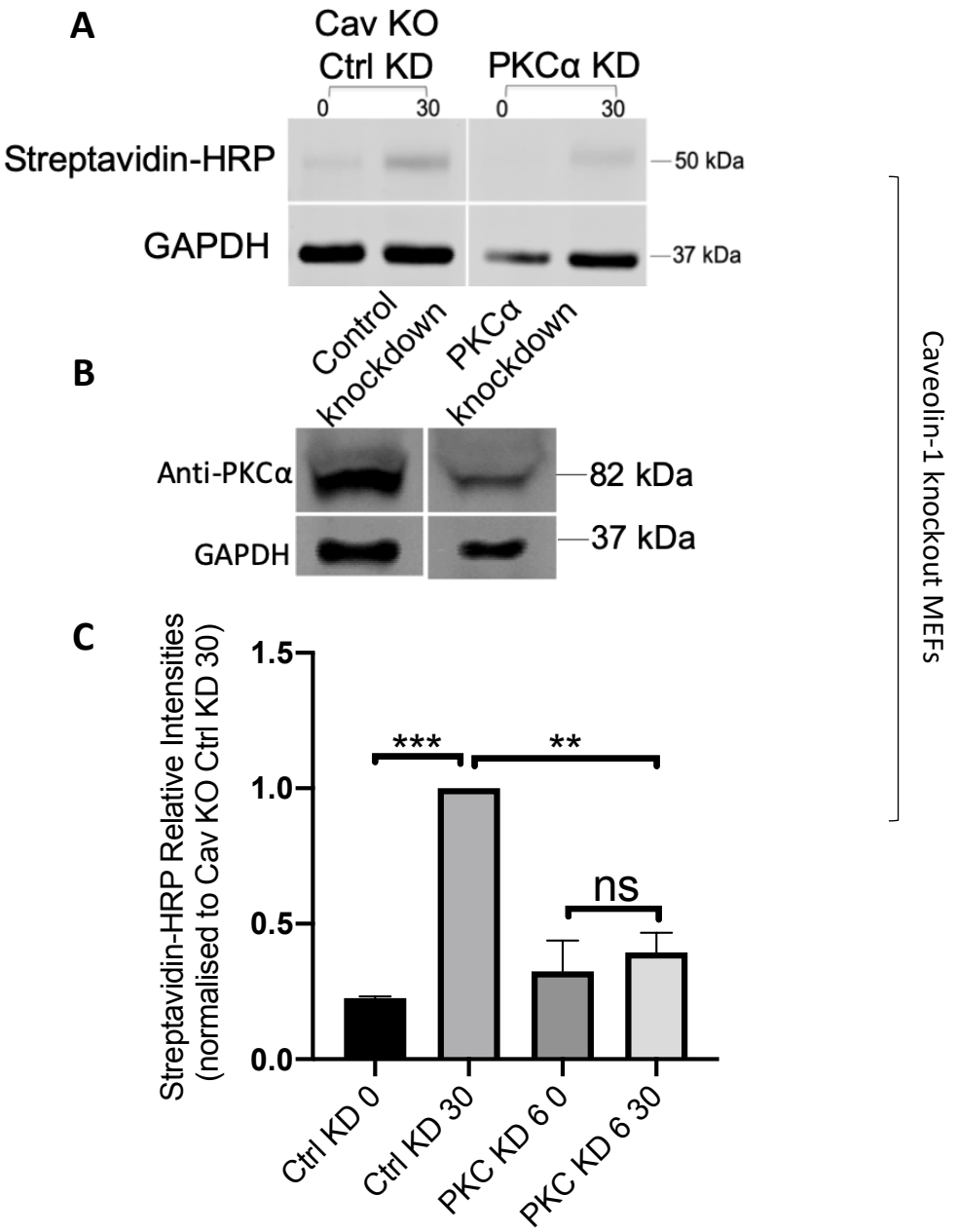


Figure 6.7: PKCα knockdown (using oligonucleotide 6) in caveolin-1 knockout MEFs blocks syndecan-4 internalisation. Syndecan-4 uptake was measured in control knockdown cells and PKCα knockdown (using oligonucleotide 6) using 10 µg/ml biotinylated syndecan-4 antibody and allow 30 minutes of internalisation on ice (0) or 37°C (30) followed by lysing the cells. Cell lysates containing internalised syndecan-4 were analysed by western blotting to compare biotinylated syndecan-4 in each condition using HRP-conjugated streptavidin antibody. Syndecan-4 uptake was blocked in PKCα knockdown 30 MEFs when compared to control knockdown 30. **(A)** Representative western blot showing cell lysate of control knockdown cells demonstrating a difference in syndecan-4 antibody uptake reflected by reduced anti-streptavidin antibody staining in PKCα knockdown 30 when compared to control knockdown 30. **(B)** Western blot lysate showing PKCα levels in control and PKCα knockdown cells (oligonucleotide 6) showing 20% PKCα expression. **(C)** Graph summarising the quantification of internalised anti-streptavidin antibody demonstrating syndecan-4 antibody in control knockdown and PKCα knockdown for 30 minutes at 0 (on ice) and 30 (37°C). GAPDH was used as loading control. Data were normalised to loading control (GAPDH) and caveolin-1 knockout control knockdown 30. (n=7 from independent experiment; Ctrl= control, KD= knockdown, ns= not significant, ** $p \leq 0.01$, *** $p \leq 0.001$). Error bars represent SEM. Statistical significance was calculated using One-way ANOVA with Tukey's post hoc test.

6.2 Syndecan-4 receptor clustering is required for syndecan-4 internalisation

Receptor engagement of syndecan-4 with fibronectin enables fibroblasts to detect minimal changes in the surrounding environment. Subsequent interaction and coordination between syndecan-4 and integrin are key elements in regulating cell adhesion and migration for effective wound healing. Syndecan-4 can stabilise interactions between ECM and cytoskeletal-bound proteins via clustering, similar to integrin. Syndecan-4 mediates its surface expression and receptor clustering induces its redistribution as reported previously (Tkachenko & Simons, 2002), therefore, the balance of syndecan-4 surface expression, internalisation and recycling back to the plasma membrane by endosomal compartment is essential and contribute to vital cell processes including cell adhesion, migration and polarisation. Generally, surface receptors can be internalised by two mechanisms: constitutive endocytosis where no ligand interaction is required, like transferrin in clathrin-mediated endocytosis (Cao et al., 2016), or ligand dependant where endocytosis occurs only when the receptor is bound to the corresponding ligand, like fibroblast growth factor binding to fibroblast growth factor receptor (Haugsten et al., 2011). Syndecan-4 receptor engagement and clustering by the biotinylated antibody was sufficient to trigger receptor internalisation, mimicking ligand-dependant endocytosis.

Syndecan-4 antibody (as well as other antibodies) are composed of two regions: antigen binding region (fab) and the fragment of crystallisation (FC) region, and together the antibody promotes receptor binding, clustering and triggering receptor-mediated activity. However, it is not known if syndecan-4 fab fragment can engage the receptor by itself to trigger receptor clustering, or if the fab fragment is internalised in a ligand dependant manner. Therefore, to determine if syndecan-4 fab fragment can act as a ligand to trigger receptor clustering, syndecan-4 antibody was enzymatically cleaved, and the produced fab fragment was biotinylated for endocytic testing in syndecan-4 rescue and knockout MEFs.

Surface staining of unquenched fab fragment represented fragment which was bound to the cell surface but not internalised in syndecan-4 rescue MEFs demonstrating that the biotinylated fab fragment produced was functionally stable and specific to the syndecan-4 receptor as it showed no binding to syndecan-4 knockout MEFs (Figure 6.8 A). On the other hand, quenching free biotin after adding the biotinylated fab fragment to cells reflected internalised biotinylated fab fragments which were not seen in both cell types used. To discriminate the difference between internalised material and surface staining, syndecan-4 antibody was used in syndecan-4 rescue demonstrating more surface staining (unquenched biotin) when compared to internalised (quenched biotin) syndecan-4 (Figure 6.8 B). Hence, the fab fragment was insufficient to trigger syndecan-4 receptor clustering in syndecan-4

rescue to promote receptor internalisation. Taken together, the full length of syndecan-4 antibody was required for receptor engagement to cluster syndecan-4 mimicking ligand-dependant endocytosis and the fab fragment was not enough despite its binding.

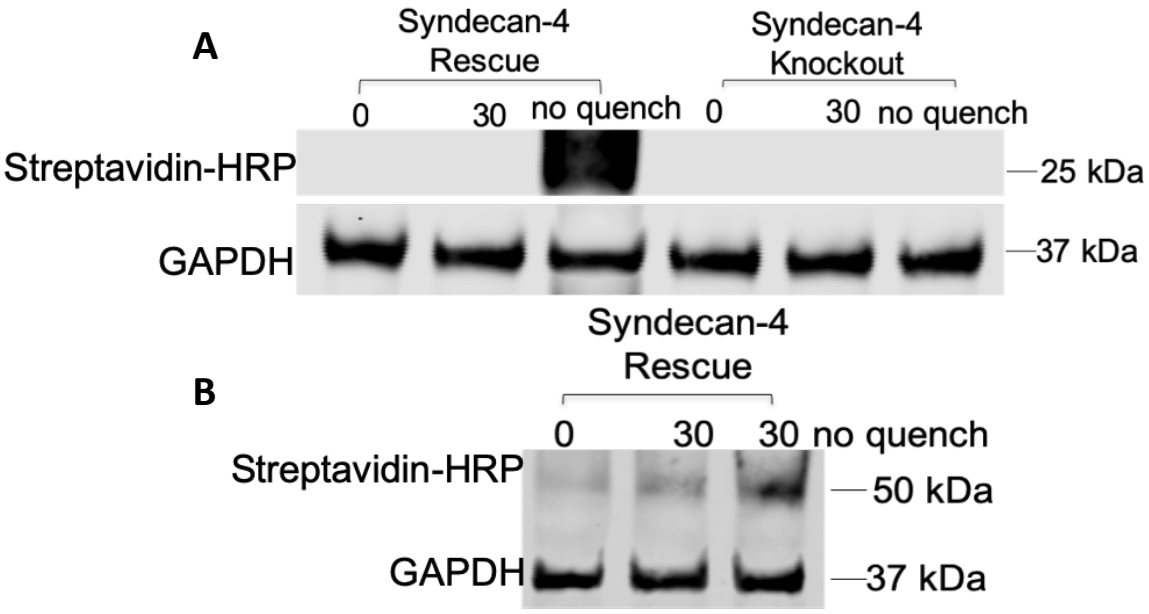


Figure 6.8: Syndecan-4 endocytosis is ligand-dependant. Syndecan-4 antibody was digested to produce fab and FC fragments. Fab uptake was measured in syndecan-4 knockout and rescue MEFs using 20 μg/ml biotinylated fab fragment of syndecan-4 antibody and allow 30 minutes of internalisation on ice (0) or 37°C (30) followed by lysing of cells. Cell lysates containing internalised fab fragment were analysed by western blotting to compare biotinylated fab in each condition using HRP-conjugated streptavidin antibody. Both MEFs failed to internalise fab fragment within 30 minutes with surface staining in syndecan-4 rescue MEFs when free biotin of fab fragment was not quenched with 200 μg/ml avidin indicating that syndecan-4 internalisation is ligand-dependant. **(A)** Representative western blot showing cell lysate of syndecan-4 knockout and rescue MEFs with no fab endocytosis within 30 minutes as reflected by anti-streptavidin antibody. When biotinylated fab was not quenched, surface staining was present in syndecan-4 rescue, but not in knockout MEFs indicating that fab fragment binding specificity was not compromised. **(B)** Syndecan-4 rescue MEFs showed syndecan-4 uptake reflected by higher anti-streptavidin surface staining when the antibody was not quenched with 200 μg/ml avidin (n=9 from independent experiments).

Data in this chapter demonstrate that upon engagement of syndecan-4 with the antibody, syndecan-4 is clustered and internalised in a ligand-dependant manner. The clustering of the syndecan-4 receptor activates PKCα downstream signalling. The results indicate that PKCα plays a central role in caveolin- and clathrin endocytosis of syndecan-4, as receptor internalisation was markedly affected when PKCα was perturbed. However, syndecan-4 downstream signalling to PKCα is dependent on the availability of its binding site on syndecan-4 V region to be activated, as PKCα-binding mutant was incapable to internalise syndecan-4.

Biotinylation of syndecan-4 fab fragment did not affect ligand binding nor specificity as demonstrated in Figure 6.8. When the pre-biotinylated syndecan-4 antibody (BAF2918) was used to produce fab fragment, both fab and FC fragments were found to be biotinylated. Hence, it is recommended for future work to use the pre-biotinylated antibody for such experiments instead of enzymatically digesting the unbiotinylated antibody (AF2918) and biotinylate it.

Chapter 7: Discussion

In the intact skin, fibroblasts populate the dermis in a collagen-rich environment. Upon wounding fibronectin leakage from damaged blood vessels and growth factors release during clot formation stimulate fibroblast activation, differentiation and migration to wound bed to promote healing. Syndecan-4 engagement with fibronectin is essential to promote cell migration to the injury site during the process of wound healing. Syndecan-4 requires the involvement of integrin, a coreceptor required by syndecan-4 during the process, as together they initiate a physical linkage between ECM and cell cytoskeleton mediating PKCα activation and focal adhesion formation at the leading edge of migrating cells. Focal adhesion dynamics in migrating fibroblast was reported to be mediated by syndecan-4 dependant endocytosis of integrin. For example, syndecan-4 engagement activates Arf6 activation driving α5β1 integrin recycling back to plasma membrane mediating focal adhesion turnover, whereas c-Src mediated phosphorylation of syndecan-4 leads to inactivation of Arf6 driving αVβ3 recycling to stabilise focal adhesion (Morgan et al., 2013). Therefore, syndecan-4 plays a central role in fibroblast activation and migration to wound bed contracting to reduce wound size, close it and secrete new matrix during wound healing.

On the contrary, chronic wounds show delayed healing due to the lower number of fibroblasts being activated and recruited at the injury site (Bass et al., 2011; Shaw & Martin, 2009). Therefore, understanding the mechanism of fibroblast activation and syndecan-4 trafficking is essential for the development of prospective healing therapeutics to improve the quality of life of affected patients.

7.1 Syndecan-4 internalisation requires syndecan-4 receptor clustering

Although some aspects of syndecan-4 receptor engagement by fibronectin were reported to drive rapid internalisation of α5β1-integrin through caveolin, PKCα and RhoG (Bass et al., 2011), syndecan-4 regulation and internalisation have not been investigated. The hypothesis was based on the clustering effect mediated by syndecan-4 antibody directed against the ectodomain mimicking receptor engagement with a ligand, including fibronectin, triggering syndecan-4 clustering and downstream signalling. The antibody used in this project induced receptor clustering and oligomerisation leading to internalisation of receptor-bound antibody, which is quantifiable using standard western blotting. Clustering syndecan-4 ectodomain using an antibody was an approach used by some groups when studying syndecan-integrin signal mechanotransduction (Bellin et al., 2009; Saoncella et al., 1999), cell migration (Araki et al., 2009; Tkachenko et al., 2006) and focal adhesion formation (Echtermeyer et al., 1999). Inducing syndecan-4 receptor clustering using an antibody directed against syndecan-4 ectodomain resulted in effective syndecan-4 downstream signalling including integrin engagement as

observed by vinculin containing adhesion (Figure 3.2), which was similar to stimulating fibroblasts with H/0 matching previous studies. Although the clustering hypothesis used syndecan-4 knockout as a negative control to test the downstream effect, additional testing like non-specific IgG antibody directed against other syndecans, 1,2 or 3, or mutated H/0 could be used to test syndecan-4 clustering effect.

The clustering of syndecan-4 upon activation has been reported to mediate several events in cells: for example, fibroblast spreading on fibronectin (Oh et al., 1997a), better downstream signalling and overall efficient biological effect compared to syndecan-4 monomer (Choi et al., 2005), Rac1 activation to mediate cytoskeleton reorganisation (Tkachenko et al., 2006), better PKCα signalling as it was found to be doubled when syndecan-4 was in multimeric form compared to syndecan-4 monomers (Oh et al., 1997a).

Therefore, data in this project proposes that syndecan-4 receptor clustering is the first step for receptor internalisation as seen in Chapter 3 where receptor clustering induced focal adhesion formation (Figure 3.2), and when syndecan-4 antibody was validated for endocytosis (Figure 3.4). The clustering hypothesis was tested using syndecan-4 fab fragment to trigger receptor clustering. Fab fragment failed to trigger syndecan-4 clustering and was not taken up by cells (Figure 6.8 A) supporting the hypothesis.

Syndecans heparan sulphate is essential for interaction with the extracellular environment. The modification of glycosaminoglycan sugar chains of syndecan-4 adds more complexity to the receptor and allows selective cell engagement with distant diluted ligands. Although most of the added sugar to the glycosaminoglycan chains in syndecan-4 is heparan sulphate, chondroitin sulphate chain can be found in glycosaminoglycan of syndecan-4. A functional analysis study demonstrated that heparan sulphate can bind extracellular growth factors, including basic fibroblast growth factor, and extracellular proteins involved in wound healing like fibronectin and collagen type I, III and IV (Koda et al., 1985; Saunders & Bernfield, 1988). Chondroitin sulphate was demonstrated to bind to the heparin-binding growth factors midkine and pleiotrophin, low molecular weight proteins of the midkine/pleiotrophin family involved in neuronal development. However, chondroitin sulphate failed to bind basic fibroblast growth factor when heparan sulphate was enzymatically digested suggesting binding specificity within syndecan-4 ectodomain (Deepa et al., 2004). The removal of either chondroitin or heparan sulphate was proposed to affect the kinetics of syndecan-4 binding to basic fibroblast growth factor, midkine and pleiotrophin indicating better association and dissociation of growth factors tested compared to binding to heparan sulphate chains alone. It was agreed that HepII

domain type III repeat 13 of fibronectin was a heparin-binding site containing arginine, threonine and proline amino acids. A mutation of arginine was sufficient to prevent further binding of heparin and chondroitin sulphate chains to fibronectin, hence inhibiting cell surface proteoglycan interaction with fibronectin (Barkalow & Schwarzbauer, 1991; Mostafavi-Pour et al., 2001).

Syndecan HSPG chains act as receptors that bind a wide range of ligands through the sugar chains within the sulphated domain. Heparan chains demonstrated specificity and binding sites to some ligands. Syndecan-1 demonstrated specific binding to triglyceride-rich lipoproteins through heparan sulphate chains which triggered receptor binding, clustering and internalisation of triglyceride. Syndecan-1 knockout mice or selective inactivation of the sulphate modification of the heparan sulphate chains resulted in accumulation of triglyceride-rich lipoproteins particles demonstrating ligand-target specificity (MacArthur et al., 2007; Stanford et al., 2010). A study explored the specificity of heparan sulphate chains of syndecan-4 in focal adhesion formation demonstrating that in mutated syndecan-4 cells at least two heparan sulphate chains were required to restore normal actin cytoskeletal phenotype to those of syndecan-4 wild type. Syndecan-4 with one chain of heparan sulphate were unable to restore normal cytoskeletal phenotype, unless syndecan-4 was clustered using an antibody directed against that single heparan sulphate chain demonstrating two outcomes: (A) the specific binding of the heparan sulphate chains to members of the focal adhesion (αSMA) in order to restore normal actin cytoskeletal phenotype and (B): the essential role of syndecan-4 clustering in mediating downstream effect (Gopal et al., 2010).

The use of heparin-binding fragment (H/0), fibronectin fragment comprised of type III repeats 12-15, to cluster syndecan-4 receptor, would be valuable to study syndecan-4 downstream effect as performed previously in our group (Bass et al., 2007). H/0 was prepared from fibronectin (Figure 1.7), hence had the advantage of using the natural ligand to stimulate fibroblasts. The disadvantage of using H/0 was the probability of H/0 binding other targets as the fibroblasts used in this projects did not exclusively express syndecan-4 only, but expressed syndecan-1 and syndecan-2 as well (Bass et al., 2007). This may lead to H/0 binding other syndecans resulting in a false interpretation of the results. Therefore, this technique was abandoned, and the biotinylated anti-syndecan-4 antibody was utilised instead as a ligand to cluster syndecan-4 promoting antibody bound receptor uptake, which will target the syndecan-4 receptor more specifically (Figure 3.4) to mediate downstream effects.

7.2 Caveolin solely is not responsible for syndecan-4 internalisation and clathrin could be important in syndecan-4 regulation

Receptors can be internalised continuously or after binding a ligand, which is referred to as constitutive and stimulated endocytosis, respectively. Syndecan-4 was reported to regulate integrin endocytosis and recycling (Morgan et al., 2013). Many studies have demonstrated that β1-integrin, as well as other integrins, undergoes constitutive endocytosis and recycling (Upla et al., 2004; Wary et al., 1996; White et al., 2007). Some agreed that caveolin constitutively regulates β1-integrin endocytosis and recycling in the absence of fibronectin and matrix (Shi & Sottile, 2008; Upla et al., 2004). The Bass research group has shown that syndecan-4 activation leads to induced caveolin-dependent endocytosis of integrin, an example of endocytosis following ligand binding (Bass et al., 2011). β-integrin showed clathrin-involvement in regulating matrix turnover as well. αVβ5 integrin was reported to be internalised in an active vitronectin-bound form via clathrin-mediated endocytosis regulating its internalisation (Panetti & McKeown-Longo, 1993). Therefore, integrin is internalised by both caveolin and clathrin endocytic pathway in a regulated and constitutive manner (Bass et al., 2011; Chao et al., 2009; Shi & Sottile, 2008; White et al., 2007).

Given the same analogy, syndecan-4 could be subjected to both caveolin- and clathrin-mediated endocytosis in a stimulated or constitutive manner. All the data presented in Chapters 3-6 support the idea of syndecan-4 internalisation in a stimulated manner as the pre-biotinylated antibody clustered the receptor mimicking receptor activation. Since both caveolin and clathrin contributed to syndecan-4 endocytosis, it was interesting to investigate the fate of internalised syndecan-4 vesicle. Internalised caveolin vesicles containing endocytosed material, including syndecan-4, pass through to caveosome, a neutral pH compartment with no lysosomes, before fusing with early endosome in a Rab5-dependant manner (Pelkmans et al., 2004), but it was not known if syndecan-4 could be trafficked to late endosome or rescued from lysosomal degradation by sorting nexins, as in integrin, or recycled back to the plasma membrane. A study demonstrated that internalised caveolae were trafficked to late endosomal compartment to which caveolin-1 was restricted and undergo lysosomal degradation (Botos et al., 2008). Degraded caveolin-1 in the study was replenished by newly synthesised caveolin-1 at plasma membrane instead of recycled caveolin-1. Other studies disagreed and found that caveolin-1 was enriched in recycling endosome suggesting that caveolae content could undergo recycling (Gagescu et al., 2000). Interestingly, a group demonstrated that caveolae containing material cannot be trafficked to intracellular caveosome compartment without a stimulus (Thomsen et al., 2002). Antibody clustering of syndecan-4 receptor in this project might fit the proposed model by Thomsen group as data suggested that syndecan-4 clustering leads to receptor internalisation via caveolin-

dependant endocytosis. Internalised syndecan-4 enters caveolin vesicles first prior trafficking to early endosome as proposed in (Figure 5.3). However, the fate of internalised syndecan-4 in caveolin vesicle is not known and needs further investigation.

Clathrin-mediated endocytosis is another endocytic mechanism involved in syndecan-4 receptor trafficking from extracellular to the intracellular environment. Upon syndecan-4 engagement with a ligand, it is proposed that clathrin triskelia, AP-2 and regulatory proteins are assembled at the inner leaflet of the cell membrane to mediate receptor internalisation via clathrin-coated vesicle. However, clathrin triskelia cannot bind directly to the plasma membrane (Maldonado-Báez & Wendland, 2006). A study has investigated the mechanistic of clathrin-mediated endocytosis using live-cell TIRF imaging with high temporal resolution and reported that clathrin-coated pits are initiated by the arrival of one clathrin triskelia and 2 AP-2 complexes, or 2 clathrin triskelias and 4 AP-2 complexes (Cocucci et al., 2012). Cocucci research group described the Initiation of a clathrin-coated vesicle by the AP-2 binding to plasma membrane containing PIP2. The weak, but stable, binding of PIP2 to plasma membrane provided more stability of the newly formed vesicle.

The fate of internalised syndecan-4 in clathrin-containing vesicles might be speculated by comparing the fate of internalised integrin and other receptors. A study showed that during cell migration, clathrin vesicles containing active integrin were recycled back to the plasma membrane in a Rab11-dependant manner to reassemble new focal adhesion (Nader et al., 2016). Some of the clathrin vesicles containing growth factor bound to their corresponding receptors, for example, epidermal growth factor-bound receptor and insulin-bound receptor, internalised in clathrin-mediated endocytosis were reported to traffic to early endosome where the receptors were recycled back to the plasma membrane when stimulated with insulin or low dose of epidermal growth factor instead of receptor degradation (Carpentier, 1994; Sigismund et al., 2005). A higher dose of the epidermal growth factor resulted in internalisation of receptor-bound in a caveolin-dependant and clathrin-dependant endocytosis. However, caveolin-dependant endocytosis of epidermal growth factor was reported to progress to late endosomal compartment for receptor degradation (Sigismund et al., 2008). Syndecan-4 might follow similar internalisation mechanism as epidermal growth factor receptor described by Sigismund research group, but no lysosome marker (such as lysosomal associated membrane protein 1) was used in this project, thus worth pursuing forward. It is well documented that fibronectin activates fibroblasts and that both integrin and syndecan-4 are central to fibroblast function, but it is unclear if the fate of internalised integrin or syndecan-4 or surface availability of syndecan-4 is dependent on the concentration of receptor engagement. Since both caveolin and clathrin contributed to syndecan-4

uptake, it is not known which endocytic mechanism works normally to internalise syndecan-4 following ligand engagement. If the same fate of internalised syndecan-4 by either endocytic mechanism was applied to the fate described by Sigismund, internalised syndecan-4 from either mechanism could meet at the early endosomal compartment. Therefore, it is challenging to predict the fate of internalised syndecan-4 if presumed that both mechanisms work normally to internalise syndecan-4.

To conclude, internalised syndecan-4 receptor demonstrated contribution by both caveolin and clathrin (Figure 5.3) as well as compensation of caveolin by clathrin when caveolin knockout MEFs were utilised as demonstrated by biochemical and fluorescence assays (Figure 4.7-4.8); however, the fate of syndecan-4-containing vesicle is unclear and worth further investigation. Designing syndecan-4 chimeric receptor could help determine the fate of internalised receptor. There is a possibility that caveolin independent mechanism may affect syndecan-4 endocytosis, which both biochemical and immunofluorescence experiments did not cover and worth further investigation.

7.3 Syndecan-4 ectodomain shedding and antibody binding

Syndecan-4 receptor is a large molecule and requires ectodomain receptor engagement and cytoplasmic domain to initiate cellular response. A study suggested that the role of syndecan ectodomain could be in enhancing receptor activation and downstream effect during engagement with low ligand concentration (Carey et al., 1997). However, it is widely accepted that syndecan ectodomain, including syndecan-4, undergoes a highly regulated proteolytic shedding in body fluids or culture media (Jalkanen et al., 1987). This shedding can be enhanced in acute injury resulted from binding of ectodomain to growth factor, including epidermal growth factor family members, released during injury (Subramanian et al., 1997) or blocked by inhibiting some enzymes suggested to be involved in ectodomain shedding including matrix metalloproteinase (Arribas et al., 1997). Soluble shed proteoglycan of ectodomain demonstrated to retain the binding properties of its cell surface precursor and suggested to have a physiological role in regulating cellular events (Subramanian et al., 1997). In fact, shed ectodomain of syndecan-4 was found in the fluids of acute dermal injury patient suggesting syndecan-4 upregulation, shedding and contribution of shed domain during injury (Bernfield et al., 1999). A study argued that tagging the C-terminus could interfere with the functionality of the receptor (Maday et al., 2008), where tagging the N-terminus of syndecan-1 showed that the receptor activity was retained outlining a better labelling site for receptor-downstream signalling studies (Fujikura et al., 2017). Thus, the ligand-binding site of the HSPG could be near the N-terminus.

Shedding of heparan sulphate proteoglycan can indeed be induced during inflammation, but internalisation of such large molecules have been demonstrated (Yanagishita, 1992). It was proposed that each heparan sulphate proteoglycan was internalised according to which protein they were anchored to. This means that internalisation, trafficking and degradation of transmembrane linked proteoglycan of syndecans and glycosylphosphatidylinositol-anchored could be different from each other. The antibody used in this project targeted syndecan-4 ectodomain (Glu-19 – Glu-145), a site reported to bind with specificity to ECM proteins (Carulli et al., 2012), to cluster syndecan-4 receptor and increase surface interaction enhancing receptor engagement to mediate interaction with effector proteins (Klemm et al., 1998). The transmembrane domain of syndecans bound to heparan sulphate chains was demonstrated to play a role in anchoring the receptor and forming a stable syndecan oligomer upon receptor engagement (Asundi & Carey, 1995). In addition to the key roles of ectodomain and transmembrane domain, research groups successfully used a heparin-binding fragment of fibronectin or antibody directed against syndecan ectodomain to induce receptor clustering and mediated downstream signalling events including focal adhesion formation demonstrating the importance of each domain during receptor engagement (Bass et al., 2007; Ishiguro et al., 2000; Saoncella et al., 1999). The antibody signal was optimised by quenching surface staining resulted from bound but not internalised antibody (Figure 6.8). However, further investigation is needed to determine which region of the syndecan-4 receptor was internalised as the antibody signal represents internalised antibody which could be bound to either syndecan-4 ectodomain only, ectodomain linked to the transmembrane region or the entire syndecan-4 receptor. Integrin is a heavily glycosylated surface protein that is endocytosed entirely, hence it is plausible to think that syndecan-4 could be internalised as a whole despite the size of the receptor. Therefore, tracking internalised syndecan-4 by tagging and tracking labelled syndecan-4 using chimera could provide a detailed answer to the specific region(s) of the receptor being taken up to mediate an effect.

Syndecan-4 receptor engagement with the antibody showed better focal adhesion formation (Figure 3.2) compared to heparin-binding fibronectin fragment (Figure 3.1), hence cleaving syndecan-4 polysaccharide chains using enzymatic approach (heparinase) could help in determining if the remaining syndecan-4 can still be engaged with the antibody to initiate focal adhesion. It would provide some insight on the time required for heparan sulphate proteoglycan to be delivered to the cell surface when syndecan-4 ectodomain shedding is upregulated as in acute injury or downregulated as in cancer patients where heparan sulphate modification enzymes are dysregulated (Nagarajan et al., 2018). Patient with chronic wounds is known to have elevated levels of proinflammatory cytokines and proteases impeding the function of other growth factors released during the healing process (Lei et al.,

2017). It seems plausible to think that therapeutic enhancement of fibroblast activation at injury sites by increasing syndecan-4 expression or using shed ectodomain in patients with chronic wounds could be beneficial in patients affected by high proteases released.

7.4 Syndecan-4 cytoplasmic domain is essential for PKCα activation to trigger endocytosis in a ligand-dependant manner

Syndecan-4 dependant endocytosis and activation of PKCα and Rac1 in focal adhesion have been explored in vivo and in vitro by different groups (Bass et al., 2007, 2011; Chronopoulos et al., 2020; Keum et al., 2004; Lim et al., 2003; Oh et al., 1997a; Oh et al., 1997b; Tkachenko et al., 2004). The groups agree that syndecan-4 is known to bind and activate PKCα at the cytoplasmic domain in the presence of phosphatidylinositol 4,5-bisphosphate, which is essential for adhesion-mediated signalling and stress fibre assembly. The role of syndecan-4 receptor engagement and PKCα activation in focal adhesion is well established as integrin engagement and signalling alone can promote cell spreading, but not focal adhesion formation, which requires syndecan-4 signalling (Woods et al., 1986). In addition, PKCα activation of Rac1 demonstrates the essential role of syndecan-4 engagement in cell adhesion and focal adhesion complex formation (Bass et al., 2007). Lastly, syndecan-4 activation drives the endocytosis of integrin via PKCα and RhoG to regulate integrin trafficking and surface availability demonstrating the importance of PKCα in multiple cell processes. Collectively, mediating a cellular event in syndecan-4 dependant manner suggests a direct link between syndecan-4 receptor and PKCα activation. Syndecan-4 endocytosis was not investigated before and the agreement of syndecan-4 dependant activation of PKCα drives the hypothesis of PKCα in mediating syndecan-4 endocytosis following receptor activation (Chapter 6).

PKCα is central to many events involved in cell polarisation and migration in a syndecan-4 dependant manner (Figure 1.4). Following syndecan-4 receptor engagement, activated PKCα phosphorylates RhoGDI (RhoA sequestering molecule) causing the release of RhoA, which contributes to the stabilisation of focal adhesion and cell contraction. Released RhoA undergoes circles of suppression and reactivation controlled by GAP and GEF (Dovas et al., 2010). Maintaining cell membrane protrusion in cells was reported to occur in a PKCα-dependant phosphorylation of p190RhoGAP (Bass et al., 2008). The phosphorylation of p190RhoGAP was mentioned to occur upon syndecan-4 receptor engagement where PKCα-mediated phosphorylation of p190RhoGAP via serine/threonine, but integrin engagement can stimulate serine phosphorylation of p190RhoGAP as well. A study using fluorescence lifetime imaging microscopy demonstrated an association between PKCα active β1-integrin recycling (Ng et al., 1999). The study agreed with previous observations that β1-integrin was internalised (endocytic

mechanism was not investigated but nominated caveolin- and clathrin-mediated endocytosis) and recycled upon PKCα activation highlighting the role of recycling GTPase Arf6 and PKCα activation. Arf6 is known to be activated upon syndecan-4 receptor engagement with ECM and regulate integrin recycling but has no role on syndecan-4-dependant internalisation of integrin stressing the role of RhoG in integrin endocytosis. Arf6 activity was demonstrated in modulating focal adhesion dynamics by regulating integrin recycling during cell motility (Morgan et al., 2013). Following syndecan-4 receptor engagement, Arf6 activity is increased leading to increase αVβ3-integrin recycling and stable focal adhesion. Whereas c-Src-mediated syndecan-4 phosphorylation leads to lower Arf6 activity and focal adhesion turnover as a result of increase recycling of α5β1-integrin. Not only receptor engagement with ECM involves in Arf6 activation, but the syndecans-binding adaptor syntenin plays a role in Arf6 activity as well. The EFYA motif on syndecans can bind PDZ-containing proteins including syntenin to mediate cellular activity. Phosphorylated syndecan-4 enhanced syntenin binding and inhibited Arf6 activity, which would result in low receptor recycling. In addition to syntenin, synectin is another EFYA-binding protein and was reported to contribute to syndecan-4 signalling complex (Gao et al., 2000). Synectin plays a balancing role at cell membrane where it binds, suppress and sequesters other Rho GTPases incorporated into syndecan-4- synectin-RhoGDI complex when the syndecan-4 receptor is not engaged with ECM (Elfenbein et al., 2009). Hence, syndecan-4-dependant activation of PKCα and regulation of integrin surface expression are equally crucial during tissue injury.

PKCα activity was demonstrated to regulate caveolin-dependant endocytosis via protein kinase C and casein kinase substrate in neurons protein 2 (PACSIN-2) (Senju et al., 2015). PACSIN-2 is a surface membrane sculpting F-BAR domain-containing protein involved in caveolae synthesis and internalisation. Both caveolin-1 and PACSIN-2 are required to enable membrane tubulation (Hansen et al., 2011). Senju research group investigated the role of F-BAR domain of PACSIN-2 and showed that it was responsible for the flask-shaped of the invagination as the knockdown of PACSIN-2 demonstrated a different shape of invagination at the plasma membrane of caveolin-1 containing vesicle compared to control knockdown (Senju et al., 2015). Furthermore, the phosphorylation of PACSIN-2 by PKCα demonstrated a central role of PKCα in caveolin-dependant internalisation. The phosphorylation of PACSIN-2 reduced its ability to bind and form membrane tubulation at cell surface suggesting dissociation of PACSIN-2 from caveolae. Hence, caveola was no longer attached to the plasma membrane following the phosphorylation of PACSIN-2 at serine 313 providing a direct link between caveolin-dependant endocytosis and PKCα-dependant phosphorylation of PACSIN-2 (Senju et al., 2015). In this study, the group used 12-myristate 13-acetate (PMA) and BIM-I to activate and suppress PKCα activity, respectively in order to study the role of PACSIN-2. Although the role of PACSIN-2 was

not evaluated in this project, syndecan-4 was reported to stimulate PKCα more effectively compared to PMA (Bass et al., 2011), thus the role of PACSIN-2 in caveolar endocytosis of syndecan-4 is a possibility that needs exploring further.

Filamin is a family of proteins that cooperates with PKCα to regulate cellular activity via associating with integrin to bind and anchor actin cytoskeleton to the plasma membrane (Glogauer et al., 1998). Similar to PACSIN-2, filamin A was reported to be phosphorylated by PKCα and linked to caveolin-dependant endocytosis mechanism (Smart et al., 1995). The N-terminus of filamin was demonstrated to harbour actin-binding domain while the C-terminus containing the dimerisation site which reported to bind a variety of targets including PKCα and small Rho GTPases (Ohta et al., 1999). PKCα-dependant phosphorylation of filamin A at serine 2152 regulates filamin function (Van der Flier & Sonnenberg, 2001; Jay et al., 2000; Muriel et al., 2011; Tigges et al., 2003). Moreover, filamin A was demonstrated to stabilise caveolin-1 containing vesicle by anchoring caveolae to actin stress fibre at the plasma membrane. Phosphorylation of filamin A at serine 2152 by PKCα mediated caveolae detachment from the cell surface and inward trafficking of caveolae linked to actin filaments in a filamin A-dependent manner (Muriel et al., 2011). Caveolae at plasma membrane demonstrated uncontrolled and non-linear movement when filamin A was depleted in cells used suggesting a central role of PKCα-mediated phosphorylation of filamin in caveolar endocytic mechanism. Filamin demonstrated a central role in caveolar endocytosis and investigating its role in syndecan-4 endocytosis could provide some ideas to improve our understanding of endocytic regulation of cellular trafficking.

Although the activity of PKCα was not measured during syndecan-4 fab fragment stimulation, it would be interesting to support the hypothesis and previous studies regarding the role syndecan-4 dependant activation of PKCα to mediate cellular events including Rac1 activation, focal adhesion formation and maturation. Fab fragment data could suggest that without syndecan-4 receptor engagement, cytoplasmic signalling and PKCα activation there would be no syndecan-4 receptor uptake (Figure 6.8 A). The incapability to mediate syndecan-4 internalisation demonstrates that syndecan-4 uptake is ligand-dependant and supports the role of syndecan-4 dependant activation of PKCα in the hypothesis. The idea of syndecan-4 internalisation in a ligand-dependant manner was not discussed in the literature, a possibility that would be worth pursuing further.

The linked role of syndecan-4 dependant activation of PKCα in this project stems from in vivo and in vitro studies demonstrating the role of PKCα in wound healing (Chandrasekher et al., 1998; Cooper et al., 2015; Keum et al., 2004; Thomason et al., 2012). These studies agree that PKCα-/- mice

demonstrated delayed healing rate post wounding compared to PKCα wild type. In addition, PKCα overexpression resulted in accelerated tissue re-epithelisation. Their data were in line with the hypothesis in which they found that the knockdown of PKCα in murine resulted in delayed wound healing, while PKCα activation demonstrated improved wound healing. Establishing the role of PKCα in wound healing. PKCα could provide a therapeutic target to promote effective wound healing for chronic wounds. Although functional analysis of fibroblast upon PKCα stimulation with extracellular matrix was not performed, it would provide more evidence of syndecan-4 dependant activation of PKCα in wound healing.

7.5 Consequence of syndecan-4 internalisation

Syndecan-4 regulates many cellular events as demonstrated above, but it is not clear what the consequences are following syndecan-4 internalisation. For example following stimulation of epidermal growth factor receptor with its corresponding ligand in rats, the receptor was rapidly phosphorylated and accumulated in the endosomal compartment. Furthermore, the phosphorylation level of internalised epidermal growth factor was increased suggesting ongoing phosphorylation of the receptor within the endosome, suggesting continuous receptor-mediated signalling (Di Guglielmo et al., 1994). Another in vitro study demonstrated continuous signalling of epidermal growth factor receptor from early endosome strengthening the concept of signalling after internalisation, which was generally accepted (Sorkin et al., 2000).

In recent years, internalised active integrin following engagement with ECM was demonstrated to colocalised with active focal adhesion kinase (non-receptor tyrosine kinase ubiquitously expressed and involved in many processes including cell migration in wound healing) on the endosomal compartment. Focal adhesion kinase activity was induced by integrin endocytosis in an early endosome antigen-1-dependant manner demonstrating that internalised integrin continues signalling on endosome via focal adhesion kinase (Alanko et al., 2015). Endosomal signalling of integrin via focal adhesion kinase on endosome was linked to anoikis resistance and anchorage-independent growth of cancerous cells (Frisch et al., 1996). Collectively, this could suggest that syndecan-4, as integrins and other surface receptors, might continue signalling following receptor internalisation. Moreover, syndecan-4 could play a role in regulating other cellular cues during wound healing via signalling on endosome. It is not known if syndecan-4 signalling from endosome could affect syndecan-4 expression on cell surface, recycling nor receptor redistribution. Another question to answer is the similarity between downstream syndecan-4 signalling on the cell surface and signalling from endosome as focal adhesion kinase targeting to focal adhesion recruitment upon surface engagement of integrin demonstrated to

be different from the endosomal compartment (Alanko et al., 2015), hence additional investigation is required. Additionally, it is yet to investigate if syndecan-4 endosomal signalling could affect the fate of the receptor in the endosomal compartment to progress to either recycling or lysosomal compartment, a point that could improve our comprehension of endosomal-mediated signalling.

7.6 Summary model for syndecan-4 internalisation

Data in this thesis when combined with current understanding of syndecan-4 role in wound healing suggests that syndecan-4 receptor engagement with a ligand triggers syndecan-4 oligomerisation and clustering, which activates Rac1 and PKCα. Rac1 activation promotes polymerisation and reorganisation of actin cytoskeleton, whereas syndecan-4 dependant activation of PKCα activates RhoG mediating: (1) syndecan-4 internalisation via caveolin and clathrin endocytic mechanisms prior fusing with early endosomal compartment; (2) integrin internalisation via caveolar endocytosis. In addition, PKCα phosphorylates RhoGDI and p190RhoGAP resulting in cycles of RhoA suppression and reactivation promoting membrane protrusion, focal adhesion stabilisation and cell contraction. The fate of internalised syndecan-4 is unknown and would be ideal to use live-cell imaging approaches with image reconstructing techniques to define whether syndecan-4 undergoes receptor recycling or lysosomal degradation. These events are central in initiating efficient cell migration toward wound bed to promote wound healing and restore anatomical structural of affected area within a reasonable period of time. Figure 7.1 highlights main findings in of this project.

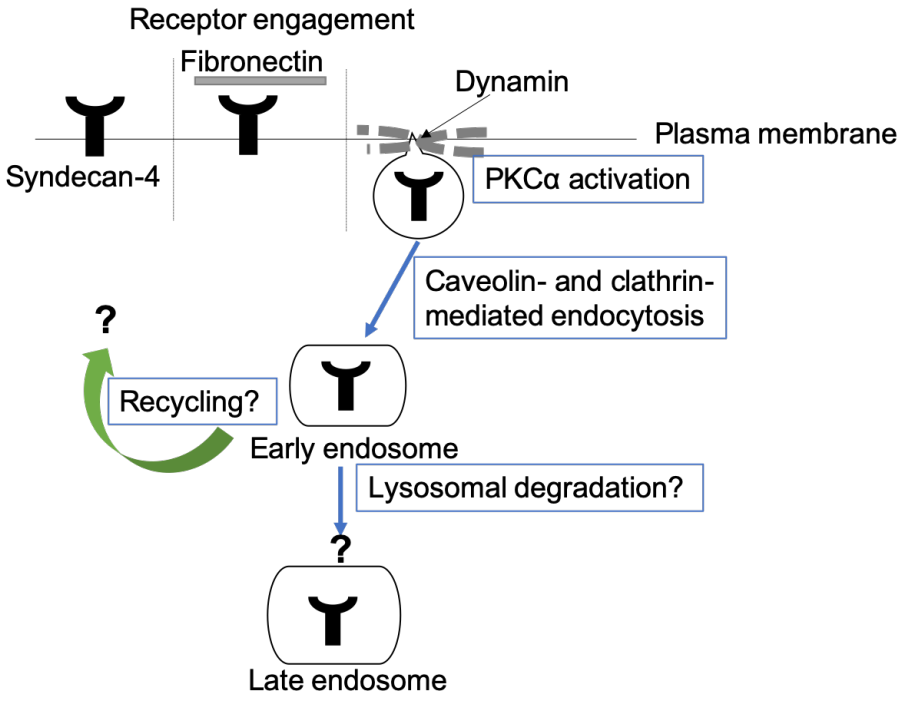


Figure 7.1: Summary model highlighting main findings of this project. Syndecan-4 receptor engagement with a ligand triggers receptor clustering and subsequently activating PKCα. Syndecan-4-dependant activation of PKCα is required to mediate syndecan-4 internalisation by caveolin and clathrin endocytic mechanisms. The fate of internalised syndecan-4 remains unidentified and requires further investigation to define it.

Chapter 8: Concluding remarks and future work

The goal of this project was to assess the trafficking of syndecan-4 receptor and signalling during wound healing to evaluate the role of syndecan-4 in fibroblast migration. The aim of this project stems from previous work in the lab which was focused on the role of syndecan-4 engagement with extracellular matrix to initiate cell migration and wound healing by regulating integrin endocytosis. The mechanism of syndecan-4-dependant signalling during wound healing has been studied extensively, but syndecan-4 regulation upon wounding remains unclear.

The data in this project demonstrates that syndecan-4 receptor engagement with a ligand is the first step in receptor uptake. Data in this project suggests that regulation of syndecan-4 is likely to be mediated by PKCα with the contribution of both caveolin and clathrin. The reduced expression of either of the mentioned, substitution of PKCα-binding motif or pharmacological agent resulted in blockade of syndecan-4 endocytosis and downstream signalling. When placing PKCα findings in this project with current understanding in cell migration, it could mean that syndecan-4 receptor activation by fibronectin drives syndecan-4 dependant-endocytosis of integrin and possibly syndecan-4 via PKCα and RhoG activation which recruits elements of focal adhesion, including integrin and syndecan-4, at wound site forming a bridge connecting extracellular matrix to the actin cytoskeleton. The crosstalk between syndecan-4 and integrin during focal adhesion formation leads to maturation of the nascent adhesion at the leading edge of migrating fibroblasts. The knockout of any of the following: syndecan-4 (Echtermeyer et al., 2001), PKCα (Thomason et al., 2012), RhoG (Bass et al., 2011), Rac1 (Liu et al., 2009), and caveolin-1 (Grande-García et al., 2007), has been reported to cause a delay in wound healing in vivo and in vitro models demonstrating the essential part of each .

Future studies should study the role of PKCα and caveolin upon syndecan-4 activation in wounds. PKCα is known to modulate not only cell migration but cell-cell adhesion and re-epithelisation to promote efficient wound closure. The role of PKCα and caveolin in fibroblast migration upon stimulation of fibroblasts with a soluble heparin-binding fragment of fibronectin (H/0) was assessed previously (Bass et al., 2011) and therefore fibroblast migration assay was not performed in this project. Although caveolin was found to be upregulated in chronic wounds causing fibroblast hyperproliferation, PKCα was not assessed. It would be worth investigating PKCα level in fibroblasts extracted from chronic wounds and determine if the upregulation in caveolin was PKCα-dependant. Hence, targeting PKCα and caveolin in chronic wounds could represent a therapeutic potential to promote efficient wound healing as both showed to be downstream to syndecan-4 activation.

Although syndecan-4 regulation in this project was simplified by using caveolin wild type and knockout MEFs, functional assessment of syndecan-4 in regulating cell adhesion and migration was not performed and worth pursuing in future studies to explore caveolin-dependant and independent association upon extracellular stimulation of syndecan-4. Similarly, previous reports suggested that integrin and syndecan-4 receptors in addition to signalling intermediaries are located in close proximity (Bass et al., 2011). This hypothesis needs further pursuing as data in this thesis suggests that, much like integrin, syndecan-4 requires syndecan-4 receptor engagement and PKCα activity for signalling and use the same mediators, caveolin and clathrin (Ng et al., 1999; Caswell et al., 2006), for receptor uptake. Antibody feeding experiment with staining for integrin and early endosomal marker EEA1 could present some information regarding special proximity. Colocalisation analysis is equally essential to provide any evidence of coreceptors trafficking and define any point of segregations if any were found.

In chronic wounds and diabetic patients, there is a notable reduction in fibroblast migration, differentiation and senescence leading to delay in wound closure (Harding, Moore & Phillips 2005; Roper et al., 2015). This hallmark is suggested to be occurring in a syndecan-4 and Rac1 dependant mechanism, as demonstrated in syndecan-4 knockout mice previously in our lab (Bass et al., 2007). Since Rac1 activation is downstream of syndecan-4 engagement, our lab has used low-intensity ultrasonic stimulation of Rac1 and showed to improve fibroblast migration in diabetic and old mice without fibronectin engagement (Roper et al., 2015). The restoration of wound healing in syndecan-4 knockout, aged and diabetic mice by ultrasound are Rac1 dependant, and independent of fibronectin-dependant activation pathway. Developing ultrasound device criteria, based on previous work, to promote healing in chronic wounds is ongoing in the lab. Therefore, targeting Rac1 to promote wound healing in chronic wounds and elderly patient could be beneficial whether by ultrasound or topical application (Fan et al. 2018). Although this project did not cover the role of senescence in wound healing, ongoing investigations in the lab are continuing to improve understanding of cellular senescence in wound chronicity using senescent-induced fibroblasts.

In conclusion, syndecan-4 is a unique receptor and with integrin as a coreceptor, it enables fibroblasts to operate as a sensor for changes in extracellular matrix and cytoskeleton to promote cell adhesion, migration and tissue regeneration. Data in this project demonstrates that in addition to what is currently known about syndecan-4-dependant signalling during wound healing phases, activation of PKCα shows potentials in playing a key role during wound healing, in addition to Rac1, clathrin and caveolin which could help to provide treatment in chronic wounds of old and diabetic patients. Patients with chronic wounds are often presented with inflammation and hyperproliferation which impedes the

healing process. Assessment of syndecan-4 mediators, mentioned in this thesis, using fibroblasts driven from these wounds, in parallel to ultrasonic stimulation could help in promoting healing. Therefore, future studies must advance our understanding of syndecan-4 downstream signalling events to promote efficient wound healing strategies providing relief for affected patients with chronic wounds and reducing the cost of annual expenditure.

Chapter 9: References

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