Regulation of C-type lectin-like receptors dectin-1 and CLEC-2 by tetraspanins

by

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ABSTRACT

Tetraspanins are a superfamily of glycoproteins that function as 'organisers' of membranes by clustering with each other to form tetraspanin-enriched microdomains, into which certain other receptors and signalling proteins are recruited and regulated. Tetraspanin microdomains have been implicated in a range of biological processes including cell signalling, adhesion, intracellular trafficking, cell-cell fusion and viral entry. The tetraspanin CD37 was recently shown to negatively regulate the C-type lectin-like receptor dectin-1, which is essential for innate immune responses to fungal pathogens. The aim of this thesis was to firstly develop a cell line model system to investigate the mechanism by which tetraspanins inhibit dectin-1, and to secondly extend this work to the dectin-1-related CLEC-2, which is essential for platelet thrombus formation and stability. Using a nuclear factor of activated T-cells (NFAT) transcriptional reporter assay in the Jurkat T-cell line, transient over-expression of CD37 was found to powerfully inhibit dectin-1 signalling following stimulation with its ligand, β-glucan. Over-expression of other tetraspanins also inhibited dectin-1 signalling, but did not globally inhibit receptor signalling because the platelet collagen receptor, GPVI, was unaffected. Similar to dectin-1, CLEC-2 signalling in response to its ligand, the snake venom toxin rhodocytin, was also abrogated following tetraspanin over-expression. However, stable tetraspanin over-expression only partially reduced signalling. Moreover, knockdown of the major Jurkat cell tetraspanin, CD81, and deletion of the major platelet tetraspanin, CD9, did not affect dectin-1 and CLEC-2 signalling, respectively. In summary, the importance of transient tetraspanin over-expression for dectin-1 and CLEC-2 inhibition, and the fact that any tetraspanin can inhibit, suggests that tetraspanin microdomains are disrupted

by the presence of one over-expressed tetraspanin. This leads to a failure of dectin-1 and CLEC-2 signalling by a mechanism that is not clear, but suggests that tetraspanin microdomains are important for signalling by these C-type lectin-like receptors.

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ABBREVIATIONS

ACD - Acid citrate dextrose
AEBSF - 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride
ANOVA - Analysis of variance
AP-1 - Activator protein-1
APC - Antigen-presenting cell
BRET - Bioluminescence resonance energy transfer
C3d - Complement 3d
cDNA - Complementary DNA
COOH - Carboxy-terminus
CRAC - Calcium release activated calcium channel
CRD - Carbohydrate recognition domain
CTLD - C-type lectin-like domain
CXCL2 - CXC-chemokine ligand 2
DAG - 1,2-diacylglycerol
EAP - Endothelial adhesive platform
EC1 - Small extracellular loop
EC2 - Large extracellular loop
EGFR - Epidermal growth factor receptor
ER - Endoplasmic reticulum
FRET - Fluorescence resonance energy transfer
GFP - Green fluorescent protein
GPCR - G-protein coupled receptor
GPI - Glycosylphosphatidylinositol
HBS - HEPES-buffered saline
HCV - Hepatitis C virus
HIV-1 - Human immunodeficiency virus-1
HUVEC - Human umbilical vein endothelial cell
ICAM-1 - Inter-cellular adhesion molecule-1
Ig - Immunoglobulin
IgSF - Immunoglobulin superfamily
IL - Interleukin
IP - Immunoprecipitation
IP_3 - Inositol 1,4,5-trisphosphate
IP_3R - Inositol 1,4,5-trisphosphate receptor
ITAM - Immunoreceptor tyrosine-based activation motif
KO - Knockout
LB - Luria-Bertani
lbm - late bloomer

MAPK - Mitogen-activated protein kinase
MFI - Mean fluorescence intensity
NFAT - Nuclear factor of activated T-cell
NF-κB - Nuclear factor-κB
NH_2 - Amino-terminus
NK - Natural killer
PAMP - Pathogen-associated molecular pattern
PCR - Polymerase chain reaction
PGI_2 - Prostacyclin
PGS - Protein G sepharose
PI4K - Phosphatidylinositol 4-kinase
PIP_2 - Phoshatidylinositol 4,5-bisphosphate
PKC - Protein kinase C
PLCγ - Phospholipase C γ
PMA - Phorbol 12-myristate 13-acetate
PRP - Platelet-rich plasma
PVDF - Polyvinylidene difluoride
RNAi - RNA interference
SAGE -Serial analysis of gene expression
SDS-PAGE - Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH2 - Src homology 2
Sun - Sunglasses
TIRF - total internal reflection fluorescence
TLR - Toll-like receptor
TNF - Tumour necrosis factor
UP - Uroplakin
UV - Ultra-violet
VCAM-1 - Vascular cell adhesion molecule-1
WCL - whole cell lysate
WT - Wild-type

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CHAPTER 1 –

GENERAL INTRODUCTION

1.1 The tetraspanin family of proteins

1.1.1 Overview

Tetraspanins are widely expressed membrane glycoproteins of 20-50 kilo Daltons involved in a number of biological processes, for example, cell signalling, adhesion, migration, proliferation, tumour metastasis and cell-cell fusion (Charrin et al., 2009; Hemler, 2005; Levy and Shoham, 2005). Their expression in a diverse range of organisms including mammals (33 family members), *Caenorhabditis elegans* (20 members), *Drosophila melanogaster* (36 members), schistosomes (>25 members) and some fungi (Garcia-Espana et al., 2008), suggests a fundamental biological role. A phylogenetic tree of human tetraspanins illustrates their large number and the existence of sub-groups that share relatively high sequence similarity, such as the CD9/CD81/Tspan2 sub-group that are approximately 50% identical to each other at the protein level (Figure 1.1). Tetraspanins have proposed roles in interacting with specific partner proteins and regulating their plasma membrane organization, biosynthetic maturation and trafficking (Berditchevski and Odintsova, 2007) (Figure 1.2). For example, the tetraspanin CD37 negatively regulates dectin-1 signalling (Meyer-Wentrup et al., 2007). Attention is now being directed towards their interaction with proteins such as the C-type lectins, integrins, G-protein coupled receptors (GPCRs) and the immunoglobulin superfamily (IgSF). This is of particular relevance as tetraspanins are thought to interact laterally with receptors and other tetraspanins forming distinct tetraspanin-enriched microdomains to organise the cell membrane (Charrin et al., 2009; Hemler, 2005; Le Naour et al., 2006b).

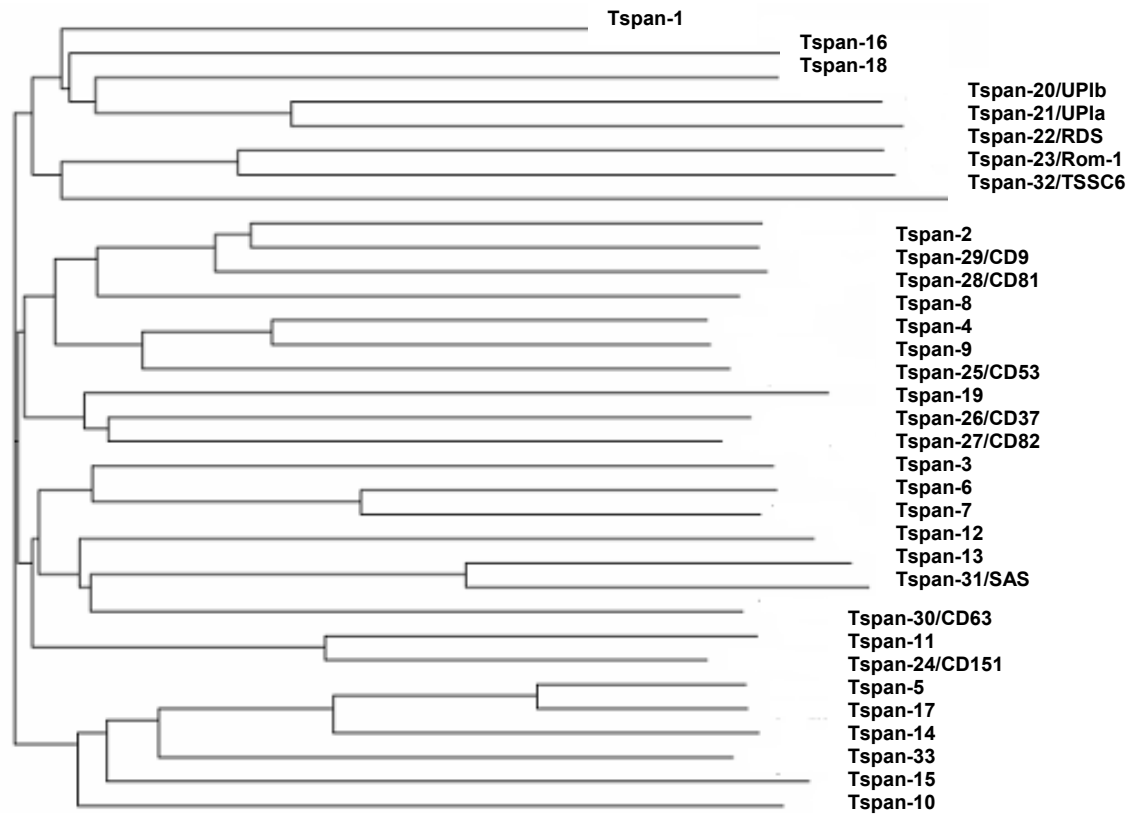


Figure 1.1 A phylogenetic tree of human tetraspanins

Human tetraspanin amino-acid sequences were aligned and a phyologram was prepared using the Clustalw alignment programme (www.ebi.ac.uk/custalw) to determine similarity between superfamily members.

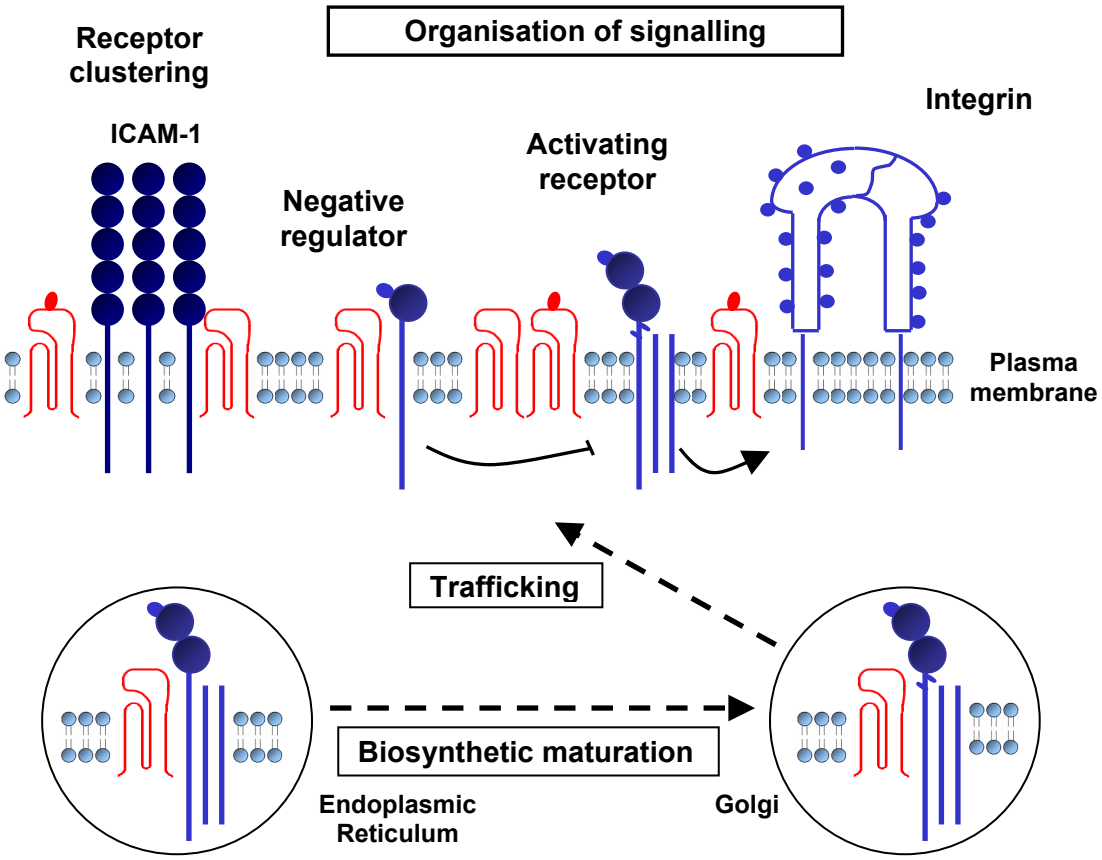


Figure 1.2 A hypothetical model for tetraspanin function

Diagrammatic representation of the proposed roles for the tetraspanin superfamily of proteins. It is thought that tetraspanins interact with specific partner proteins modulating their biosynthetic maturation and trafficking, as well as their organisation at the plasma membrane which may facilitate the regulation of signalling and clustering.

1.1.2 Structural features of a tetraspanin

1.1.2.1 Overview

Tetraspanins are characterised by the presence of four transmembrane domains, a small extracellular loop (EC1), a large extracellular loop (EC2) and intracellular amino- (NH_2) and carboxy-termini (COOH) (Figure 1.3A). In addition, these transmembrane proteins have a CCG motif and at least two other cysteine residues that contribute to disulphide bond formation in the large extracellular loop that characterise this particular superfamily of proteins (Kitadokoro et al., 2001b; Seigneuret et al., 2001). The crystal structures of the tetraspanin uroplakins (UP) Ia and Ib, along with their respective non-tetraspanin partner proteins, have been solved and a schematic is shown in Figure 1.3B (Min et al., 2006). They are small and compact, projecting only 4-5 nm from the plasma membrane (Kitadokoro et al., 2001b; Min et al., 2006). The crystal structure has also revealed that tetraspanins are rod-shaped and that the large extracellular loop is folded over the small extracellular loop (Min et al., 2006).

1.1.2.2 Transmembrane regions

The first, third and fourth transmembrane domains often contain a polar amino-acid residue. These include a conserved asparagine residue in the first transmembrane, and conserved glutamic acid or glutamine in the third and fourth. The function of these residues is not known (Hemler, 2005). In addition, tetraspanins have membrane-proximal cysteine residues that undergo palmitoylation which is necessary for tetraspanin-tetraspanin interactions. Palmitoylation is the post-translational *S*-acylation of proteins with palmitate and is thought to be important for protein stability, localization, trafficking and protein-protein interactions (Berditchevski et al., 2002). Indeed, palmitoylation is necessary for tetraspanin-tetraspanin secondary

interactions, as mutation of intracellular juxtamembrane cysteine residues proximal to all four transmembrane domains of CD9, CD81 and CD151 decreases lateral associations with other tetraspanins (Berditchevski et al., 2002; Charrin et al., 2002; Delandre et al., 2009; Yang et al., 2002). Conversely, the strong and direct primary interaction between CD151 and integrin $\alpha_3\beta_1$ is not affected by mutation of palmitoylation sites (Berditchevski et al., 2002; Yang et al., 2002). Metabolic labelling with ^{3}H-palmitate has been shown to be a useful tool in studying tetraspanin-enriched microdomains (Yang et al., 2004; Yang et al., 2008). Indeed, it has been identified that the palmitate transferase, DHHC2, is the likely candidate to be involved in palmitoylation of tetraspanins, as it promotes the palmitoylation, stability and function of both CD9 and CD151, and localizes to tetraspanin microdomains (Sharma et al., 2008). Inactive mutants of DHHC2 failed to promote tetraspanin palmitoylation, and RNA interference (RNAi) knockdown of DHHC2, but not six other DHHC family members, substantially impaired tetraspanin stability, as detected by western blotting (Sharma et al., 2008). In summary, palmitoylation appears fundamental to the formation of tetraspanin-tetraspanin interactions and the integrity of tetraspanin-enriched microdomains.

1.1.2.3 The small extracellular loop

Relatively little is known about the small extracellular loop, but it has been proposed that it is required for stabilisation of the large extracellular loop (Hemler, 2005). This may be particularly relevant in light of the group that crystallized the bladder-specific tetraspanins, the uroplakins, where the large extracellular loop appears to cover the small loop as shown in Figure 1.3B (Min et al., 2006).

1.1.2.4 The large extracellular loop

The large extracellular loop has been shown to be necessary for protein-protein interactions, with a relatively high degree of variability between individual family members, and so may be important for specific tetraspanin functions within a particular cell type (Stipp et al., 2003). It is subdivided into a constant region that contains three α-helices denoted A, B and E and a variable region (Figure 1.4), where most of the tetraspanin-partner protein interactions occur (Charrin et al., 2009; Hemler, 2005). In addition, this region has a number of conserved residues including a CCG motif and at least a further two cysteine residues involved in disulphide bond formation. The model of the large extracellular loop has been determined by crystallographic studies of CD81 and the bladder-specific tetraspanins, the uroplakins (Kitadokoro et al., 2001a; Min et al., 2006). In addition, the majority of tetraspanins have between one and three potential *N*-linked glycosylation sites in this region, but their role in tetraspanin function has not been investigated. There are some exceptions, for example, CD9 has a single glycosylation site in the small extracellular loop and CD81 is not glycosylated. However, the glycosylation status is dependent on both the cell type and tissue in which the tetraspanin is expressed. The role of the large extracellular loop in tetraspanin function and interaction with partner proteins is discussed in more detail below.

1.1.2.5 The carboxy- & amino- termini

Tetraspanins have both intracellular amino- and carboxy-termini. They are relatively short in length, apart from a few exceptions, for example, Tspan32 and retina-specific tetraspanins, peripherin and Rom-1. For most tetraspanins, the role of these regions is not well understood. However, the role of the carboxy-terminus of CD63 has been characterised. It has a tyrosine-based lysosomal targeting motif (G-Y-E-V-M) that

plays a role in trafficking of this tetraspanin by interacting with adaptor-protein-2 (AP-2) and AP-3 proteins, which promote clathrin-mediated endocytosis (Berditchevski and Odintsova, 2007). Mutation of the tyrosine residue to alanine abolishes trafficking of CD63 to lysosomes in rat fibroblasts (Rous et al., 2002). However, this has been shown to be cell type-dependent, as CD63 trafficking to lysosomes in mouse fibroblasts is independent of the motif (Ryu et al., 2000). In addition, it is noteworthy that other tetraspanins have a similar tyrosine-based motif including CD37, CD82 and CD151 (Berditchevski and Odintsova, 2007).

The tetraspanin CD63 has also been shown to interact directly with the PDZ (Postsynaptic density 95, PSD-85; Discs large, Dlg; Zonula occludens-1, ZO-1) domain-containing protein syntenin-1 (Latysheva et al., 2006). In common with most PDZ domain interactions, which involve binding of the globular PDZ domain to a four amino acid region at the carboxy terminus of a partner protein, this has been shown to be dependent on the carboxy-terminus of CD63. Syntenin-1 appears to negatively regulate CD63 internalisation, thus opposing the AP-2/AP-3 clathrin-dependent endocytosis (Latysheva et al., 2006). Some other tetraspanins have predicted PDZ-binding motifs in their COOH-termini (Berditchevski and Odintsova, 2007). Since many PDZ domain-containing proteins have more than one PDZ domain and function as scaffolds, it is possible that specific tetraspanin-tetraspanin interactions might be promoted by such scaffolds.

There are other cytoplasmic proteins that are tetraspanin-associated and are likely to bind to the cytoplasmic regions of the tetraspanin. These include small G-proteins, phosphatidylinositol 4-kinase (PI4K) and protein kinase C (PKC) α, the latter of which is closely associated with the tetraspanins CD9 and CD81 (Berditchevski,

2001; Le Naour et al., 2006b; Zhang et al., 2001). However, the functional consequences of these interactions have not been elucidated.

The role of the amino-terminus is less well-defined in tetraspanin biology, and for this reason this is the most commonly-used site for epiptope tagging of tetraspanins. It has been suggested that ubiquitination, a post-translational modification whereby the attachment of ubiquitin to lysine residues labels proteins for internalisation and/or proteosomal degradation (Hershko et al., 2000), occurs on the amino-terminus of some tetraspanins (Lineberry et al., 2008). Indeed, the GRAIL E3 ligase has been shown to be involved in conjugating ubiquitin to the tetraspanins CD151 and CD81 via lysine residues in the amino-terminus, promoting proteosomal degradation and tetraspanin down-regulation (Lineberry et al., 2008).

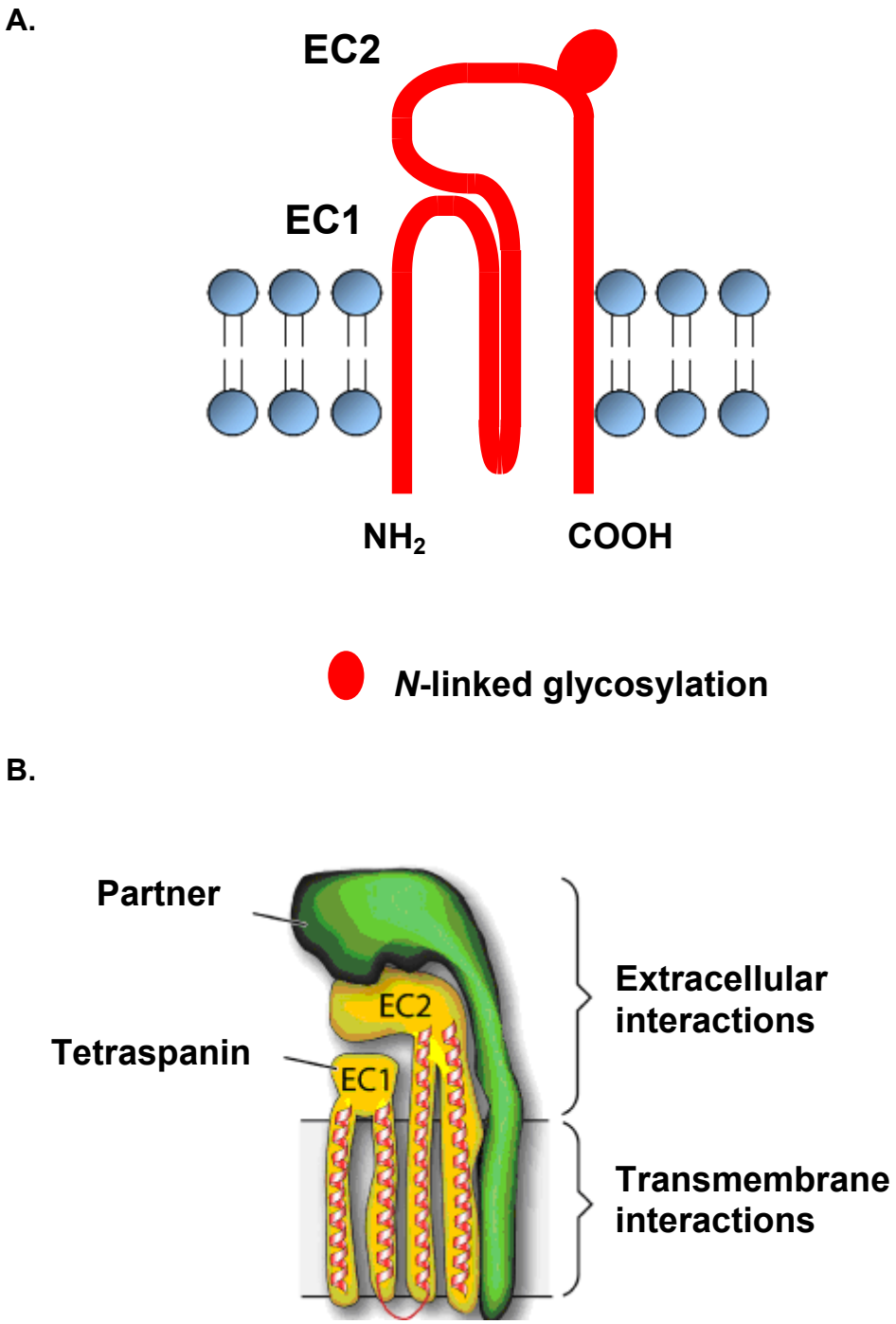


Figure 1.3 Diagrammatic representation of a tetraspanin and a tetraspanin/partner protein interaction

(A) Tetraspanins have four transmembrane domains, a small extracellular loop (EC1) and a large extracellular loop (EC2), along with intracellular amino- and carboxy-termini. In addition, the majority of tetraspanins have *N*-linked glycosylation sites. (B) Schematic representation of the structure of one of the tetraspanin uroplakins in complex with its non-tetraspanin partner protein uroplakin. Adapted from Min et al. 2006.

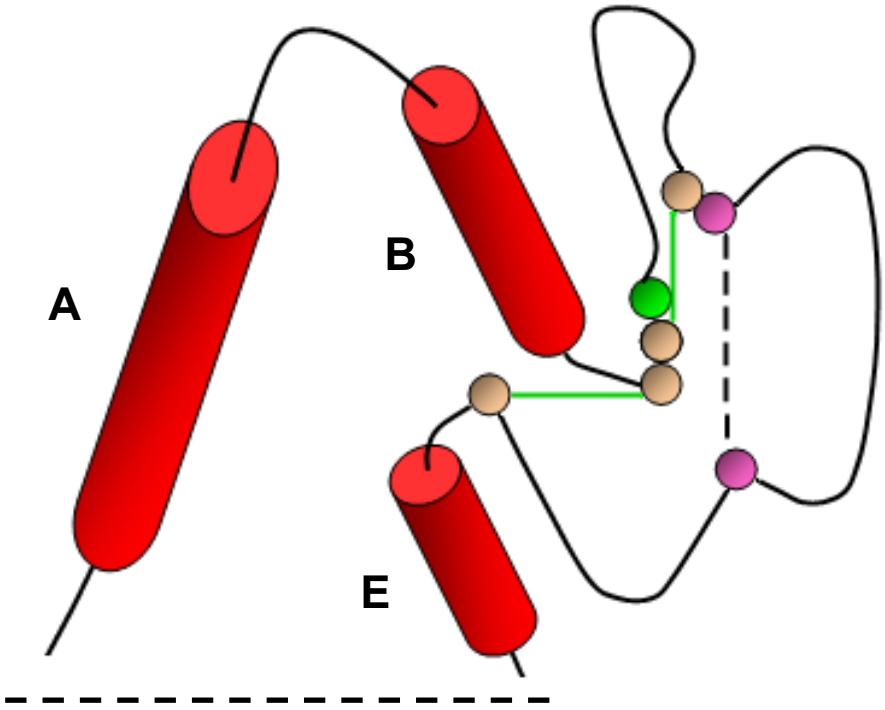


Figure 1.4 Diagrammatic representation of the large extracellular loop of a tetraspanin

The large extracellular loop (EC2) of a tetraspanin is composed of a constant region that contains three α-helices denoted A, B and E. There is a CCG motif after the B helix and two other conserved cysteines (brown circles) that form two intramolecular disulphide bonds (green lines). A significant number of tetraspanins contain a further two cysteine residues that form another disulphide bond (dotted black line). In relatively few tetraspanins, there may be seven or eight cysteine residues, the former of which allows the formation of an intermolecular disulphide bond. Adapted from Hemler, 2005.

1.1.3 Tetraspanin-enriched microdomains

1.1.3.1 Overview

The general consensus is that tetraspanins are thought to function by self-association to form tetraspanin-enriched microdomains that may act as molecular organisers of the cell surface by modifying the signalling and adhesion of other receptors. This concept was first introduced by Maecker et al. (1997) who termed tetraspanins as "molecular facilitators" of the cell surface. Since then, it has been shown that tetraspanins have specific binding partners (Table 1.1) allowing the formation of tetraspanin networks across the cell membrane that control the presentation and spatial organisation of membrane complexes (Charrin et al., 2009; Hemler, 2003; Hemler, 2005; Le Naour et al., 2006a). Recent evidence has highlighted the possibility of discrete tetraspanin microdomains, for example, fluorescence and immunoelectron microscopy revealed the presence of several hundred distinct tetraspanin-enriched microdomains upon the surface of HeLa cells, each with an individual size of 0.2 μm^2 (Nydegger et al., 2006). Moreover, individual tetraspanins are often expressed at 30,000-100,000 per cell (Hemler, 2003) and each cell type generally expresses multiple tetraspanins, for example, megakaryocytes express 19 different tetraspanins (Protty et al., 2009).

Table 1.1 Tetraspanins and examples of their specific binding partners

Tetraspanin	Partner protein	Function	Reference
CD9	CD9	Structural unit	(Kovalenko et al., 2004)
	CD9P-1 (EWI-F)	Not known	(Charrin et al., 2001; Stipp et al., 2001)
	EWI-2	Integrin-dependent modulation of cell motility and spreading	(Stipp et al., 2001)
	Claudin-1	CD9 stabilises expression of claudin-1	(Kovalenko et al., 2007)
CD63	Syntenin-1	Regulation of endocytosis	(Latysheva et al., 2006)
CD81	CD81	Structural unit	(Kitadokoro et al., 2001a)
	EWI-2	CD81 involved in maturation and surface expression of EWI-2	(Stipp et al., 2001)
	CD19	CD81 involved in maturation and surface expression of CD19	(Cherukuri et al., 2004b)
	$\alpha_4\beta_1$ integrin	Supports integrin adhesion strengthening	(Feigelson et al., 2003; Serru et al., 1999)
CD151	CD151	Structural unit	(Kovalenko et al., 2004)
	$\alpha_3\beta_1$ integrin	CD151 affects integrin-dependent adhesion and motility	(Serru et al., 1999; Yauch and Hemler, 2000)
	$\alpha_6\beta_1$ integrin	CD151 affects integrin adhesion strengthening	(Lammerding et al., 2003)
	$\alpha_6\beta_4$ integrin	CD151 affects integrin-dependent adhesion and motility	(Sterk et al., 2002; Winterwood et al., 2006)
	$\alpha_7\beta_1$ integrin	Not known	(Sterk et al., 2002)
UPIa and UPIb	UPII and UPIII	Uroepithelial plaque formation	(Min et al., 2006)

1.1.3.2 Molecular interactions of tetraspanins

There are three levels of tetraspanin interaction: primary, secondary and tertiary (Figure 1.5). Tetraspanin interactions with their partner proteins have been characterised according to their maintenance in detergents and/or covalent cross-linking (Charrin et al., 2009; Hemler, 2005).

Primary associations are direct, maintained in stringent detergents (Triton X-100), occur at a relatively high stoichiometry and are captured by covalent cross-linking (Charrin et al., 2009; Hemler, 2005; Le Naour et al., 2006a). The association between the tetraspanin CD151 and integrin $\alpha_3\beta_1$ is an example of a primary interaction that occurs at a high stoichiometry of almost 100% (Figure 1.5) (Serru et al., 1999; Yauch and Hemler, 2000). Indeed, the association between CD151 and integrin $\alpha_3\beta_1$ occurs early in biosynthesis whilst the integrin is still in the endoplasmic reticulum (ER) (Kazarov et al., 2002). There are numerous other examples of primary interactions including the tetraspanin uroplakins and the relevant non-tetraspanin uroplakin partner (Wu et al., 1995) (Table 1.1).

Secondary associations are indirect and typically involve tetraspanin association with other tetraspanins that link primary complexes together. Such interactions are generally only maintained in less stringent detergents (Brij97) and occur late in biosynthesis (Yang et al., 2002). Tetraspanin interactions are promoted or stabilised by protein palmitoylation as described in section 1.1.2.2. For example, mutation of cysteine residues in CD9, CD81 and CD151 decreases lateral associations with other tetraspanins (Berditchevski et al., 2002; Charrin et al., 2002; Delandre et al., 2009; Yang et al., 2002). Such data highlights the importance of palmitoylation in organising tetraspanins into microdomains.

A third level exists which is a looser network of tetraspanins and their associated proteins, whereby larger complexes are formed that are only stable in relatively weak detergents such as CHAPS and Brij58 (Charrin et al., 2009; Hemler, 2005). However, tetraspanins may not be completely solubilised in such detergents and so the relevance of third level interactions is not clear. The biochemical studies discussed above regarding the nature of tetraspanin microdomains have been developed further using single-molecule particle tracking of CD9 (Espenel et al., 2008). This has revealed that tetraspanin-tetraspanin interactions are transient, highly dynamic, with microdomains in distinct clusters (Espenel et al., 2008).

1.1.3.3 Comparison of tetraspanin-enriched microdomains and lipid rafts

The view of biological membranes has changed considerably since the proposal of the fluid mosaic model (Singer and Nicolson, 1972). It is now generally believed that the biological membrane is composed of different compartments, or microdomains, containing various lipid and protein compositions (Brown, 2006; Brown and London, 1998). Indeed, the use of proteomics has facilitated the understanding of the protein composition of the biological membrane (Foster et al., 2009). Tetraspanin-enriched microdomains are distinct from lipid rafts, a second, better known type of microdomain. Lipid rafts are membrane domains that are comprised of cholesterol, sphingolipids and saturated phospholipids (Brown, 2006; Brown and London, 1998). Tetraspanin-enriched microdomains and lipid rafts do have a number of similarities, for example, tetraspanins can associate with cholesterol and are found in low-density fractions of sucrose gradients (Charrin et al., 2003; Le Naour et al., 2006a). However, only the tetraspanin CD81 has been shown to reside in lipid rafts, to which it translocates following co-ligation of the B-cell receptor and CD19/CD21/CD81 co-receptor complex, and will be discussed in section 1.1.4.3 (Cherukuri et al., 2004a;

Cherukuri et al., 2004b). There are a number of differences between tetraspanin-enriched microdomains and lipid rafts which suggests they should be considered as separate types of microdomain, for example, lipid rafts contain Src family kinases, glycosylphosphatidylinositol (GPI)-linked proteins and caveolin which have not been observed in tetraspanin-enriched microdomains (Brown and London, 1998). A more detailed overview of the similarities and differences between tetraspanin-enriched microdomains and lipid rafts is provided in Table 1.2.

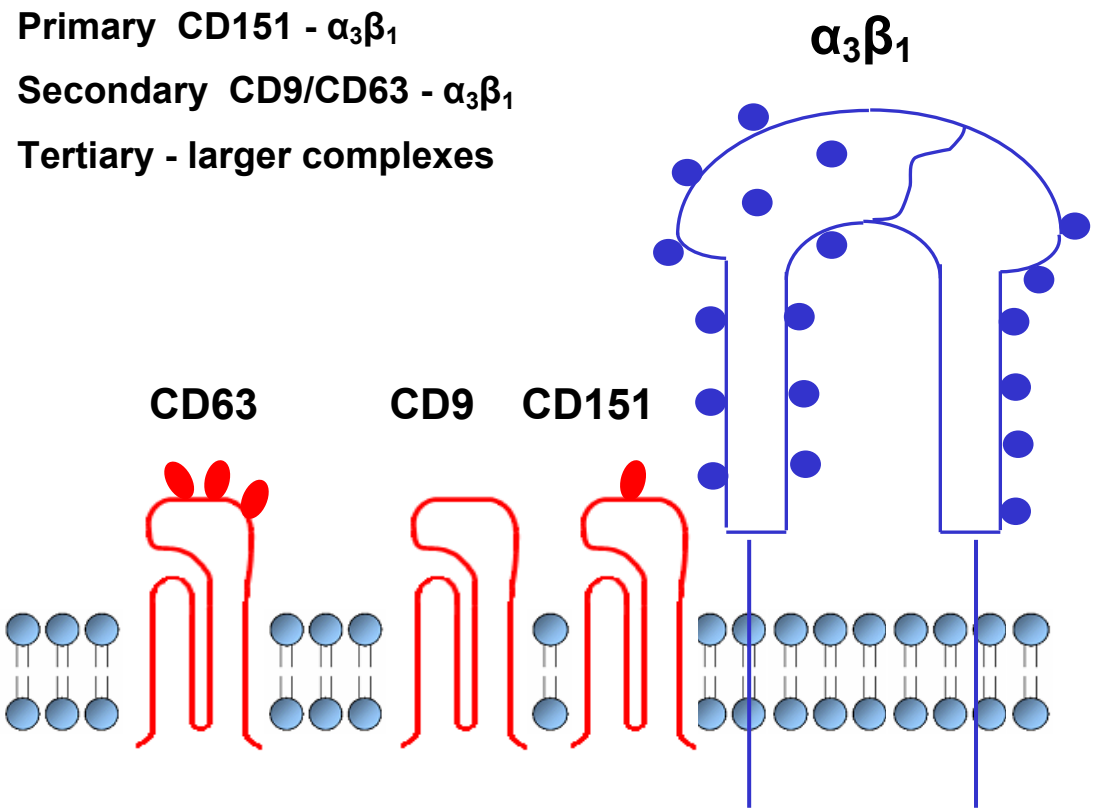


Figure 1.5 Primary, secondary and tertiary interactions of tetraspanins with partner proteins

There are three levels of tetraspanin interactions with partner proteins: primary, secondary and tertiary. Primary interactions include CD151 and integrin $\alpha_3\beta_1$, and secondary associations include CD9 and integrin $\alpha_3\beta_1$, and CD63 and integrin $\alpha_3\beta_1$ (Hemler, 2005; Serru et al., 1999; Yauch and Hemler, 2000). Tertiary interactions are a looser network of tetraspanins and their associated proteins, whereby larger complexes are formed (Hemler, 2005).

Table 1.2 Comparison between tetraspanin-enriched microdomains and lipid rafts

	Lipid rafts	Tetraspanin-enriched microdomains	Reference
Components	Src family kinases, caveolae and GPI-linked proteins present	Tetraspanins and associated proteins	(Brown, 2006; Brown and London, 1998; Hemler, 2003; Hemler, 2005)
Cholesterol	Disrupted following cholesterol depletion	Generally resistant to cholesterol depletion	(Charrin et al., 2003; Claas et al., 2001)
Extraction temperature	Disrupted at 37 °C	Maintained at 37 °C	(Hemler, 2005)
Detergent solubility	Generally insoluble in non-ionic detergents	Generally soluble in non-ionic detergents	(Claas et al., 2001)
Palmitoylation	Protein palmitoylation of raft proteins promotes insolubility	Protein palmitoylation of tetraspanin-enriched microdomain proteins does not promote insolubility	(Berditchevski et al., 2002; Yang et al., 2002)

1.1.4 Tetraspanin function

1.1.4.1 Overview

The function of the majority of tetraspanins remains to be elucidated. However, there are a few notable exceptions which have provided valuable insights into the role of tetraspanins in a number of cell types and species (Table 1.3). Some of the best characterised tetraspanins in relation to their function are CD151 and the regulation of integrins (Kagan et al., 1988; Karamatic Crew et al., 2004; Winterwood et al., 2006; Wright et al., 2004; Yang et al., 2008), altered signalling of the B-cell receptor in CD81-deficient mice (Cherukuri et al., 2004a; Cherukuri et al., 2004b), T-cell hyperproliferation in CD37-, CD81-, CD151- and Tspan32-deficient mice (Knobeloch et al., 2000; Miyazaki et al., 1997; Tarrant et al., 2002; van Spriel et al., 2004; Wright et al., 2004) and unstable haemostasis in CD151- and Tspan32-deficient mice (Goschnick et al., 2006; Lau et al., 2004; Orlowski et al., 2009). Other examples of tetraspanin function, including those in lower organisms, are discussed in later sections.

Table 1.3 Examples of tetraspanin function in different species

Species	Tetraspanin (s)	Loss of function phenotype	Reference
Human	Peripherin & ROM-1	Retinal degeneration	(Loewen et al., 2001)
	CD151	Kidney & skin defects, deafness & ß-thalassemia	(Kagan et al., 1988; Karamatic Crew et al., 2004)
	CD81	Hepatocytes no longer susceptible to hepatitis C virus (HCV) infection	(Cocquerel et al., 2006; Flint et al., 2006; Pileri et al., 1998)
Mouse	Peripherin & ROM-1	Retinal degeneration	(Clarke et al., 2000; Connell et al., 1991)
	CD9	Impaired sperm-egg fusion	(Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000)
	CD37, CD81, CD151 & Tspan32	T-cell hyperproliferation	(Knobeloch et al., 2000; Miyazaki et al., 1997; Tarrant et al., 2002; van Spriel et al., 2004; Wright et al., 2004)
	CD81	Impaired B-cell receptor signalling & sperm-egg fusion	(Cherukuri et al., 2004a; Cherukuri et al., 2004b; Miyazaki et al., 1997; Rubinstein et al., 2006b)
	CD151	Kidney, skin & platelet defects & lymphocyte hyperproliferation	(Baleato et al., 2008; Lau et al., 2004; Orlowski et al., 2009; Sachs et al., 2006; Wright et al., 2004)
Fruit-fly (*Drosophila melanogaster*)	Late bloomer	Delayed synaptic contact formation in embryos	(Kopczynski et al., 1996)
	Sunglasses	Retinal degeneration due to an inability to downregulate rhodopsin	(Han et al., 2007; Xu et al., 2004)
Worm (*Caenorhabditis elegans*)	TSP-15	Impaired epidermal integrity	(Moribe et al., 2004)
Fungi			
Magnaporthe grisea	MgPls1	Impaired host leaf penetration	(Clergeot et al., 2001)
Botrytis cincerea	BcPls1	Impaired host leaf penetration	(Gourgues et al., 2004)
Podospora anserina	PaPls1	Impaired host leaf penetration	(Lambou et al., 2008b)
Plants (*Arabidopsis thaliana*)	TORNADO2	Impaired leaf development & altered cellular distribution of shoot apical meristem	(Chiu et al., 2007; Cnops et al., 2006)

1.1.4.2 CD151 and the regulation of integrins

CD151 is one of the best studied tetraspanins and is expressed on a number of cell types including epithelial and endothelial cells, Schwann cells, muscle cells, megakaryocytes and platelets (Sincock et al., 1997). Mice deficient in CD151 are viable and fertile, but do have a number of phenotypes including unstable haemostasis, hyper-proliferative T-cells, impaired keratinocyte migration and kidney failure (Baleato et al., 2008; Sachs et al., 2006; Wright et al., 2004). CD151 and its association with the laminin-binding integrins $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$ and $\alpha_6\beta_4$ are particularly well characterised (Table 1.1) (Charrin et al., 2009; Hemler, 2005). For example, the CD151 and integrin $\alpha_3\beta_1$ association is a strong, direct, primary interaction of high stoichiometry and it has been shown that CD151 affects integrin-dependent adhesion and motility (Serru et al., 1999; Yauch and Hemler, 2000). Further studies using CD151 RNAi on carcinoma cells show impaired motility on laminin, along with impaired internalisation of integrin $\alpha_3\beta_1$ (Winterwood et al., 2006; Yang et al., 2008). Moreover, deletion of CD151 in mice has been shown to decrease pathologic angiogenesis both *in vivo* and *in vitro* by impaired integrin signalling and a failure to associate with tetraspanin-enriched microdomains (Takeda et al., 2007b). The interaction between CD151 and integrins $\alpha_3\beta_1$ and $\alpha_6\beta_1$ is dependent on a QRD $^{(194\text{-}196)}$ motif present in the large extracellular loop of CD151 (Kazarov et al., 2002). In addition, mutation of the tyrosine-based sorting motif (Y-R-S-L) in the cytoplasmic tail of CD151 reduces its internalisation and also impairs the internalisation of associated integrins on laminin (Liu et al., 2007). This reinforces the role of CD151 in promoting cell migration by regulating integrin trafficking (Berditchevski and Odintsova, 2007; Charrin et al., 2009).

The role of CD151 in human disease is highlighted as a mutation of this tetraspanin has been described (Karamatic Crew et al., 2004). This is not a common affliction, as only three patients with this mutation have been identified at present. Such a mutation would result in a truncated CD151 lacking most of the large extracellular loop including its integrin binding domain (QRD $^{(194\text{-}196)}$ motif), if the protein is even expressed, and the patients have a skin blistering disease, known as pretibial epidermolysis bullosa, and end-stage hereditary nephritis (Karamatic Crew et al., 2004). This is consistent with CD151-$\alpha_3\beta_1$ and CD151-$\alpha_6\beta_1$ complexes having roles in the normal organization of the epidermal basement membrane (Karamatic Crew et al., 2004; Lammerding et al., 2003; Nishiuchi et al., 2005). In addition, two of the patients who are siblings have sensorineural deafness and β-thalassaemia minor (Kagan et al., 1988). Interestingly, glomerular disease of the kidney is observed in mouse models due to severe alterations of the glomerular basement membrane; however, this has been shown to be dependent upon the mouse genetic background (Baleato et al., 2008). Similar kidney disruption and/or skin blistering diseases are observed upon deletion of the mouse integrin α_3 subunit, highlighting the close association between integrins and CD151 (Kreidberg, 2000).

1.1.4.3 CD81 is required for B-cell receptor signalling

The role of CD81 in B-cell receptor signalling is one of the best studied examples of a tetraspanin having a specific function (Levy and Shoham, 2005; Levy et al., 1998). An overview of the B-cell receptor, its signalling pathway, and the role of CD81 is provided in Figure 1.6. CD81 functions to enhance B-cell receptor signalling by reducing the threshold for B-cell activation. Moreover, this is the only tetraspanin identified at present to enter lipid rafts to perform this role (Levy and Shoham, 2005; Levy et al., 1998). CD81 is expressed at the surface of B-cells and is directly

associated with CD19, a signalling molecule containing tyrosine-based signalling motifs. CD81 is essential for normal glycosylation of CD19 and its trafficking to the plasma membrane (Cherukuri et al., 2004b). CD19 associates with CD21, the complement 3d (C3d) receptor. Co-ligation of the CD81/CD19/CD21 complex with the B-cell receptor is induced by simultaneous binding of complement-tagged antigens to CD21 and the B-cell receptor. This signalling complex translocates to lipid rafts where CD81 becomes inducibly palmitoylated, which is essential for sustained localization of the B-cell receptor-CD19/CD21/CD81 complex to lipid rafts and for sustained signalling, probably due to the concentration of certain signalling molecules in lipid rafts (Figure 1.6A) (Cherukuri et al., 2004a). This has been illustrated by utilising mice deficient in CD81, where co-ligation of the B-cell receptor and the CD19/CD21 complex fails to partition normally into lipid rafts (Figure 1.6B) (Cherukuri et al., 2004a; Cherukuri et al., 2004b). In addition, it was shown that the distribution and trafficking of CD19 from the ER to the Golgi body was slower than in CD81-positive cells (Shoham et al., 2003). In summary, CD81 is required for normal glycosylation of CD19 and trafficking to the plasma membrane, and is inducibly palmitoylated, which is required for the B-cell receptor-CD19/CD21/CD81 complex to localize to lipid rafts and to signal in a sustained manner (Cherukuri et al., 2004a; Cherukuri et al., 2004b).

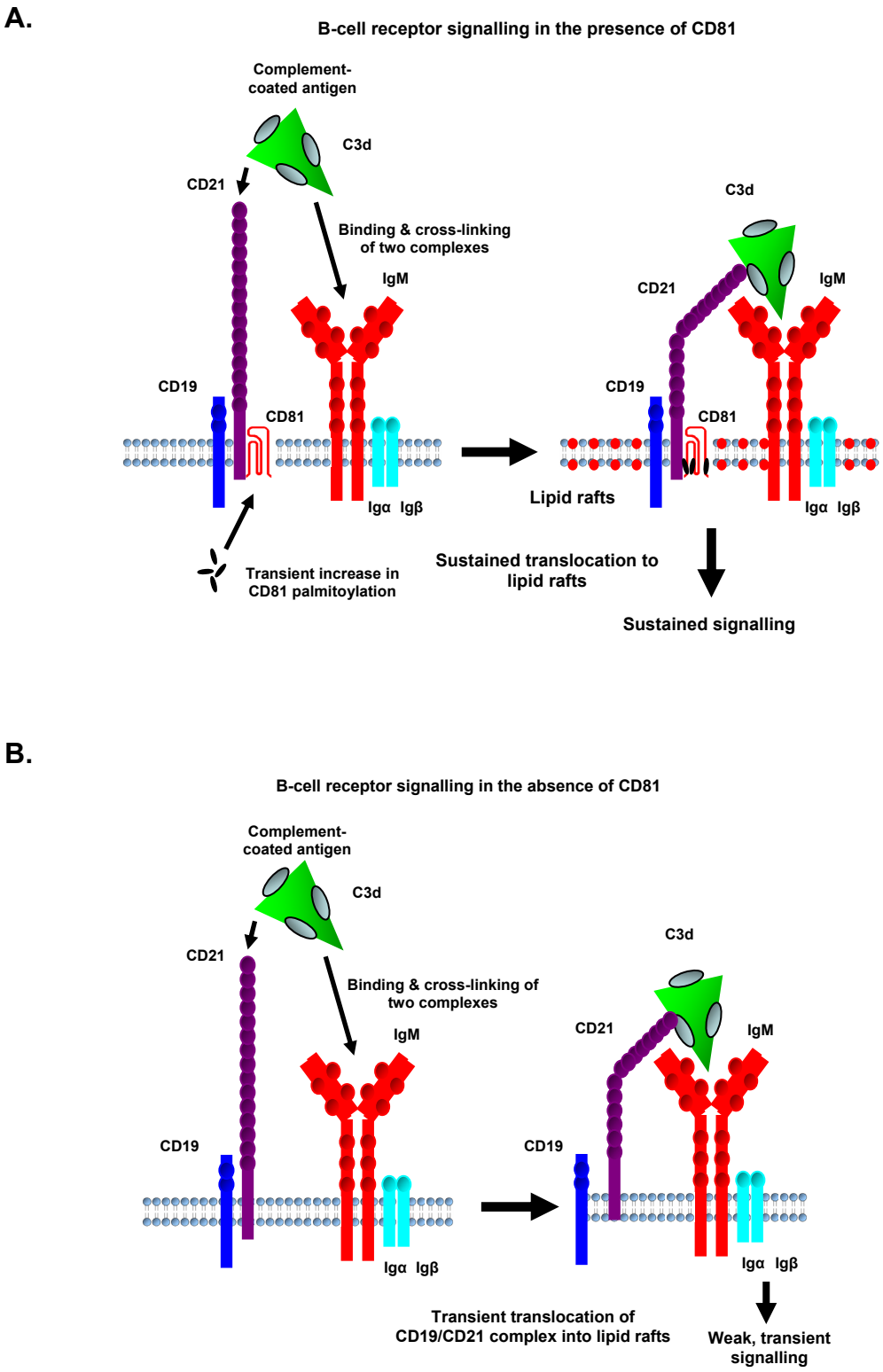


Figure 1.6 B-cell receptor signalling is impaired in the absence of CD81

CD81 deletion results in failure of the CD19-CD21 co-receptor complex to partition into lipid rafts leading to reduced B-cell signalling (Cherukuri et al., 2004a; Cherukuri et al., 2004b). Adapted from Levy & Shoham, 2005.

1.1.4.4 Hyperproliferative T-cells in tetraspanin-deficient mice

Tetraspanins have proposed roles in T-cell function which have been illustrated using mice deficient in CD37, CD81, CD151 and Tspan32 (Knobeloch et al., 2000; Miyazaki et al., 1997; Tarrant et al., 2002; van Spriel et al., 2004; Wright et al., 2004). They all show a remarkably similar phenotype of T-cell hyperproliferation to *in vitro* stimulation using anti-CD3 antibodies. CD3 are signalling units of the T-cell receptor which contain immunoreceptor tyrosine-based activation motifs (ITAMs) that undergo phosphorylation leading to recruitment and activation of Zap-70/Syk tyrosine kinases (Abraham and Weiss, 2004). A more detailed overview of T-cell receptor signalling is provided in section 1.3. Such data regarding the T-cell receptor and tetraspanins suggests that tetraspanin microdomains negatively regulate T-cell receptor signalling, and deletion of any tetraspanin might disrupt the microdomains and thus the ability to negatively regulate. It has been hypothesised that tetraspanins may recruit a negatively regulatory phosphatase or sequester key molecules into tetraspanin-enriched microdomains therefore accounting for the effects observed (Hemler, 2008).

1.1.4.5 The platelets of tetraspanin-deficient mice

The published literature has identified five tetraspanins expressed in platelets: CD9, CD63, CD151, Tspan32 and Tspan9 (Goschnick and Jackson, 2007; Protty et al., 2009; Tomlinson, 2009). Of these, CD151- and Tspan32-deficient mice have a bleeding phenotype that the authors propose to be due to defective "outside-in" signalling of the major platelet integrin $\alpha_{IIb}\beta_3$ (Goschnick et al., 2006; Lau et al., 2004). Platelets deficient in either of these two tetraspanins have a remarkably similar phenotype of defective spreading on fibrinogen, delayed clot retraction and a tendency to re-bleed in tail-bleeding assays. In addition, an *in vivo* thrombus

formation defect is observed in the Tspan32 knockout (Goschnick et al., 2006). Further to this, CD151, unlike Tspan32, is expressed on endothelial cells, and the Jackson group have recently confirmed that platelet CD151 is more important for thrombus formation than endothelial CD151 using a platelet reconstitution model (Orlowski et al., 2009). Moreover, the association between CD151 and Tspan32 and $\alpha_{IIb}\beta_3$ is supported by co-immunoprecipitation data in mouse platelets using the stringent detergent Triton X-100 (Goschnick et al., 2006; Lau et al., 2004). However, this is not supported by recent evidence in human platelets by Protty et al. (2009). Nevertheless, the functional data indicate a role for tetraspanin microdomains in platelet activation.

A mild platelet phenotype has recently been reported in CD9-deficient mouse platelets (Mangin et al., 2009), despite the fact that CD9 is expressed at approximately 49,000 copies per human platelet (Higashihara et al., 1990; Protty et al., 2009), second only to the major platelet integrin $\alpha_{IIb}\beta_3$ at 80,000 copies per platelet (Wagner et al., 1996). Tail bleeding assays in mice revealed a shorter bleeding time and reduced blood loss in the CD9 knockout compared to wild-type controls (Mangin et al., 2009). This data suggests that CD9 does not play a major role in primary haemostasis. Indeed, this data is in contrast to that of CD151- and Tspan32-deficient mouse platelets, where there is an increase in bleeding and a tendancy to re-bleed (Goschnick et al., 2006; Lau et al., 2004). Further experiments by Mangin et al. show no difference in aggregation responses in CD9-deficient platelets. However, there was increased $\alpha_{IIb}\beta_3$ activation as revealed by a significant increase in the binding of its ligand fibrinogen (Mangin et al., 2009). It would appear that CD9 may act as a repressor of $\alpha_{IIb}\beta_3$ "inside-out" signalling, which is in contrast to the role of CD151 and Tspan32. This hypothesis is supported by *in vivo* thrombosis data which have

larger thrombi in CD9-deficient mice, and suggests that in wild-type mice CD9 may act as a negative regulator of platelet recruitment, thus preventing excessive thrombus growth (Mangin et al., 2009).

Mice deficient in the tetraspanin CD63 have been reported with only minor differences in platelet function. Slighty stronger aggregation responses are observed in the CD63 knockout compared to wild-type controls *in vitro* (Schroder et al., 2009). However, CD63 has been shown to associate with integrin $\alpha_{IIb}\beta_3$ and may regulate platelet spreading and signalling on fibrinogen (Israels and McMillan-Ward, 2005).

1.1.4.6 Tetraspanins and their role in infectious disease

1.1.4.6.1 HIV-1

Tetraspanins appear to play a role in human immunodeficiency virus-1 (HIV-1) infection of human macrophages. Once inside macrophages, the virus assembles within a membrane domain containing the tetraspanins CD9, CD53, CD63 and CD81 (Nydegger et al., 2006). Moreover, HIV-1 release occurs at tetraspanin microdomains (Nydegger et al., 2006). However, knockdown of CD63 in macrophages does not affect the productivity or infectivity of HIV-1 in tissue culture (Ruiz-Mateos et al., 2008). It is possible that other tetraspanins and/or other molecules present in tetraspanin-enriched microdomains may be more important in HIV-1 productivity/infectivity. Therefore, the exact mechanism by which tetraspanins are involved in HIV-1 infection has yet to be fully determined.

1.1.4.6.2 Hepatitis C virus and CD81

Hepatitis C virus (HCV) infectivity is supported by the tetraspanin CD81 where it is an essential part of the HCV receptor complex that also includes the scavenger receptor class B type I, claudin-1 and occludin (Ploss et al., 2009). The virus E2

glycoprotein binds to the large extracellular loop of CD81 (Pileri et al., 1998). Mutations of CD81 that prevent the binding to E2 reduce, but do not completely prevent, infectivity of HCV (Flint et al., 2006). This data suggests that CD81 binds to to the virus glycoprotein as a co-receptor, as this binding is not essential for infection (Cocquerel et al., 2006). Interestingly, the association of CD81 with tetraspanin-enriched microdomains is not necessary for HCV infection (Rocha-Perugini et al., 2009).

1.1.4.7 Tetraspanin association with receptor tyrosine kinases

The tetraspanin CD82 has been shown to associate with receptor tyrosine kinases of the ErbB family, in particular epidermal growth factor receptor (EGFR), whose activity alters depending upon expression level of the tetraspanin, since CD82 diminishes ligand-induced dimerization and endocytosis (Berditchevski and Odintsova, 2007; Hemler, 2005). Experiments showed that CD82 expression led to composition changes in glycosphingolipids on the plasma membrane and also affected EGFR compartmentalization (Odintsova et al., 2006; Odintsova et al., 2000; Odintsova et al., 2003). This suggests a role for CD82 in EGFR trafficking to and from the membrane. CD82 has a tyrosine-based sorting motif (Y-S-K-V) in the cytoplasmic tail. Mutation of the tyrosine to alanine prevents antibody-induced internalisation of CD82, suggesting that the residue is important for endocytosis of the tetraspanin (Berditchevski and Odintsova, 2007).

1.1.4.8 Tetraspanin microdomains as endothelial adhesive platforms (EAPs)

A recent idea has emerged regarding the formation of endothelial adhesive platforms (EAPs) by tetraspanin-enriched microdomains (Barreiro et al., 2008). Leukocytes bind to activated endothelial cells, the latter of which possess specialized

microdomains that contain the leukocyte integrin adhesion receptors vascular cell adhesion molecule-1 (VCAM-1) and inter-cellular adhesion molecule-1 (ICAM-1), along with the tetraspanins CD9 and CD151. Using fluorescence microscopy, along with tetraspanin-blocking peptides, the authors show that such EAPs induce "nanoclustering" of adhesion receptors to enhance adhesive properties during leukocyte adhesion to the endothelium (Barreiro et al., 2008). The tetraspanin-blocking peptides were soluble forms of the large extracellular loop expressed as a GST-fusion protein, and have previously been used to perturb functions regulated by tetraspanin microdomains, for example, sperm-egg fusion (Higginbottom et al., 2003).

1.1.4.9 Impaired oocyte fertilisation in CD9-deficient mice

CD9-deficient mice have been generated and three groups used such mice to show that CD9 has a profound effect upon mouse reproductive biology (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000). CD9-deficient females have severely reduced fertility as a result of impaired sperm-egg fusion. In addition, CD9-null oocytes have altered length, thickness and density of microvilli which may prevent them from fusing with sperm (Runge et al., 2007). Further studies have suggested that CD9 controls the distribution of some membrane proteins, including the laminin-binding integrin $\alpha_6\beta_1$, into clusters, which may play a role in gamete fusion (Ziyyat et al., 2006). Subsequent data has shown that the tetraspanin CD81 is expressed on mouse eggs and is similarly involved in sperm-egg fusion, as mice deficient in this tetraspanin have reduced female fertility (Rubinstein et al., 2006a). However, it is not as severe as the CD9 phenotype. Interestingly, CD81 over-expression rescues the fertility defect in CD9-deficient eggs (Higginbottom et al., 2003). CD9 and CD81 show 45% identity across the protein, but CD81 lacks a motif in the large extracellular loop that has been shown to be critical for sperm-egg fusion in CD9.

Impressively, mice have been generated deficient in both of these tetraspanins and female mice are completely infertile (Rubinstein et al., 2006a). This is clear evidence that tetraspanins can functionally substitute for one another and suggests the need to generate mice deficient in more than one tetraspanin to overcome the subtle phenotypes seen in some particular cell types at present.

1.1.4.10 Tetraspanin function in lower organisms

1.1.4.10.1 *Drosophila melanogaster*

The role of tetraspanins in the fruit-fly is highlighted as the tetraspanin gene, *late bloomer* (*lbm*), is linked to delayed synaptic contact formation in embryos (Kopczynski et al., 1996). Deletion of a further two tetraspanins, CG10106 and CG12143, delays formation even further (Fradkin et al., 2002). These studies suggest that more than one tetraspanin can perform a similar role in a cell type. In addition, a lysosomal tetraspanin, sunglasses (sun), is required for downregulation of the light-sensing GPCR rhodopsin upon prolonged stimulation, presumably by playing a role in its lysosomal degradation (Han et al., 2007; Xu et al., 2004). Rhodopsin is also important for the formation of photoreceptor cells. Indeed, in mutant flies that lack sun, the eyes undergo light-induced retinal degeneration. (Han et al., 2007; Xu et al., 2004).

1.1.4.10.2 *Caenorhabditis elegans*

In *Caenorhabditis elegans* there are 20 tetraspanins. However, only Tsp-15 has been linked to a specific function (Todres et al., 2000). This particular tetraspanin is necessary for normal epidermal integrity, as knockdown of Tsp-15 resulted in degeneration of the hypodermis, an epidermal layer of cells, and blistering of the cuticle, an extracellular structure shed during moulting (Moribe et al., 2004).

Interestingly, Tsp-15 is 24% similar to CD151 according to amino-acid identity, consistent with the hypothesis that CD151 plays a role in epidermal integrity in mammals (Hemler, 2005).

1.1.4.10.3 *Magnaporthe grisea*, *Botrytis cincerea* and *Podospora anserina*

Three tetraspanin families have been identified in fungi: Pls1, Tspan2 and Tspan3. The fungi, *Magnaporthe grisea*, *Botrytis cincerea* and *Podospora anserina* and their respective tetraspanin proteins, MgPls1, BcPls1 and PaPls1 have been studied and highlight the role of tetraspanin proteins in fungal pathogenicity. Pls1, also known as punchless, is required for the pathogenicity of *Magnaporthe grisea*, *Botrytis cincerea* and *Podospora anserine,* in particular their penetration into host plant leaves (Clergeot et al., 2001; Gourgues et al., 2004; Lambou et al., 2008a). Other tetraspanins include Tspan2, whose function remains to be elucidated, and Tspan3, which has been shown to be involved in the infection of the plant pathogenic fungus *Magnaporthe grisea* (Lambou et al., 2008b).

1.1.4.10.4 *Arabidopsis thaliana*

The plant, *Arabidopsis thaliana,* is a model organism in which the tetraspanin TORNADO2 has been shown to be essential for normal function. Mutants of this tetraspanin result in defective leaves in both symmetry and size, a defective venation network and an altered distribution of the plant hormone auxin (Cnops et al., 2006). In addition, mutations of the *tornado2* gene affect the cellular distribution of the shoot apical meristem in *Arabidopsis thaliana* highlighting the role of tetraspanins in plants (Chiu et al., 2007).

1.2 C-type lectins

1.2.1 Overview

The C-type lectin receptors were originally classified as proteins that bind to carbohydrates via a distinct fold, referred to as a carbohydrate recognition domain (CRD) in a calcium-dependent manner (Drickamer, 1999). This superfamily, which includes both classical and non-classical members, consists of over 75 separate gene products and is sub-divided into 17 sub-groups depending on their domain architecture (Drickamer, 1999; Zelensky and Gready, 2005). Proteins that bind in a calcium-dependent manner are considered 'classical' C-type lectins and examples include DC-SIGN and langerin. Protein sequence alignment suggested that other members were calcium-independent and could not bind carbohydrate. In this case, the domain is referred to as a C-type lectin-like domain (CTLD) (Drickamer, 1999). These proteins are considered 'non-classical' C-type lectins and examples include dectin-1, CLEC-2 and CLEC9A.

1.2.2 Group V C-type lectin-like receptors

Dectin-1, CLEC-2 and CLEC9A (Figure 1.7) belong to group V of non-classical C-type lectin-like receptors, as they share the same domain architecture and, generally speaking, are encoded within the same natural killer (NK) gene complex (Sobanov et al., 2001). Other members of the group include CLEC-1, LOX-1, MICL and AICL (Sobanov et al., 2001). All members are type II transmembrane proteins and have a single CTLD, stalk region, transmembrane region and a cytoplasmic tail that often contains a signalling or internalisation motif (Figure 1.8). Table 1.4 provides an overview of some of the most thoroughly characterised group V C-type lectin receptors.

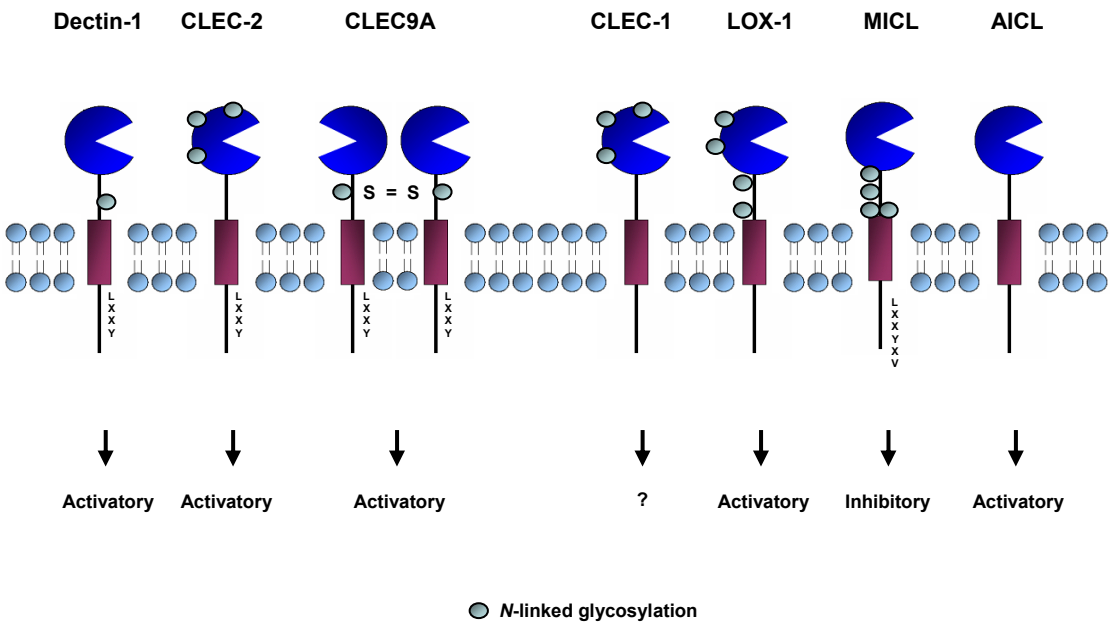


Figure 1.7 Members of the group V C-type lectin-like family of receptors

Diagrammatic representation of the members of the group V C-type lectin-like family of proteins including dectin-1, CLEC-2 and CLEC9A.

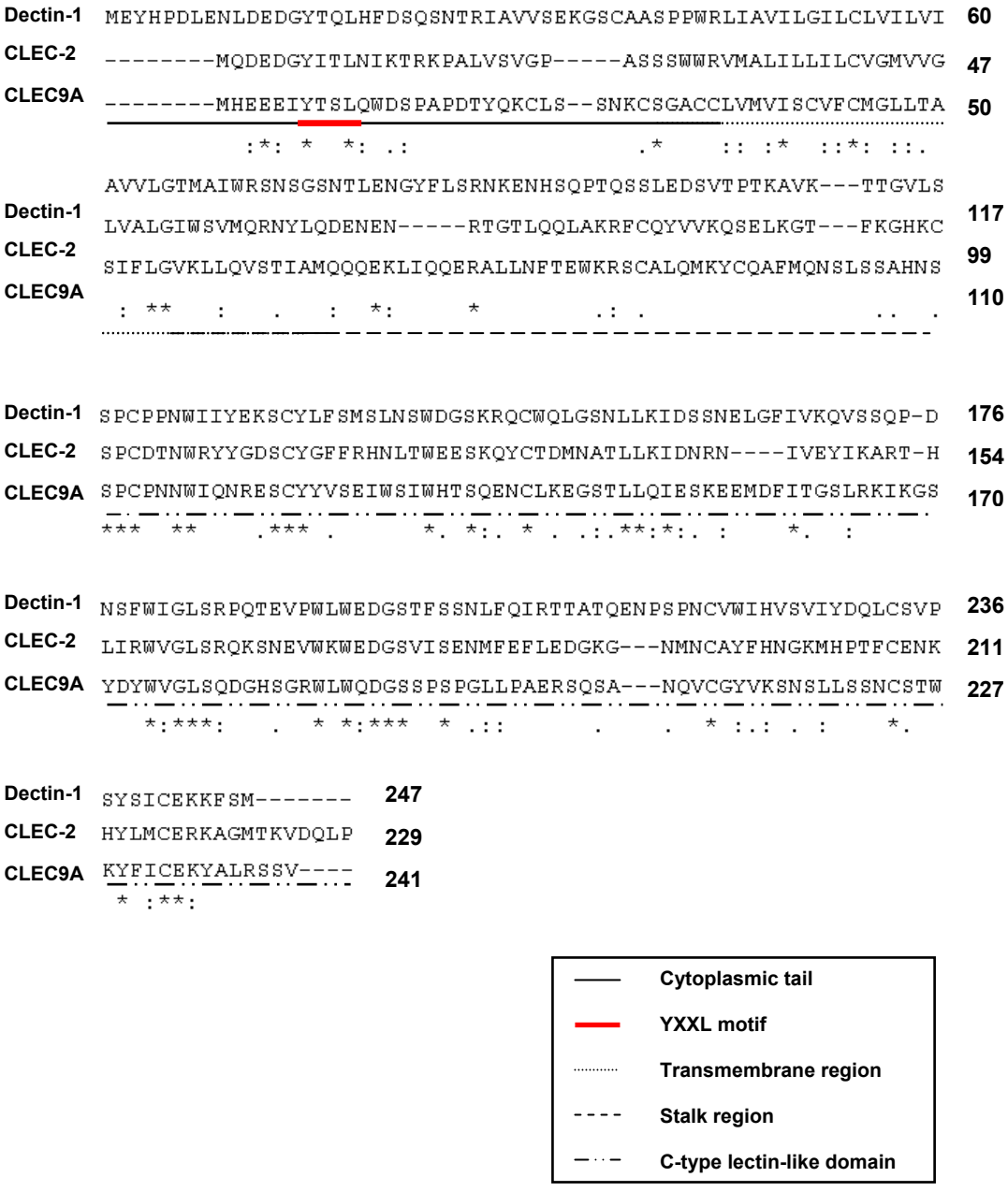


Figure 1.8 Sequence alignment of dectin-1, CLEC-2 and CLEC9A

Human dectin-1, CLEC-2 and CLEC9A amino-acid sequences were aligned using the Clustalw alignment programme (www.ebi.ac.uk/clustalw). Sequence alignment symbols denote degree of conservation: "*" = identical residues, ":" = conserved substitution and "." = semi-conserved substitution.

Table 1.4 Group V C-type lectin-like proteins

Name	Expression	Ligand	Function	Reference
Dectin-1	Dendritic cells, macrophages, monocytes	β-glucan	Anti-fungal immunity	(Brown, 2006; Willment and Brown, 2008)
CLEC-2	Platelets, monocytes, dendritic cells, granulocytes	Receptor for rhodocytin, podoplanin	Platelet activation	(Colonna et al., 2000; May et al., 2009; Suzuki-Inoue et al., 2006; Suzuki-Inoue et al., 2007)
CLEC9A	Dendritic cells, subset of monocytes	?	Activation receptor, role in cross-presentation of necrotic cells	(Huysamen et al., 2008; Sancho et al., 2009)
CLEC-1	Dendritic cells	?	?	(Colonna et al., 2000)
LOX-1	Vascular endothelial cells	Oxidised LDL	Platelet-endothelial interactions	(Dunn et al., 2008)
MICL	Dendritic cells, moncytes, granulocytes	?	Signals via an ITIM sequence in the cytoplasmic tail	(Marshall et al., 2004; Pyz et al., 2008)
AICL	Lymphocytes, monocytes, granulocytes	NKp80	Cytolysis of malignant myeloid cells & stimulates release of cytokines from NK cells & monocytes	(Welte et al., 2006)

1.2.3 Dectin-1

1.2.3.1 Overview

Antigen-presenting cells (APCs) express pattern-recognition receptors, such as the Toll-like receptors (TLRs) and the C-type lectins that recognise pathogen-associated molecular patterns (PAMPs) upon microbial structures (Brown, 2006; Willment and Brown, 2008). These highly conserved PAMP microbial structures are generally essential for micro-organism survival and so are immutable. Dectin-1 is a pattern recognition receptor that is expressed at high levels on APCs in portals of pathogen entry such as the lung and intestine, supporting the hypothesis that it has a role in immune responses against pathogens (Reid et al., 2004; Taylor et al., 2002). Recent evidence has shown the importance of dectin-1 in host defence, as mice deficient in this receptor are immuno-compromised in response to pathogenic fungi such as *Candida albicans* (Taylor et al., 2007) and *Pneumocystis carinii* (Saijo et al., 2007). A key difference between the papers is that Saijo et al. found no requirement of dectin-1 for protection against *Candida albicans.* This could be explained by the differing routes of infection, fungal strains used and genetic background of the mice (Dostert and Tschopp, 2007).

1.2.3.2 The dectin-1 ligand, β-glucan

The dectin-1 ligand β-glucan, a polymer of *D*-glucose, is a major component of fungal cell walls. Indeed, it is estimated that more than 50% of the cell wall of *Saccharmoyces cerevisiae* is made up of β-glucan polymers (Klis et al., 2002). A number of dectin-1 ligands, such as curdlan and zymosan, contain high amounts of β-glucan and are used to investigate the role of this receptor in immune response against pathogenic fungi both *in vitro* and *in vivo*. Curdlan is isolated from *Alcaligenes faecalis* and is composed of linear glucan polymers. In comparison, zymosan is

isolated from *Saccharmoyces cerevisiae*, but is less specific than curdlan, as it contains mannan, chitin and other proteins and has the ability to activate receptors other than dectin-1 (McGreal et al., 2006).

1.2.3.3 Dectin-1 structure and signalling

The dectin-1 CTLD has a binding site for β-glucan, as a mutation in this region (W^{221}→A & H^{223}→A) impairs binding of the dectin-1 ligand (Adachi et al., 2004). The cytoplasmic tail of dectin-1 contains a signalling motif that is made up of one tyrosine residue in a YXXL motif. This undergoes tyrosine phosphorylation by Src family kinases and is required for signalling events via phospholipase C (PLC) γ2 (Figure 1.9) (Rogers et al., 2005; Xu et al., 2009b). This was supported by Fuller et al. who used cell line reporter assays in DT40 B- and Jurkat T-cells to show that dectin-1 signals via the YXXL motif through interaction with a Syk/Zap-70 family kinase (Fuller et al., 2007).

When β-glucan binds to dectin-1 induction of various cytokines and chemokines ensues, including the interleukins (IL)-2, -6, -10, -12, tumour necrosis factor (TNF) and CXC-chemokine ligand 2 (CXCL2), in addition to respiratory burst, phagocytosis and possibly endocytosis (Brown, 2006; Willment and Brown, 2008). There is a plethora of evidence to suggest that the above are important in the control of fungal infection. For example, IL-6 is secreted by T-cells and macrophages in response to tissue damage, leading to inflammation, and mice deficient in this interleukin have increased susceptibility to systemic candidiasis and *Streptococcus pneumoniae* (van Enckevort et al., 1999). However, IL-12 and TNF production are not solely dependent on dectin-1, but can be activated via signals from TLR2 and TLR6 via the adaptor protein MyD88 and the transcription factor nuclear factor-κB (NF-κB). The caspase-

recruitment domain adaptor protein CARD9 is also involved in linking dectin-1-Syk activation to adaptor protein Bcl-10-MALT1-dependent activation of transcription factor NF-κB and cytokine production following stimulation with *Candida albicans* or zymosan in dendritic cells (Figure 1.9) (Gross et al., 2006; LeibundGut-Landmann et al., 2007). CARD9 plays an intermediate role in linking dectin-1 signalling to NF-κB activation and is involved in additional secretion of IL-12 (Colonna, 2007; Gross et al., 2006). Moreover, the importance of CARD9 is illustrated as mice deficient in this protein have reduced survival following *Candida albicans* infection (Gross et al., 2006). However, the mechanism by which CARD9 activates downstream signalling events is yet to be determined, but it is hypothesised that other adaptor proteins/mediators are involved (Colonna, 2007). This indicates that the interaction between these proteins is complex and not only dependent on dectin-1. However, it does illustrate the importance of this C-type lectin-like receptor in generating an appropriate immune response.

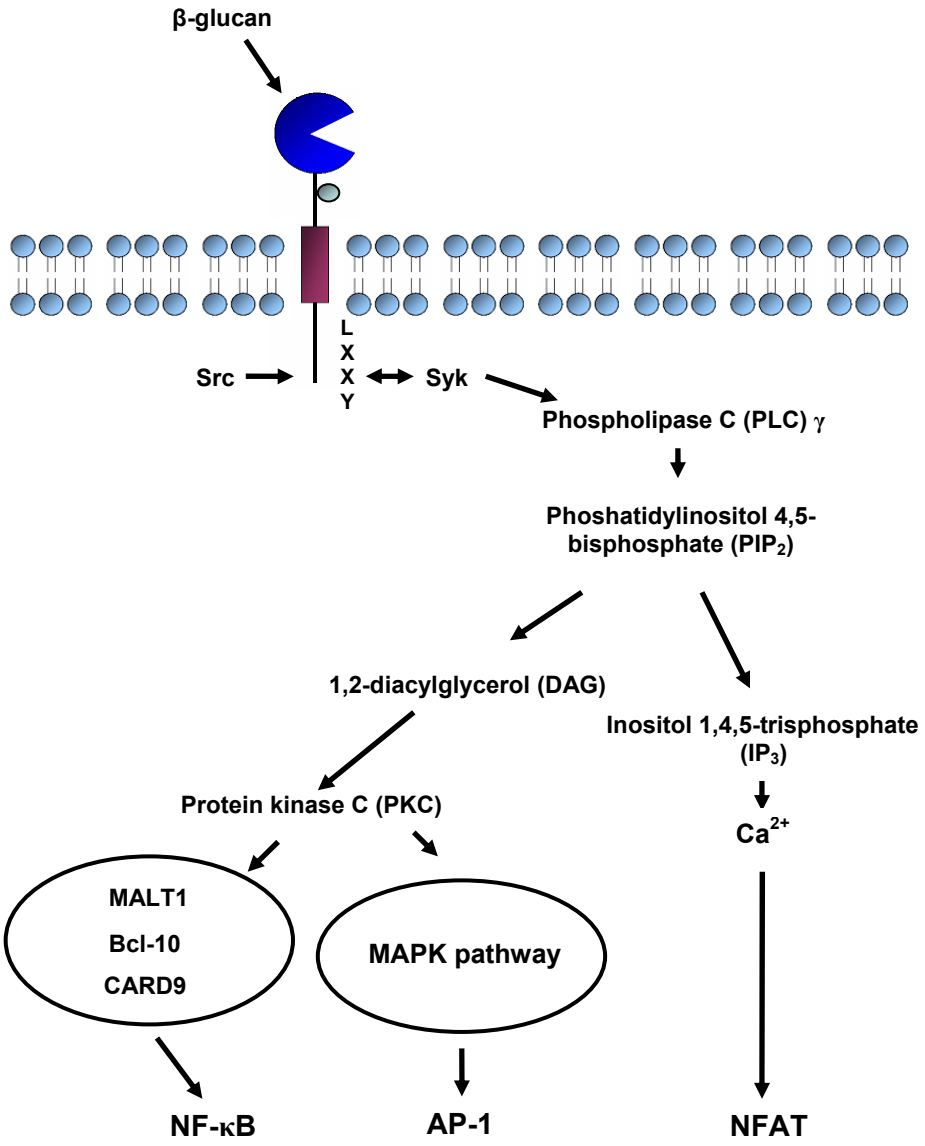


Figure 1.9 Diagrammatic representation of dectin-1 signalling

Dectin-1 signals following stimulation with β-glucan. The tyrosine residue in the cytoplasmic tail of dectin-1 undergoes phosphorylation and subsequently signals via Syk. This ultimately results in NF-κB, AP-1 and NFAT activation.

1.2.3.4 CD37 inhibits dectin-1-mediated IL-6 production

The van Spriel group has shown that CD37 plays a role in dectin-1-mediated signalling, as macrophages from CD37-null mice have increased dectin-1-mediated IL-6 production despite decreased cell surface dectin-1 expression (Meyer-Wentrup et al., 2007). Moreover, the relatively low endogenous dectin-1 expression on RAW mouse macrophages increases by transfection of CD37 into this cell line. In addition, a co-immunoprecipitation between dectin-1 and CD37 was observed in transfected HEK293T cells using the relatively stringent detergent Triton X-100 (Meyer-Wentrup et al., 2007). This suggests a strong, direct tetraspanin-partner protein interaction (Claas et al., 2001) and was the first published report of a tetraspanin to functionally interact with a C-type lectin-like receptor. In addition, an interaction between the tetraspanin CD63 and dectin-1 on dendritic cells was reported using the relatively weak detergent CHAPS (Mantegazza et al., 2004). Nevertheless, this is consistent with the idea that dectin-1 interacts with tetraspanin microdomains via CD37. The Meyer-Wentrup et al. (2007) paper suggests that CD37 is involved in dectin-1 stabilisation at the cell surface and negative regulation of dectin-1-mediated signalling and these were key findings that stimulated the studies in this thesis. In addition, further studies by the van Spriel group show that CD37-deficient mice exposed to *Candida albicans* have increased IL-6 production and immunoglobulin (Ig) A levels, and are therefore better protected from this fungal species compared to wild-type mice (van Spriel et al., 2009).

1.2.4 CLEC-2

1.2.4.1 CLEC-2 expression pattern

The C-type lectin-like receptor CLEC-2 is expressed on a number of human haematopoietic cells, namely monocytes, granulocytes, dendritic cells and platelets

(Colonna et al., 2000; Suzuki-Inoue et al., 2006). A mouse homologue has also been identified (Colonna et al., 2000; Xie et al., 2008). However, CLEC-2 may not be expressed on mouse dendritic cells as serial analysis of gene expression (SAGE) of six different dendritic cell libraries revealed no SAGE tags out of 162,312 total compared to 52 SAGE tags in a mouse megakaryocyte library of 53,046 total tags (Senis et al., 2007). The physiological role of CLEC-2 has yet to be determined and there were no functional reports of CLEC-2 until it was identified on platelets as a receptor for rhodocytin (Suzuki-Inoue et al., 2006).

1.2.4.2 The identification of CLEC-2 as a platelet receptor for rhodocytin

Rhodocytin is purified from the venom of the Malayan pit viper, *Calloselasma rhodostoma,* and induces platelet activation with a distinct lag phase (Suzuki-Inoue et al., 2006). There were previous reports that rhodocytin could bind to the collagen binding integrin $\alpha_2\beta_1$ and the GPIb/IX/V complex (Shin and Morita, 1998). This was questioned by Bergmeier et al., since platelets from mice deficient in the integrin β_1 subunit and treated with *O*-sialoglycoprotein endopeptidase, to remove surface expression of GPIbα, could still respond to rhodocytin (Bergmeier et al., 2001). This suggested that there was another receptor on platelets that was activated following rhodocytin stimulation. CLEC-2 was identified as a candidate by rhodocytin affinity chromatography and confirmed as a rhodocytin receptor using cell line models (Fuller et al., 2007; Mori et al., 2008; Suzuki-Inoue et al., 2006). Interestingly, recent evidence has suggested that CLEC-2 acts as an activation receptor on neutrophils as it induced the production of pro-inflammatory cytokines including TNFα (Kerrigan et al., 2009).

1.2.4.3 CLEC-2 is an essential platelet activating receptor in haemostasis and thrombosis

During the writing of this thesis the Nieswandt group have shown that CLEC-2 is essential in haemostasis and thrombosis (May et al., 2009). CLEC-2-deficient platelets were generated by treatment of mice with a CLEC-2 antibody that leads to a specific loss of CLEC-2 from the surface of circulating platelets for several days, which may be attributable to the shedding or internalisation of the receptor. Such platelets have normal adhesion under flow but aggregate formation is defective both *in vitro* and *in vivo* leading to increased bleeding times and impaired occlusive thrombus formation (May et al., 2009). The authors suggest that the ligand (s) of CLEC-2 may be present in the plasma, on the surface of other platelets or released upon platelet activation (May et al., 2009). Such data highlights the role of CLEC-2 in thrombosis and haemostasis, with the generation of CLEC-2-deficient mice the next logical step in the study of this receptor, and is discussed in more detail in the discussion of this thesis.

1.2.4.4 CLEC-2 structure and signalling

CLEC-2 has a C-type lectin-like domain, a stalk region, transmembrane region and a cytoplasmic tail. In common with dectin-1, the latter contains a YXXL signalling motif that undergoes phosphorylation to generate downstream signalling events. This motif is highly conserved between a number of species (Figure 1.10). The signalling of CLEC-2 is dependent on Syk and PLCγ2 in murine platelets and in cell line reporter assays using DT40 B-cells following stimulation with rhodocytin (Fuller et al., 2007; Mori et al., 2008; Spalton et al., 2009; Suzuki-Inoue et al., 2006). In addition, there is a highly conserved DEDG motif directly upstream of the YXXL tyrosine and it has been shown that the glycine residue is important for CLEC-2

signalling (Fuller et al., 2007). Further studies conducted during the writing of this thesis have demonstrated that CLEC-2 signals as a dimer (Hughes et al., 2010; Watson et al., 2009). Peptide pull down studies, Biacore, quantitative western blotting, tryptophan fluorescence measurements and competition experiments showed that Syk activation by CLEC-2 is mediated by the cross-linking through the tandem SH2 domains with a stoichiometry of 2:1 (Hughes et al., 2010). In addition, cross-linking and electron microscopy demonstrated that CLEC-2 is present as a dimer in resting platelets and converted to larger complexes upon activation (Hughes et al., 2010). These data have shown a model by which Syk activation occurs through interaction with a single YXXL-containing receptor.

The crystal structure of the extracellular domain of CLEC-2 has been determined (Watson et al., 2008). In addition, the crystal structure of rhodoyctin has been determined and suggests that it assembles as a non-disulphide linked tetramer (Hooley et al., 2008; Watson et al., 2008). As a result, the authors hypothesise that rhodocytin binding to CLEC-2 may result in clustering of the receptor upon the platelet surface leading to platelet activation (O'Callaghan, 2009; Watson et al., 2008).

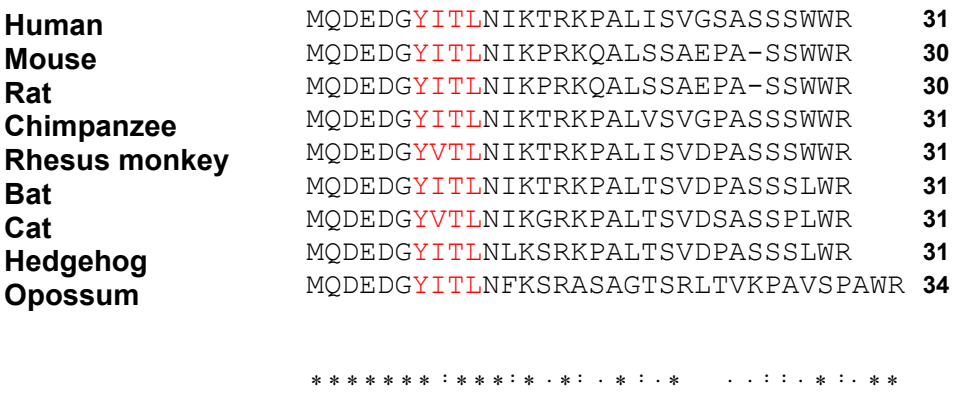


Figure 1.10 Sequence alignment of the cytoplasmic tail of CLEC-2 from different species

CLEC-2 cytoplasmic tail amino-acid sequences from various species were aligned using the Clustalw alignment programme (www.ebi.ac.uk/clustalw). The YXXL signalling motif is highlighted. Sequence alignment symbols denote degree of conservation: "*" = identical residues, ":" = conserved substitution and "." = semi-conserved substitution.

1.2.4.5 Identification of the CLEC-2 ligand, podoplanin

A ligand, podoplanin (also known as aggrus), has been identified for CLEC-2 (Ozaki et al., 2009; Suzuki-Inoue et al., 2007). It is expressed on tumour cells, lymphatic endothelial cells and kidney podocytes (Christou et al., 2008; Wicki and Christofori, 2007). Podoplanin is a transmembrane sialoglycoprotein involved in tumour cell-induced platelet aggregation and tumour metastasis (Wicki and Christofori, 2007). Suzuki-Inoue et al. (2007) show that podoplanin-induced platelet aggregation is dependent on Src family kinases and PLCγ2, and the response was similar to that observed with rhodocytin. In addition, recombinant CLEC-2 inhibits platelet activation using podoplanin-expressing tumour cells or lymphatic endothelial cells suggesting that podoplanin is a ligand for CLEC-2 (Fuller et al., 2007; Suzuki-Inoue et al., 2006). This has been confirmed using HEK293T cells, which express podoplanin endogenously, and which were able to activate platelets (Suzuki-Inoue et al., 2006; Fuller et al., 2007). Moreover, the interaction of recombinant podoplanin with CLEC-2 was confirmed by Biacore (Christou et al., 2008). These findings suggest that tumour cells might bind to platelets via podoplanin and CLEC-2 to facilitate immune evasion during metastasis, and promote subsequent tumour growth, since platelets contain several growth factors. However, this hypothesis has yet to be tested.

During the writing of this thesis, it was reported that the tetraspanin CD9 interacts with podoplanin and inhibits podoplanin-mediated platelet aggregation and suppresses pulmonary metastasis therefore suggesting a link between tetraspanins, CLEC-2 and podoplanin (Nakazawa et al., 2008). This will be further discussed in Chapter Seven.

1.2.4.6 CLEC-2, an attachment factor in HIV-1 capture by platelets

It has been reported that CLEC-2, along with the C-type lectin DC-SIGN, can facilitate the capture of HIV-1 by CLEC-2-transfected cells and platelets (Chaipan et al., 2006). A anti-CLEC-2 antibody, which binds specifically to the CLEC-2 ectodomain, potently reduces the HIV-1 association with platelets suggesting that this C-type lectin-like receptor is required for efficient binding. This may have implications in facilitating HIV-1 dissemination in patients infected with the virus (Chaipan et al., 2006).

1.2.5 CLEC9A

CLEC9A has recently been identified as a novel C-type lectin-like activation receptor (Huysamen et al., 2008). It has restricted expression being detected on small subsets of dendritic cells and monocytes. Unlike dectin-1 and CLEC-2, a ligand for CLEC9A has yet to be identified and Huysamen et al. activate the receptor by generating a dectin-1-CLEC9A chimera and stimulating with the dectin-1 ligand, β-glucan. However, it has recently been shown that CLEC9A is used as a receptor on a subset of dendritic cells for necrotic cells that regulates the cross-presentation of dead-cell-associated antigens (Sancho et al., 2009). Indeed, CLEC9A is similar to dectin-1 and CLEC-2 in its ability to signal via a YXXL motif through interaction with a Syk kinase (Huysamen et al., 2008; Sancho et al., 2009). However, a key difference is that human CLEC9A has been shown to be expressed as a disulphide-linked dimer using western blot analysis under non-reducing conditions (Huysamen et al., 2008). However, the functional significance of this has yet to be fully determined.

1.3 Tyrosine kinase-linked signalling pathways

1.3.1 Overview

As described previously, both dectin-1 and CLEC-2 signal via a single YXXL (Table 1.5) motif that undergoes tyrosine phosphorylation by Src family protein kinases to generate downstream signalling events, such as cytokine production and platelet activation respectively (Rogers et al., 2005; Suzuki-Inoue et al., 2006). This is distinct from the signalling of the major platelet collagen receptor, GPVI and the T- and B-cell antigen receptors, which utilise dually phosphorylated tyrosine residues in the form of an ITAM, which has the consensus sequence YXXL/IX_{6-12}YXXL/I (where 'X' denotes any amino-acid) (Table 1.6).

1.3.2 ITAM signalling

To generate positive signals leading to cellular activation, ITAMs play a key role. For example, the major platelet collagen receptor GPVI couples to the ITAM-containing FcRγ chain to evoke platelet activation (Bori-Sanz et al., 2003; Locke et al., 2003; Suzuki-Inoue et al., 2002; Watson et al., 2001). Similarly, the B-cell receptor couples to the ITAM-containing Igα-Igβ heterodimer, and the T-cell receptor to the ITAM-containing CD3 to induce B- and T-cell activation respectively (Table 1.6) (Abraham and Weiss, 2004; Gupta and DeFranco, 2007). Upon recognition and clustering of the receptor in question, the tyrosine residues of the ITAM become phosphorylated by Src protein tyrosine kinases, for example, Fyn and Lyn in platelets and B-cells, and Lck and Fyn in T-cells. The ITAMs act as docking sites for Syk family protein tyrosine kinases, Syk in platelets and B-cells and Zap-70 in T-cells (Barrow and Trowsdale, 2006). Both Syk and Zap-70 have two Src homology 2 (SH2) domains which are recruited to the dually phosphorylated ITAM upon activation. These tyrosine kinases then phosphorylate a number of adaptor proteins, for example,

BLNK in B-cells and LAT and SLP-76 in platelets and T-cells which then recruit other signalling molecules (Fu et al., 1998; Judd et al., 2002; Zhang et al., 1998). This forms a scaffold which controls the spatial organisation of other effector molecules to initiate a downstream signalling cascade (Smith-Garvin et al., 2009).

1.3.3 The differences between YXXL and ITAM signalling

There are some notable differences between signalling via a single YXXL motif and ITAMs (Figure 1.11). In respect of ITAMs, it has been observed that phosphorylation of both tyrosine residues is required for recruitment and full activation of Syk and Zap-70 tyrosine kinases in B- and T-cells respectively (Iwashima et al., 1994; Rowley et al., 1995). Therefore, a key question is how the two SH2 domains of Syk/Zap-70 bind to the single YXXL signalling motif. As discussed in section 1.2.4.4, CLEC-2 has recently been shown to signal as a dimer resulting in two YXXL motifs undergoing phosphorylation to recruit Syk and generate signalling events (Hughes et al., 2010; Watson et al., 2009).

Another difference between C-type lectin-like receptor single YXXL signalling and ITAMs is the only partial dependence of dectin-1 and CLEC-2 upon the SLP-76 and BLNK family of adaptor proteins (Fuller et al., 2007; Judd et al., 2002). Using nuclear factor of activated T-cell (NFAT)/activator protein (AP)-1-luciferase reporter assays in SLP-76- and BLNK-deficient Jurkat T- and DT40 B-cells respectively, the signalling of both dectin-1 and CLEC-2 is reduced by approximately 60-70% compared to wild-type cells (Fuller et al., 2007). This is distinct from GPVI signalling, where the presence of these adaptor proteins is imperative for signalling using cell line reporter assays (Fuller et al., 2007). Consistent with this, the platelets of SLP-76-deficient mice have an almost completely abolished GPVI-induced

aggregation, but only a partial reduction is observed in aggregation following CLEC-2 activation (Judd et al., 2002; Suzuki-Inoue et al., 2006).

Table 1.5 C-type lectin-like receptors that contain YXXL motifs used in this thesis

Name	Sequence	Signalling
Dectin-1	DEDG**Y**TQ**L**	Activatory
CLEC-2	DEDG**Y**IT**L**	Activatory

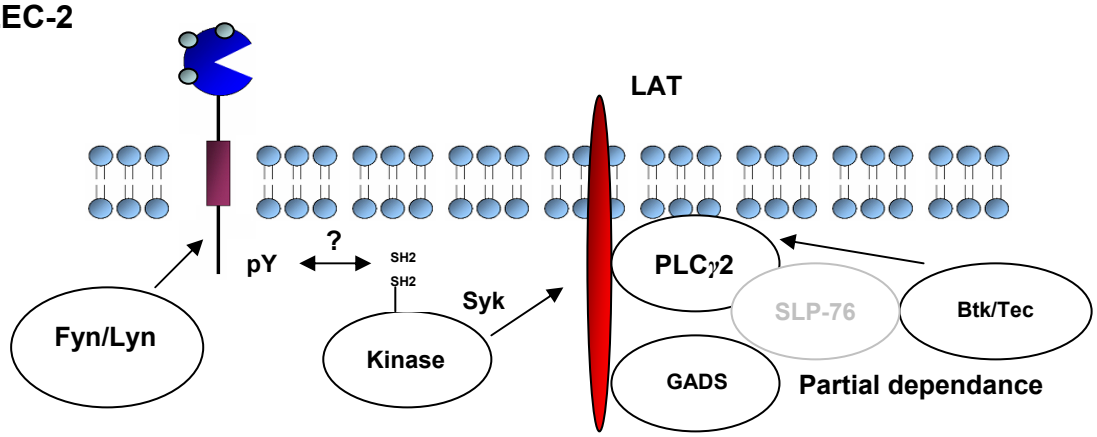
Table 1.6 ITAM receptor sequences

Receptor	Name	Sequence
T-cell	ζ chain 1	QNQL**Y**NE**L**NLGRREE**Y**DV**L**
	ζ chain 2	QEGL**Y**NE**L**QKDKMAEA**Y**SE**I**
	ζ chain 3	HGDL**Y**QG**L**STARKDT**Y**DA**L**
	CD3γ	NDQL**Y**QP**L**KDREDDQ**Y**SH**L**
	CD3δ	NDQV**Y**QP**L**RDRDDAQ**Y**SH**L**
	CD3ε	PNPD**Y**EP**I**RKGQRDL**Y**SG**L**
B-cell	Igα	DENL**Y**EG**L**NLDDCSM**Y**ED**I**
	Igβ	EDHT**Y**EG**L**DIDQTAT**Y**ED**I**
GPVI	FcR γ-chain	SDGV**Y**TG**L**STRNQET**Y**ET**L**

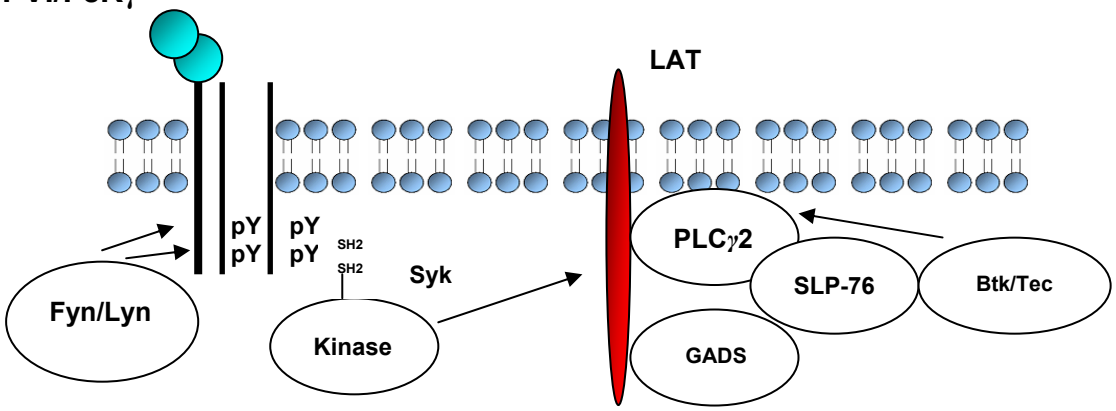
Figure 1.11 A comparison of the signalling of CLEC-2 with GPVI and the T- and B-cell antigen receptors

The key signalling and adaptor proteins involved in CLEC-2 single YXXL signalling are compared with GPVI and the T- and B-cell antigen receptors. One of the main differences is how the two SH2 domains of the Syk/Zap-70 family of tyrosine kinases bind to a single YXXL motif in the cytoplasmic tail of both CLEC-2 and dectin-1.

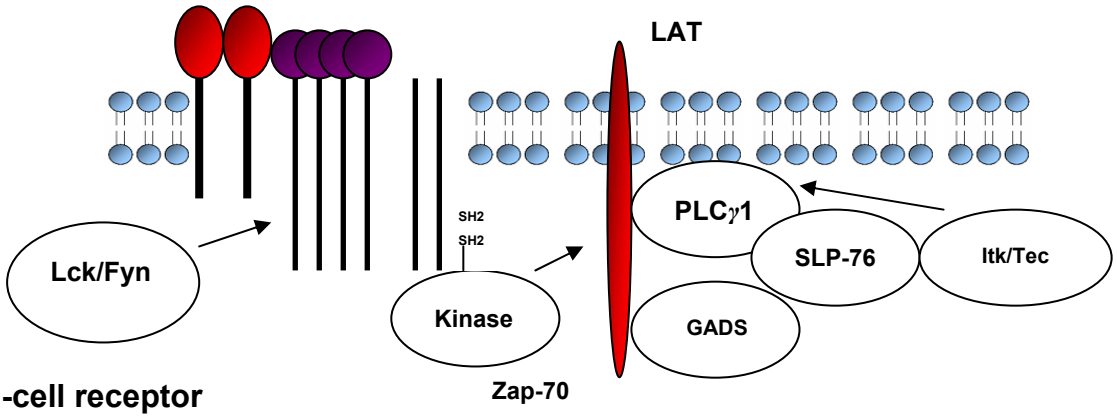
CLEC-2



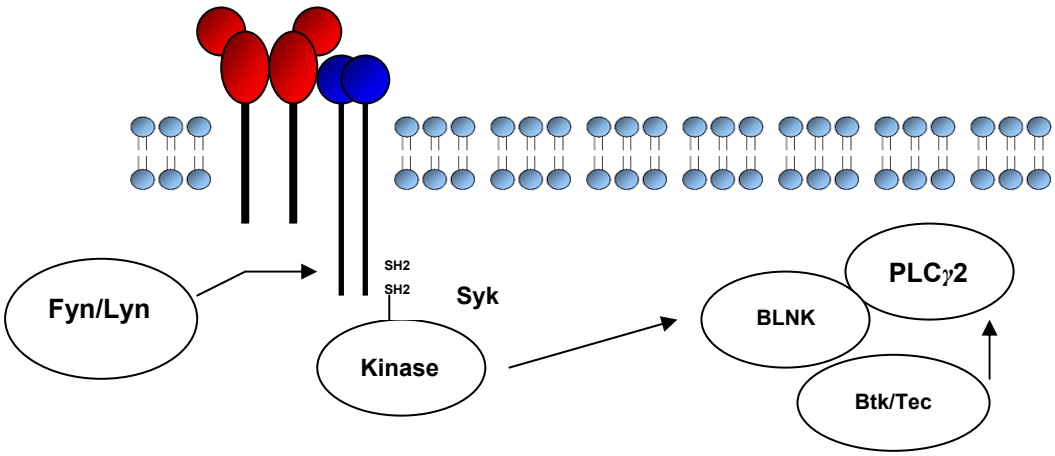
GPVI/FcRγ



T-cell receptor



B-cell receptor



1.4 Aims of this thesis

The work in this thesis was stimulated by a publication of the van Spriel group, which suggested that CD37 negatively regulates dectin-1-mediated signalling (Meyer-Wentrup et al., 2007). The mechanism of inhibition was not clear from this study, in part because of their focus on primary cells which are not readily transfectable and thus amenable to mechanistic studies. The aims are as follows:

- To determine whether CD37 and other tetraspanins negatively regulate dectin-1 signalling in model cell lines.
- To investigate the mechanism of dectin-1 regulation by tetraspanins.
- To determine whether tetraspanins also negatively regulate CLEC-2.
- To investigate CLEC-2-induced platelet aggregation in mice deficient in the major platelet tetraspanin CD9.

CHAPTER 2 –

MATERIALS AND METHODS

2.1 Agonists and antibodies

The agonists used are described in Table 2.1. The antibodies used are described in Table 2.2. All other materials were from Sigma (Poole, UK) unless otherwise stated.

Table 2.1 - Agonists

Agonist	Target receptor	Source
ADP	$P2Y_1$, $P2Y_{12}$	Sigma (Saint Quentin-Fallavier, France)
Collagen (HORM)	GPVI $\alpha_2\beta_1$	Nycomed (Munich, Germany)
Curdlan	dectin-1	Sigma (Poole, UK)
Ionomycin	Ca^{2+} pathway	Sigma (Poole, UK)
PMA	PKC/ RasGRP	Sigma (Poole, UK)
Rhodocytin	CLEC-2	Dr J.A. Eble (University of Munster, Germany)
Zymosan	dectin-1	Sigma (Poole, UK)

Table 2.2 - Antibodies

Antibody reactivity	Species of origin	Experimental uses*	Source
Primary antibodies			
FLAG	Rabbit	WB: 0.5 µg/ml	Sigma (Poole, UK)
FLAG-M2 agarose	Mouse	IP: 10 µl	Sigma (Poole, UK)
MOPC (IgG)	Mouse	FC: 10 µg/ml IP: 1 µg	Sigma (Poole, UK)
MYC (9B11)	Mouse	FC: 10 µg/ml WB: 1 µg/ml	Cell Signaling Technology (MA, USA)
Normal goat IgG	Goat	IP: 1 µg	R & D Systems (MN, USA)
4G10 anti-phosphotyrosine	Mouse	WB: 1 µg/ml	Upstate (Bucks, UK)
Mouse CLEC-2	Rabbit	FC: 10 µg/ml	Dr. K. Suzuki-Inoue
Human CLEC-2	Goat	WB: 1 µg/ml	R & D Systems (MN, USA)
Human CD9 (1AA2)	Mouse	FC: 10 µg/ml IP: 1 µg	Prof. L. Ashman
Mouse CD9	Rat	FC: 10 µg/ml WB: 1 µg/ml	Prof. B. Nieswandt
Human CD53 (MEM53)	Mouse	FC: 10 µg/ml	Dr. V. Horejsi
Human CD63 (6H1)	Mouse	FC: 10 µg/ml	Dr. F. Berditchevski
Human CD81 (M38)	Mouse	FC: 10 µg/ml	Dr. F. Berditchevski
Human CD82 (M104)	Mouse	FC: 10 µg/ml	Dr. O. Yoshie
Human CD151 (11B1)	Mouse	FC: 10 µg/ml	Prof. L. Ashman
Human Tspan4	Mouse	FC: 10 µg/ml	Dr. F. Berditchevski
Human GPVI (204-11)	Mouse	IP: 1 µg	Dr. M. Moroi
Human ADAM10 (11G2)	Mouse	IP: 1 µg	Dr. E. Rubinstein
Conjugated antibodies			
Human CD37 FITC-conjugate	Mouse	FC: 10 µg/ml	AbD Serotec (Oxford, UK)
Mouse IgG FITC-conjugate	Goat	FC: 1/100	DakoCytomation (Cambridgeshire, UK)
Mouse IgG HRP-conjugate	Sheep	WB: 1/10, 000	Amersham (Buckinghamshire, UK)
Rabbit IgG HRP-conjugate	Donkey	WB: 1/10, 000	Amersham (Buckinghamshire, UK)

*** IP: Immunoprecipitation, WB: Western Blot, FC: Flow Cytometry**

2.2 Molecular Biology

2.2.1 Cloning PCR products into vectors

The constructs used are in Table 2.3, all of which were gifts, the only exception being mouse integrin $\alpha_{IIb}\beta_3$. An overview of the general protocol to clone a polymerase chain reaction (PCR) product into the pEF6 vector with a MYC epiptope (Invitrogen, Paiseley, UK) is detailed below. Primers were designed to amplify the appropriate product from complementary DNA (cDNA) using the proofreading polymerase HighFidelity (Promega, Wisconsin, USA) or PfuTurbo (Stratagene, California, USA). An example of a thermocycling programme used for PCR is provided in Tables 2.5 and 2.6. PCR product amplification was confirmed by separating DNA using standard gel electrophoresis 1% agarose gels containing 10 ng/ml ethidium bromide in TBE (89 mM Tris, 89 mM boric acid, 20 mM EDTA, pH 8) buffer and imaged under ultra-violet (UV) light. The PCR product was purified using a QIAquick purification kit (QIAGEN, West Sussex, UK) and digested with the appropriate restriction enzymes (NEB, MA, USA) for 3 hours at 37 °C. Digested product was ligated into cut pEF6 vector (Invitrogen, Paiseley, UK) in frame with a C-terminal MYC tag using the Rapid DNA ligation kit (Roche, MA, USA). Competent DH5α *Escherichia coli* were transformed with pEF6-insert construct using the heat shock method. DNA was mixed with bacteria, incubated on ice for 30 minutes, 20 seconds at 37 °C, and 5 minutes at room temperature, before the addition of 250 µl SOC medium (Sigma, Poole, UK). This was then incubated for 30 minutes at 37 °C and plated on agar plates (22% NaCl, 22% Tryptone, 11% yeast extract, 44% agar, deionized water to 1 litre, pH 7) containing 100 µg/ml ampicillin. Individual colonies were screened by colony PCR using one insert primer and one vector primer and positive colonies were confirmed by gel electrophoresis using 1% agarose gels. A positive colony was inoculated in 250

ml Luria-Bertani (LB) broth (22% NaCl, 22% Tryptone, 11% yeast extract, deionized water to 1 litre, pH 7), grown for 20 hours at 37 °C and plasmid DNA purified according to the Plasmid Maxiprep Kit (QIAGEN, West Sussex, UK). Confirmation of the correct sequence was performed by DNA sequencing using the ABI Prism 3,700 DNA analyser (Functional Genomics Laboratory, The School of Biosciences, The University of Birmingham, UK).

Table 2.3 - Expression constructs

Insert	Vector	Epitope tag	Origin
Mouse dectin-1	pEF6	MYC	J.A. Williams (University of Birmingham)
Human CLEC-2	pEF6	MYC	Dr. K. Suzuki-Inoue (University of Yamanashi, Japan)
Human GPVI	pCDNA3	MYC	Prof. S.P. Watson
Human FcRγ	pEF6	-	Dr. M.G. Tomlinson
Mouse α_{IIb}	pEF6	MYC	N.D. Tomlinson
Mouse β_3	pEF6	MYC	N.D. Tomlinson
Human L6 antigen	pCDNA3	-	Dr. F. Berditchevski (University of Birmingham)
Mouse CD9	pEF6	FLAG	Dr. M.G. Tomlinson
Mouse CD37	pEF6	FLAG	Dr. M.G. Tomlinson
Mouse CD63	pEF6	FLAG	Dr. M.G. Tomlinson
Mouse CD81	pEF6	FLAG	Dr. M.G. Tomlinson
Human CD81	pEF6	-	Prof. J.A. McKeating (University of Birmingham)
Human CD81 P.M.	pEF6	-	Prof. J.A. McKeating
Mouse CD82	pEF6	FLAG	Dr. M.G. Tomlinson
Mouse CD151	pEF6	FLAG	Dr. M.G. Tomlinson
Human CD151	pCDNA3	FLAG	Dr. F. Berditchevski
Human CD151	pCDNA3	-	Dr. F. Berditchevski
Mouse Tspan3	pEF6	FLAG	Dr. M.G. Tomlinson
Mouse Tspan4	pEF6	FLAG	Dr. M.G. Tomlinson
Mouse Tspan5	pEF6	FLAG	Dr. M.G. Tomlinson
Mouse Tspan6	pEF6	FLAG	Dr. M.G. Tomlinson
Mouse Tspan9	pEF6	FLAG	Dr. M.G. Tomlinson
Mouse Tspan14	pEF6	FLAG	Dr. M.G. Tomlinson
Mouse Tspan15	pEF6	FLAG	Dr. M.G. Tomlinson
Mouse Tspan17	pEF6	FLAG	Dr. M.G. Tomlinson
Mouse Tspan18	pEF6	FLAG	Dr. M.G. Tomlinson
Mouse Tspan31	pEF6	FLAG	Dr. M.G. Tomlinson
Mouse Tspan32	pEF6	FLAG	Dr. M.G. Tomlinson
Mouse Tspan33	pEF6	FLAG	Dr. M.G. Tomlinson

2.3 Cell culture and functional studies

2.3.1 Cell culture

Jurkat human T-cells (clones E6.1 & DNAX) and those stably over-expressing human CD9 (clones AE9 & AE12) were cultured in RPMI supplemented with 10% foetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 20 mM GlutaMAX. DT40 chicken B-cells were cultured in RPMI supplemented with 10% foetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 20 mM GlutaMAX, 1% chicken serum and 50 μM 2β-mercaptoethanol. RBL-2H3 rat basophilic leukaemia cells and HEK293T cells were cultured in DMEM supplemented with 10% foetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 20 mM GlutaMAX.

2.3.2 Cell transfection

Jurkat were transfected by using 2 x 10^7 cells from a log phase culture per transfectant. Cells were washed in 50 ml non-supplemented RMPI, re-suspended in a volume of 400 μl non-supplemented RPMI, the DNA constructs were added and the cells electroporated using a GenePulser II (Biorad, Hertfordshire, UK) set at 250 V and 950 μF. Jurkat were then left at room temperature for 10 minutes before adding to 8 ml complete RPMI. Cells were assayed approximately 20 hours post-transfection.

DT40 were transfected by using 1 x 10^7 log phase cells per transfectant. Cells were washed in 50 ml cytomix (120 mM KCl, 0.5 mM $CaCl_2$, 10 mM K_2HPO_4, 10 mM KH_2PO_4, 25 mM HEPES, 2 mM EGTA, 5 mM $MgCl_2$, 0.04 mM ATP, 5 mM glutathione, pH 7.6) before re-suspension in a volume of 400 μl cytomix. DT40 were left at room temperature for 10 minutes, electroporated at 350 V and 500 μF and

incubated 10 minutes on ice, before adding to 8 ml complete RPMI. Cells were assayed 20 hours post-transfection.

RBL-2H3 cells were transfected by using 1 x 10^7 log phase cells per transfectant. Cells were washed in 50 ml non-supplemented RPMI before re-suspension in 400 µl non-supplemented RPMI, and electroporated at 276 V and 950 µF. RBL-2H3 cells were then left at room temperature for 10 minutes before adding to 8 ml complete RPMI. Cells were assayed 20 hours post-transfection.

HEK293T cells were counted and 3 x 10^6 cells plated per 10 cm plate. The following day, DNA was mixed with 450 µl dH_2O and 63 µl 2M calcium chloride, then 500 µl HBS (HEPES-buffered saline) (50 mM HEPES, 12 mM dextrose, 1.5 mM $Na_2HPO_4.2H_20$, 10 mM KCl, 280 mM NaCl) was added drop-wise to the solution, mixing the tube contents between drops. This was left for 5-15 minutes to allow for a calcium-phosphate precipitate to form to which air was then bubbled through the solution for 10 seconds, to break up large clumps, before adding to cells. Media was replaced 24 hours later before harvest the following day.

2.3.3 Luciferase assay

The NFAT/AP-1-luciferase reporter assay used in this thesis is a highly sensitive readout for calcium and mitogen-activated protein (MAPK) signalling and has previously been used to study a number of receptors, including the C-type lectin-like proteins, dectin-1 and CLEC-2, the major platelet collagen receptor GPVI, and the T- and B-cell antigen receptors (Abraham and Weiss, 2004; Fuller et al., 2007; Gupta and DeFranco, 2007; Tomlinson et al., 2007). The NFAT/AP-1 reporter contains three copies of a composite NFAT/AP-1 element from the human IL-2 promoter (Shapiro et al., 1996) and is maximally activated by combined calcium elevation and MAPK

signalling, which activate NFAT and AP-1 respectively (Figure 2.1). NFAT refers to a group of transcription factors that are crucial in the development and function of the immune system (Macian, 2005; Macian et al., 2001). Indeed, optimal NFAT activation requires sustained calcium signalling to maintain NFAT in the nucleus (Dolmetsch et al., 1997; Macian, 2005). AP-1 is the main transcriptional partner of NFAT that promotes gene transcription following T-cell activation (Macian, 2005; Macian et al., 2001; Rao et al., 1997).

The NFAT/AP-1-luciferase transcriptional reporter assay is a sensitive readout for activation of this calcium/MAPK signalling pathway (Figure 2.1). Cells are stimulated and incubated for six hours to allow for transcription and translation of luciferase. Luciferase activity can then be measured as light emission in a luminometer following the addition to cell lysates of luciferin, the substrate of luciferase, plus ATP and Mg^{2+}. In all of these reporter assays, the phorbol ester, phorbol 12-myristate 13-acetate (PMA), and the calcium ionophore, ionomycin, drive luciferase expression downstream of receptor-induced proximal signalling and serve as a positive control. PMA acts as an activator of PKC by mimicking DAG, and ionomycin stimulates the influx of calcium ions across the cell membrane by acting as a selective mobile ion carrier. Finally, it is important to note that the NFAT/AP-1 transcriptional reporter assay is a highly sensitive readout for sustained signalling over several hours, and does not necessarily reflect membrane-proximal signalling events immediately after receptor activation (Tomlinson et al., 2007). This is because a sustained signal is required to prevent nuclear export of NFAT proteins (Macian, 2005). Indeed, a sustained elevation of intracellular calcium, that is too weak to be detected by fluorimetry, can nevertheless induce robust NFAT activation (Dolmetsch et al., 1997).

The NFAT/AP-1 luciferase assays were performed as follows. Cells were transfected with the desired expression constructs, in addition to 20 μg of the luciferase reporter construct (pΔODLO-NFAT/AP-1) (Shapiro et al., 1996) and 2 μg of pEF6-lacZ (Invitrogen, Paiseley, UK) to control for transfection efficiency. Twenty hours after transfection, live cells were counted by Trypan blue exclusion, and samples were divided for luciferase assay, β-galactosidase assay and flow cytometry. Luciferase assays were performed in triplicate with 10^5 live cells in a total volume of 100 μl. Samples were stimulated for 6 hours with the agonist of choice, or with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) plus ionomycin (1 μM) as a positive control. The assay was harvested by adding 11 μl of harvest buffer (200 mM potassium phosphate buffer, pH 7.8, 12.5% Triton X-100 and 4 mM DTT) for 5 minutes. 100 μl of this lysate was added to 100 μl of assay buffer (200 mM potassium phosphate buffer, pH 7.8, 20 mM $MgCl_2$ and 10 mM ATP). The luciferase activity was measured using a Centro LB 960 microplate luminometer (Berthold Technologies, Germany) which injects 50 μl of 1 mM luciferin (MP Biomedicals, UK) and measures luminescence over a 10 second period. Data was expressed as luminescence units normalised to β-galactosidase activity in individual experiments. Data from two to three independent experiments was combined and shown as mean ± standard error.

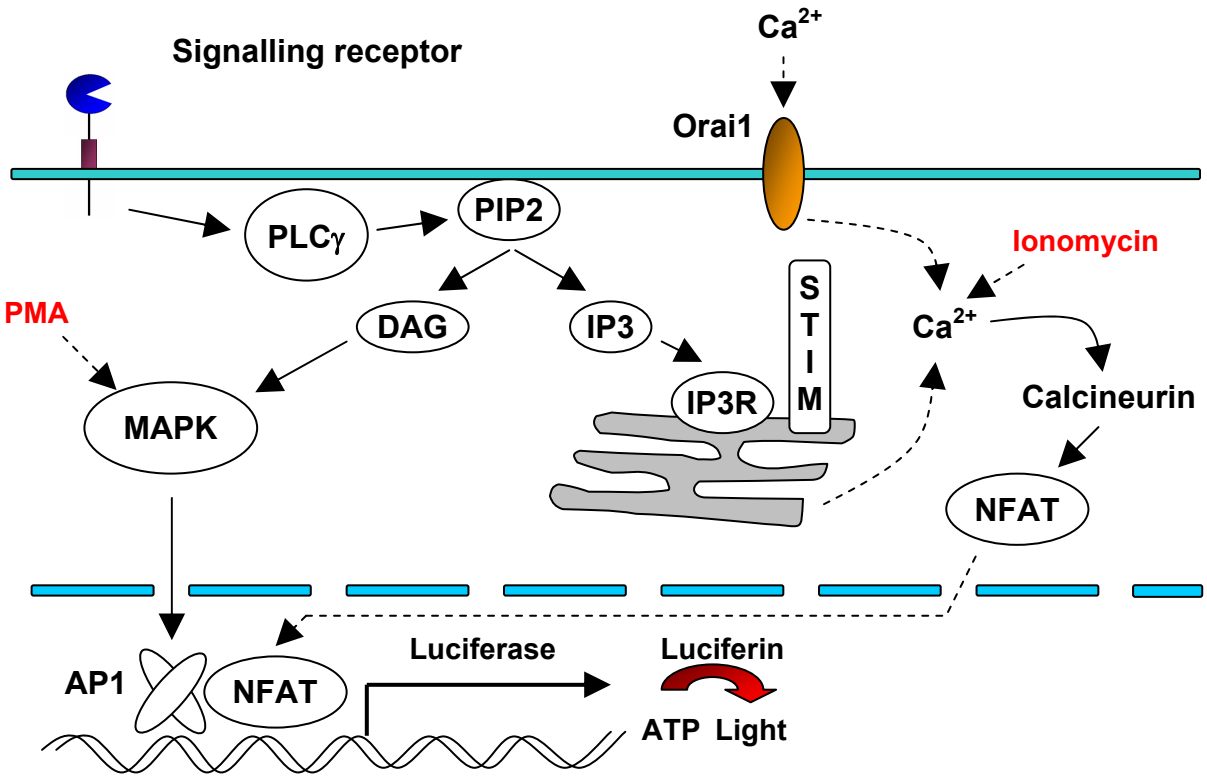


Figure 2.1 An overview of the signalling pathway of the NFAT/AP-1-luciferase reporter assay used in the Jurkat T- and DT40 B-cell lines

Following receptor activation, in this case dectin-1, the tyrosine residue in the single YXXL undergoes phosphorylation by Src family kinases (Fyn/Lyn in DT40 B-cells or Lck/Fyn in Jurkat T-cells). This leads to the recruitment of protein tyrosine kinases (Syk in DT40 B-cells or Zap-70 in Jurkat T-cells). In B-cells, Syk targets BLNK, which becomes phosphorylated. In T-cells, Zap-70 targets LAT and SLP-76, which become phosphorylated. Activation of PLCγ induces the hydrolysis of PIP_2, which generates IP_3 and DAG. IP_3 binds to its receptor, the IP_3-gated calcium channel (IP_3R), which releases calcium from ER stores. The ER transmembrane protein STIM1 senses empyting of ER stores and responds by interacting with the plasma membrane CRAC channel Orai1, which opens and leads to sustained elevation of intracellular calcium. Calcium activates calcineurin, which in turn dephosphorylates NFAT. NFAT translocates to the nucleus and interacts with AP-1, which is induced by MAPK activation. This promotes gene transcription and luciferase generation, which can be detected as light emission following addition of luciferin, ATP and Mg^{2+} to cell lysates. Stimulation with the phorbol ester, PMA, and the calcium ionophore, ionomycin, serve as a positive control in the experiments, as they drive luciferase expression downstream of dectin-1-proximal signalling. Adapted from Macian (2005).

2.3.4 β-galactosidase assay

β-galactosidase assays were performed in triplicate using half a million cells using the Light Chemiluminescent Reporter Assay Kit (Applied Biosystems, MA, USA). Cells were lysed for 5 minutes in 80 µl lysis solution with the addition of 0.5 mM DTT, 0.01 mg/ml leupeptin and 0.2 mg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF). This was then centrifuged for 2 minutes at 18,000 x *g*. Galacton, a β-galactosidase substrate, was diluted (1:100) in Galacton reaction buffer (0.5 M sodium phosphate (pH 7.1), 50 mM KCl and 5 mM $MgSO_4$) before the addition of 70 µl per well of a 96-well plate. 20 µl of cell lysate was added to each well containing the diluted galacton. Samples were left in the dark for 30 minutes before β-galactosidase activity was measured using a microplate luminometer, which injects 100 µl light emission accelerator and measures luminescence over a 1 second period. All luciferase assay data was normalised to β-galactosidase values.

2.3.5 RNAi

RNAi was performed in Jurkat using negative medium GC content control or CD81 Stealth Select siRNA oligo (sequence: GCG CUG UCA UGA UGU UCG UUG GCU U) (Invitrogen, Paisely, UK) used at a final concentration of 20 nM. This dose has been shown previously to be 100% effective in CD81 knockdown on human umbilical vein endothelial cells (HUVECs) (MGT, unpublished data). Jurkat were transfected as in section 2.3.2 but were incubated for 48 hours for maximal knockdown of CD81 compared to 24 hours in all other experiments. Luciferase assays and β-galactosidase were performed as in 2.3.3 and 2.3.4 respectively and cell surface expression of CD81 and dectin-1 was confirmed by flow cytometry as in section 2.4.

2.4 Flow cytometry studies of cell lines

Jurkat, DT40 and RBL-2H3 cells (5 x 10^5 per sample) were incubated with 50 µl FACS buffer (PBS, 0.2% BSA, 0.02% sodium azide) containing 10 µg/ml primary antibody for 30 minutes on ice. Cells were washed with 1 ml FACS buffer and then incubated with 50 µl FACS buffer containing 10 µg/ml FITC-conjugated secondary antibody for 30 minutes on ice. A volume of 250 µl of FACS buffer containing 2 µg/ml propidium iodide was added before analysis on a FACScalibur flow cytometer using Cell Quest software (Becton Dickinson, CA, USA). Dead cells, positive for propidium iodide, were gated out.

2.5 Co-immunoprecipation studies

Jurkat were isolated by centrifuging cells for 5 minutes at 200 x *g* and washed in 1 ml PBS. HEK293T cells were isolated by aspirating media, scraping the cells in 1 ml PBS and centrifuging for 5 minutes at 200 x *g*. Cell pellets were lysed on ice for 30 minutes in ice-cold lysis buffer. For Jurkat cells, 1% Brij97 (1% Brij97, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Tris, pH 7.5, 150 mM NaCl, 0.02% sodium azide) was used; for HEK293T cells either 1% Brij97 or 1% Triton X-100 (1% Triton X-100, 10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.02% sodium azide) were used. Each lysis buffer contained the protease and phosphatase inhibitors 200 µg/ml AEBSF, 10 µg/ml aprotonin, 10 µg/ml leupeptin, 1 µg/ml pepstatin A, 10 µM sodium fluoride and 2 mM sodium orthovanadate. Following lysis, cells were centrifuged for 15 minutes at 18,000 x *g* at 4 °C and a 50 µl sample was removed for whole cell lysates and mixed with an equal volume of 2 x reducing sample buffer (0.5 M Tris, pH 6.8, 5% β-mercaptoethanol, 10% sodium dodecyl sulphate (SDS), 20% glycerol, 0.05% Bromophenol blue). The remainder of the lysate was immunoprecipitated at 4 °C for

90 minutes on a rotating wheel with FLAG-M2 agarose (20 µl per immunoprecipitation) and samples were washed four times with ice-cold lysis buffer. Samples were re-suspended in 50 µl reducing sample buffer, of which 25 µl was loaded for SDS-polyacrylamide gel electrophoresis (PAGE).

2.6 Platelet studies

2.6.1 Mouse platelet studies

2.6.1.1 Animals

Genetically modified mice deficient in the tetraspanin CD9 were provided by François Lanza and Christian Gaçhet and had been back-crossed at least ten times on a C57BL/6 background. Colonies were established at the animal facilities at the Etablissement Français du Sang-Alsace (Strasbourg, France). Wild-type control mice were from Charles River (MA, USA).

2.6.1.2 Platelet preparation

Washed mouse platelets were prepared by drawing blood from the abdominal aorta into acid-citrate dextrose (ACD) (120 mM sodium citrate, 110 mM glucose, 80 mM citric acid) (1 volume ACD/6 volumes blood) and pooled (3ml) from more than one mouse. Blood was rested for 10 minutes at 37 °C before centrifugation at 200 x *g*, whereby the platelet-rich plasma (PRP) was removed and platelets re-suspended in 5 ml Tyrodes (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$, pH 7.3)-albumin buffer containing apyrase (0.02 U/ml) and prostacylcin (PGI_2) (0.5 µl/ml). A further incubation at 37 °C for 10 minutes followed before centrifugation at 1,900 x *g*. Platelets were re-suspended in 5 ml Tyrodes-albumin buffer containing PGI_2 (0.5 µl/ml) and centrifuged at 1000 x *g*. The final platelet suspension was adjusted to 2 x 10^5

platelets/µl in Tyrodes-albumin buffer containing apyrase (0.02 U/ml) and were kept at 37 °C for 30 minutes before use, to allow for the effects of prostacyclin to wear off.

2.6.1.3 Platelet aggregation

Platelets were kept at 37 °C during all experiments and aggregation was measured by turbidometry using a Payton aggregometer (Payton Associates, Ontario, Canada). Platelets were used at a concentration of 2 x 10^5/µl per aggregation and human fibrinogen (0.32 mg/ml) (Inter-Transfusion, Lingolsheim, France) was added, stirred at 1,500 x *g* for 30 seconds, before the agonist of choice was added to the suspension to a final volume of 500 µl and allowed to continue for 3 minutes in most cases.

2.6.1.4 Platelet phosphorylation studies

Aggregations were terminated by the addition of an equal volume of 2% NP-40 lysis buffer (2% NP-40, 100 mM Tris, pH 7.5, 500 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 2 mM sodium orthovanadate, 2 mM sodium fluoride) containing complete protease inhibitor cocktail (Roche, Germany). Lysates were used to determine tyrosine phosphorylation profiles and to confirm that the mice used were either CD9-deficient or wild-type controls.

2.6.1.5 Platelet flow cytometry

Flow cytometry on platelets was performed essentially as described in section 2.4 at a concentration of 2 x 10^8/ml in a volume of 50 µl in the presence of 0.1 mM EGTA to prevent aggregation.

2.6.2 Human platelet studies

2.6.2.1 Platelet preparation

Washed human platelets were prepared by drawing blood into sodium citrate (Sigma, Poole, UK) (1 volume sodium citrate/9 volumes of blood) from healthy drug-free volunteers. ACD (120 mM sodium citrate, 110 mM glucose, 80 mM citric acid) (1 volume ACD/9 volumes blood) was then added. Blood was centrifuged at 200 x *g* for 20 minutes and PRP collected. PGI_2 (0.4 μg/ml) was added and platelets centrifuged at 1,000 x *g* for 10 minutes. Platelets were washed in modified Tyrodes buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCL, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$, pH 7.3) in the presence of 0.4 μg/ml PGI_2 and ACD (1:8), then centrifuged at 1,000 x *g* for 10 minutes. Platelets were resuspended in modified Tyrodes buffer and counted using a particle counter (Beckman Coulter, CA, USA). Platelets were allowed to rest for 30 minutes prior to use to allow the effects of the prostacyclin to wear off.

2.6.2.2 Co-immunoprecipitation studies in rhodocytin-stimulated platelets

Washed human platelets were re-suspended at a concentration of 1 x 10^9/ml in modified Tyrodes buffer and stimulated in an aggregometer for 5 minutes at 37 °C with 300 nM rhodocytin or left unstimulated. Platelets were lysed/pre-cleared in ice-cold 1% Brij97 in the presence of inhibitors and 20 μl protein G sepharose (PGS) (Sigma, Poole, UK) (50% slurry PBS) and rotated for 60 minutes at 4 °C. At this point, 1 μg of isotype negative control, CLEC-2, CD9 or ADAM-10 antibodies were coated to 20 μl PGS in 1% Triton X-100 and rotated for 60 minutes at room temperature before washing three times with 1% Brij97. Following lysis/pre-clear, platelets were centrifuged for 10 minutes at 13,000 x *g* at 4 °C and a 25 μl sample was removed for whole cell lysates and mixed with an equal volume of non-reducing

sample buffer. The remainder of the lysate was immunoprecipitated at 4 °C for 90 minutes with either isotype negative control, CLEC-2, CD9 or ADAM-10 antibodies. Samples were washed four times with ice-cold lysis buffer and were re-suspended in 50 µl sample buffer, of which, 20 µl was loaded for SDS-PAGE.

2.7 Semi-quantitative PCR of mouse megakaryocytes

cDNA was provided by M.G. Tomlinson, and was generated using the first strand cDNA synthesis kit (Roche, Germany), from RNA prepared using Trizol (Invitrogen, Paiseley, UK) from mouse megakaryocytes kindly provided by P. Mangin. A semi-quantitative PCR was performed using cDNA from wild-type and CD9-deficient megakaryoctyes at three dilutions: undiluted, 1:10 and 1:100. A 2 x ReadyMix Taq PCR Reaction Mix (Sigma, Poole, UK), dH_20 and primers for tetraspanins and the GAPDH positive control (final primer concentration 20 pmol/µl) were mixed (Table 2.4). The generic thermocycling programme is provided in Table 2.5. Variations in the thermocycling programmes in regard to the annealing temperature and number of cycles is provided in Table 2.6. Following PCR, 10 µl samples were run on a 1% agarose gel to determine product amplification.

Table 2.4 Primers used for semi-quantitative PCR

Tetraspanin	Forward primer	Reverse primer
CD9	TAGTAGAGATCTCCGGTCAAAGGAGGTAGCAAG	TAGTAGGCGGCCGCCTAGACCATTTCTCGGCTCC
CD63	TAGTAGAGATCTGCGGTGGAAGGAGGAATGAAG	TAGTAGGCGGCCGCCTACATTACTTCATAGCCAC
CD81	TAGTAGGGATCCGGGGTGGAGGGCTGCACCAAATG	TAGTAGGCGGCCGCTCAGTACACGGAGCTGTTCCGG
CD151	TAGTAGAGATCTGGTGAATTCAATGAGAAGAAGG	TAGTAGGCGGCCGCTCAGTAGTGTTCCAGCTTGAG
Tspan2	TAGTAGAGATCTGGGCGTTTTCGCGGGGGCCTG	TAGTAGGCGGCCGCTCAGATCACATCGCGTGAGTTC
Tspan9	TAGTAGAGATCTGCCAGGGGCTGTCTCTGCTG	TAGTAGGCGGCCGCTCAGGCGTCGTACTTTTTACCTG
Tspan32	TAGTAGAGATCTGGGCACTGGAATCGAATCAAG	TAGTAGGCGGCCGCCTAGGTCCATCTGAAGAGGC
GAPDH control	GACCCCTTCATTGACCTCAA	GCATGGACTGTGGTCATGAGT

Table 2.5 The generic thermocycling programme used for semi-quantitative PCR

Step	Temperature °C	Time
Denaturation	94	2 minutes
Denaturation	94	30 seconds (30-35 cycles)
Annealing	See Table 2.6	30 seconds (30-35 cycles)
Elongation	72	2 minutes (30-35 cycles)

Table 2.6 The annealing temperatures and number of cycles used for semi-quantitative PCR

Tetraspanin	Annealing temperature °C	Number of cycles
CD9	55	30
GAPDH control	55	30
CD63	55	35
CD151	55	35
Tspan32	55	35
CD81	60	35
Tspan2	60	35
Tspan9	60	35

2.8 SDS-PAGE and western blotting

Proteins were resolved by running on reducing or non-reducing gradient 4-12% or 12% non-gradient SDS-PAGE, along with pre-stained molecular weight markers (NEB, Hertfordshire, UK). Gels were transferred to polyvinylidene difluoride (PVDF) membranes by the semi-dry method (25 mM Tris, 250 mM glycine, 15% methanol) at 110 mA for 35 minutes and subsequently blocked overnight at 4 °C in TBS-T (137 mM NaCl, 20 mM Tris, 10% Tween-20, pH 7.6) 5% milk. Membranes were immunoblotted with primary antibodies in blocking buffer (TBS-T containing 3% BSA and 0.1% sodium azide) for 1.5 hours at room temperature. Membranes were washed 3 times in TBS-T high salt (500 mM NaCl) for 10 minutes and subsequently incubated for 1.5 hours with HRP-conjugated antibodies in TBST-T milk or TBST. Membranes were washed 5 more times for 5 minutes each. Western blots were developed using enhanced chemiluminescence (ECL, Amersham Bioscience, Buckinghamshire, UK) and Hyperfilm (Amersham) and were developed in a Compact X4 Film Processor (Xograph Imaging Systems).

2.9 Analysis of data

Data is shown either as combined from 2-3 individual experiments for luciferase assay or as a representative of 1-3 individual experiments for western blots, flow cytometry histograms/dot plots and platelet assays. For luciferase assay, a β-galactosidase assay was performed, to which all data were normalised. β-galactosidase corrected luciferase data were then analysed, where one value was set to 100% and other values were calculated relative to this, as indicated in individual figure legends. When it was needed to determine whether there was a significant difference between data sets, an analysis of variance (ANOVA) was performed.

Where ANOVA showed a difference between data sets for luciferase assay, an unpaired Student's *t*-test was performed, with $P<0.05$ taken as significant.

CHAPTER 3 –

TETRASPANIN OVER-EXPRESSION INHIBITS DECTIN-1 SIGNALLING

3.1 INTRODUCTION

3.1.1 Dectin-1

Dectin-1 plays a key role in the immune response against fungal pathogens by inducing cytokine and chemokine production, respiratory burst, phagocytosis and possibly endocytosis (Brown, 2006; Willment and Brown, 2008). The cytoplasmic tail of dectin-1 contains one conserved tyrosine residue involved in signalling. This motif has been shown to be required for the recruitment of Syk via its tandem SH2 domains in response to zymosan, which contains high amounts of the dectin-1 ligand β-glucan, to generate signalling events (Rogers et al., 2005). The dissection of the dectin-1 signalling pathway has also been investigated by the Watson group using NFAT/AP-1-luciferase reporter assays in Jurkat T- and DT40 B-cell mutant lines. Studies using this assay have generated a model in which dectin-1 signalling is dependent on Src, Syk and Tec family tyrosine kinases and PLCγ, but is only partially dependent on the SLP-76/BLNK family of adaptor proteins (Fuller et al., 2007). Therefore, this assay is a useful model to study the signalling of dectin-1 and its regulation by tetraspanins.

3.1.2 Dectin-1 signalling is regulated by CD37

The tetraspanin CD37 has been shown to interact with dectin-1 and to negatively regulate its signalling, as macrophages of CD37-deficient mice have increased dectin-1-mediated IL-6 production despite lower levels of surface dectin-1 (Meyer-Wentrup et al., 2007). In addition, the relatively low level of endogenous of dectin-1 increases following tranfection of CD37 into the RAW 264.7 mouse macrophage cell line (Meyer-Wentrup et al., 2007). The authors hypothesise that CD37 may negatively regulate dectin-1 signalling and stabilise its expression at the cell surface. This was the first report of a tetraspanin to functionally regulate a C-type lectin-like protein.

Further studies by the van Spriel group have shown that CD37-deficient mice exposed to *Candida albicans* have increased IL-6 production and IgA levels, and are therefore better protected from this fungal species compared to controls (van Spriel et al., 2009). However, the studies did not address whether other tetraspanins could interact with and negatively regulate dectin-1. Therefore, the aim of this Chapter was to determine whether CD37 and other tetraspanins regulate the signalling of dectin-1 using an NFAT/AP-1-luciferase reporter assay in the Jurkat T-cell line.

3.2 AIMS

- To determine whether CD37 over-expression inhibits dectin-1 signalling in a model cell line.
- To determine whether other tetraspanins inhibit dectin-1 signalling.

3.3 RESULTS

3.3.1 The Jurkat T-cell line expresses relatively low levels of CD37

The initial aim was to identify a model cell line that expresses low levels of the tetraspanin CD37, such that dectin-1 signalling could be measured in the presence or absence of transfected CD37. Jurkat is a widely used model T-cell line to study T-cell receptor signalling (Abraham and Weiss, 2004) which was found to express relatively low levels of surface CD37, as detected by flow cytometry (Figure 3.1). In contrast, Jurkat expressed relatively high levels of the tetraspanins CD53, CD81 and CD82, whereas CD9, CD63, CD151 and Tspan4 were weakly expressed (Figure 3.1). Therefore, Jurkat would appear to be a useful model system to study the effect of CD37 on dectin-1 signalling.

3.3.2 Dectin-1 induces NFAT/AP-1 activation in Jurkat in response to its ligands curdlan and zymosan

The Watson group have previously demonstrated that, in Jurkat, transfected dectin-1 induces NFAT/AP-1 activation in response to its ligand zymosan (Fuller et al., 2007). In order to establish this assay to ultimately address the effect of CD37, Jurkat were initially transfected with four different amounts of a dectin-1 expression construct, and NFAT/AP-1 activation was measured in response to zymosan and curdlan, the latter of which, unlike zymosan, is a relatively pure source of the dectin-1 ligand β-glucan. For both ligands, the degree of NFAT/AP-1 activation was similar between 5 μg and 20 μg of the dectin-1 expression construct and appeared maximal at these doses (Figure 3.2A). To establish a dose response for dectin-1 stimulation, Jurkat transfected with 10 μg of dectin-1 were stimulated with different amounts of curdlan and zymosan. The degree of NFAT/AP-1 activation increased with increasing amounts of curdlan between 10 and 250 μg/ml and increased with zymosan between 1

and 10 μg/ml, at which the dose response appeared to be maximal (Figure 3.2B). As a positive control for these transfections, NFAT/AP-1 activation in response to PMA and ionomycin, which bypasses receptor proximal signalling events, was found to be similar at each dose of dectin-1 (Figure 3.2C). However, the degree of PMA and ionomycin response was relatively weak due to a suboptimal batch of the agonists. In addition, dectin-1 surface expression was also maximal between 5 μg and 20 μg of dectin-1, as measured by flow cytometry (Figure 3.2D). Together, these data suggest that for future experiments, a dose of 10 μg of dectin-1 will provide a maximal level of dectin-1 surface expression, and curdlan concentrations of 30 and 250 μg/ml will provide suboptimal and optimal levels of dectin-1 stimulation, respectively, to allow the effect of CD37 transfection to be evaluated.

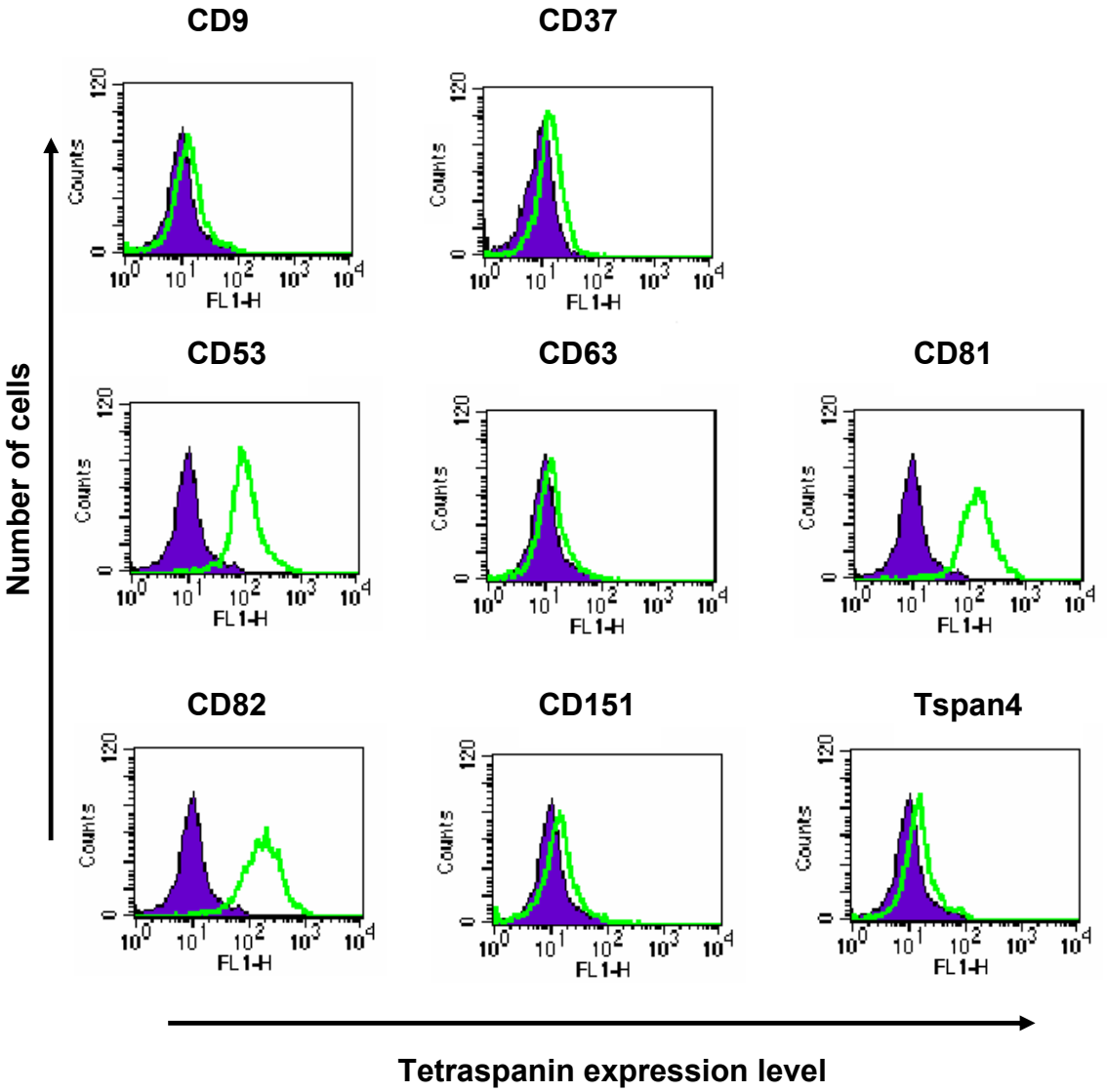
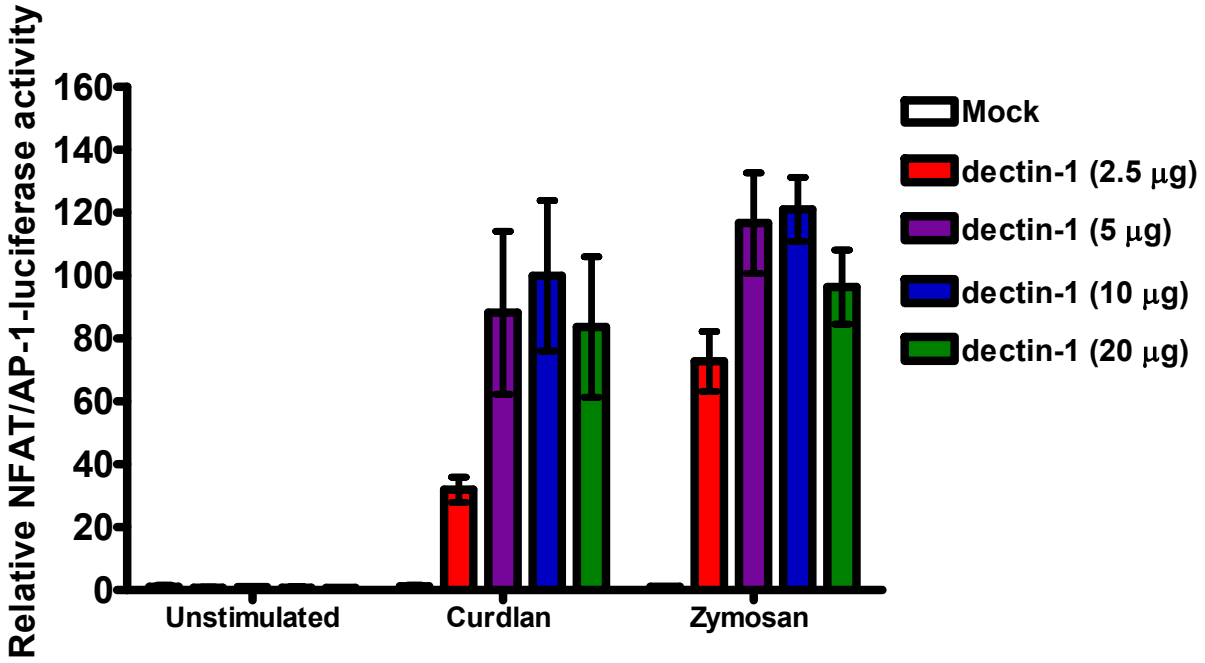


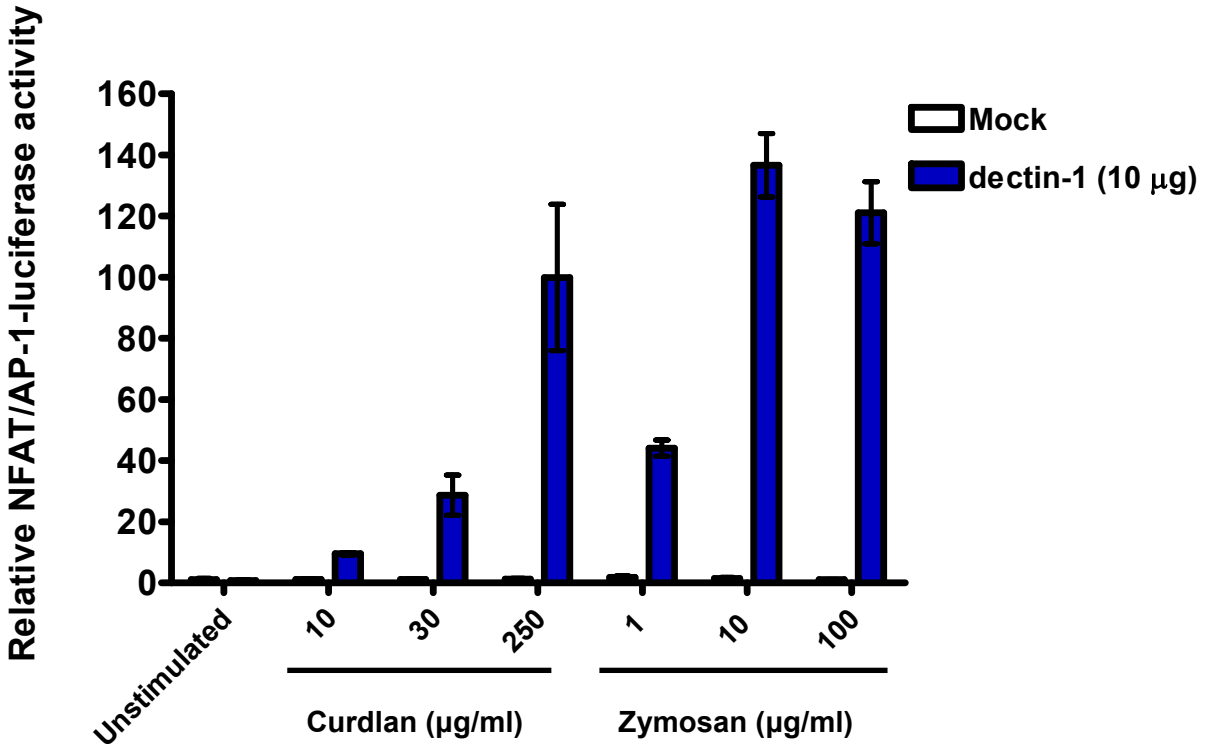
Figure 3.1 Endogenous cell surface expression of tetraspanins on Jurkat T-cells

CD37 levels were detected by flow cytometry using FITC-conjugated CD37 antibody. The other tetraspanins were stained with the indicated primary antibody and then anti-mouse FITC-conjugated secondary antibody. Filled histograms represent the negative control antibody stain and open histograms the relevant tetraspanin antibody stain. Dead cells were gated out using propidium iodide. Data is representative of at least two independent experiments.

A.


Relative NFAT/AP-1-luciferase activity
160
140
120
100
80
60
40
20
0
Unstimulated
Curdlan
Zymosan
Mock
dectin-1 (2.5 μg)
dectin-1 (5 μg)
dectin-1 (10 μg)
dectin-1 (20 μg)

B.


Relative NFAT/AP-1-luciferase activity
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140
120
100
80
60
40
20
0
Unstimulated
10
30
250
1
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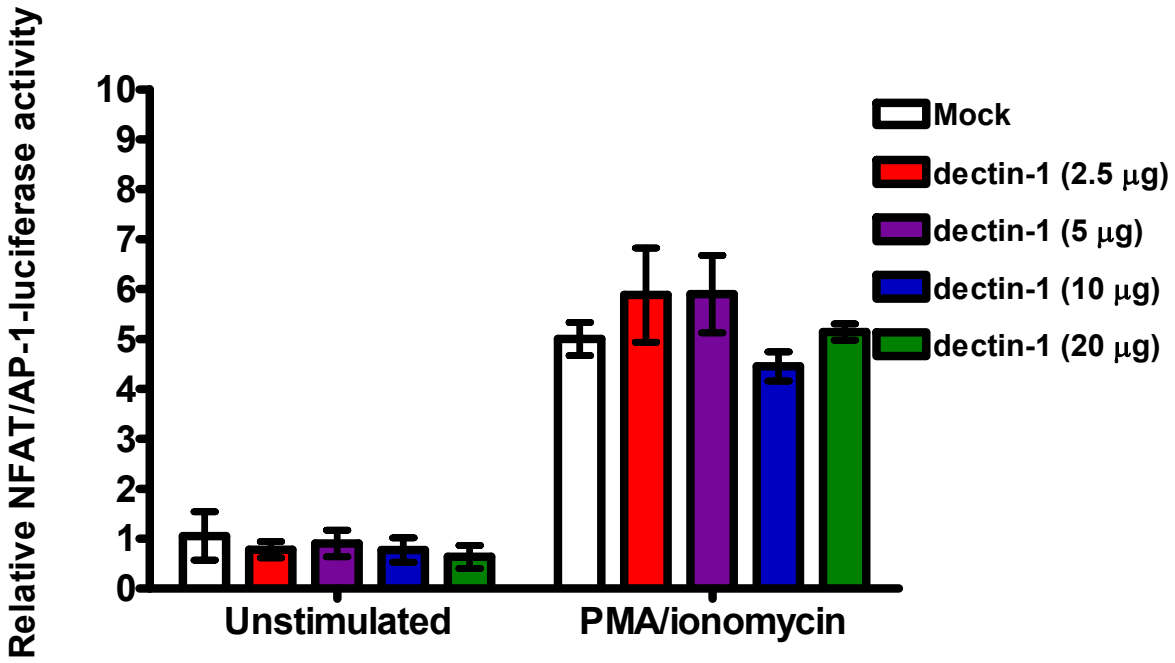
Mock
dectin-1 (10 μg)

C.


Relative NFAT/AP-1-luciferase activity
10
9
8
7
6
5
4
3
2
1
0
Unstimulated
PMA/ionomycin
Mock
dectin-1 (2.5 μg)
dectin-1 (5 μg)
dectin-1 (10 μg)
dectin-1 (20 μg)

D.

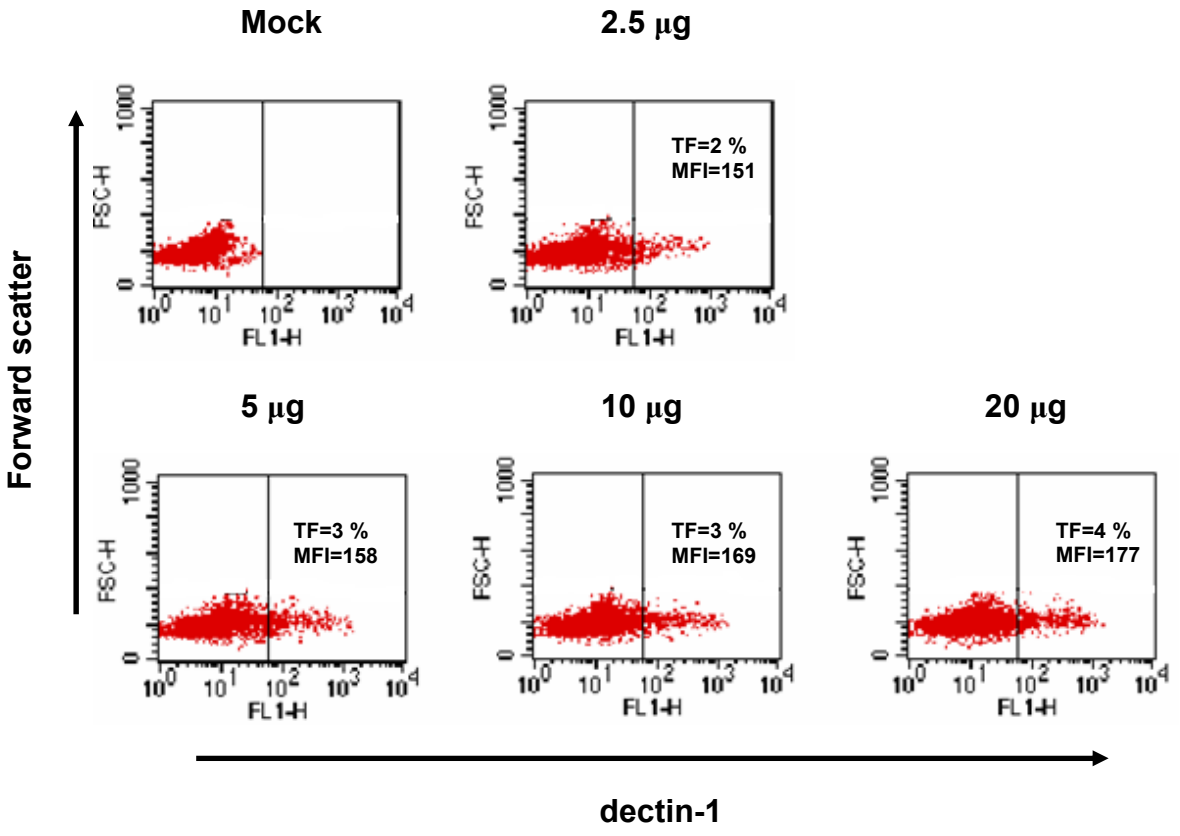

Mock
2.5 μg
5 μg
10 μg
20 μg
Forward scatter
FSC-H
FL1-H
TF=2 %
MFI=151
TF=3 %
MFI=158
TF=3 %
MFI=169
TF=4 %
MFI=177
dectin-1

Figure 3.2 Dectin-1 induces NFAT/AP-1 activation in response to curdlan and zymosan in Jurkat T-cells

Jurkat T-cells were transiently transfected with an NFAT/AP-1-luciferase reporter construct, a β-galactosidase construct to control for transfection efficiency, pEF6-MYC-dectin-1 or empty pEF6 vector (mock). Cells were stimulated with media alone (unstimulated), curdlan, zymosan (A (250 μg/ml curdlan and 100 μg/ml zymosan) & B) and PMA/ionomycin (C) for six hours as indicated. The PMA/ionomycin stimulation was weak due to a suboptimal batch of these agonists. Luciferase activity was measured following stimulation and transfection efficiency was corrected to β-galactosidase activity in individual experiments. The dectin-1 (10 μg) with 250 μg/ml curdlan stimulation value was set to 100% and other conditions presented were calculated relative to this. Error bars represent the standard error of the mean. Experiments were performed three times. (D) Cells were stained with MYC primary antibody and then anti-mouse FITC-conjugated secondary antibody. Dead cells were gated out using propidium iodide. Dot plots were analysed and values indicate the average transfection efficiency (TF) and mean fluorescence intensity (MFI) of protein on the cell surface. Data is representative of three independent experiments.

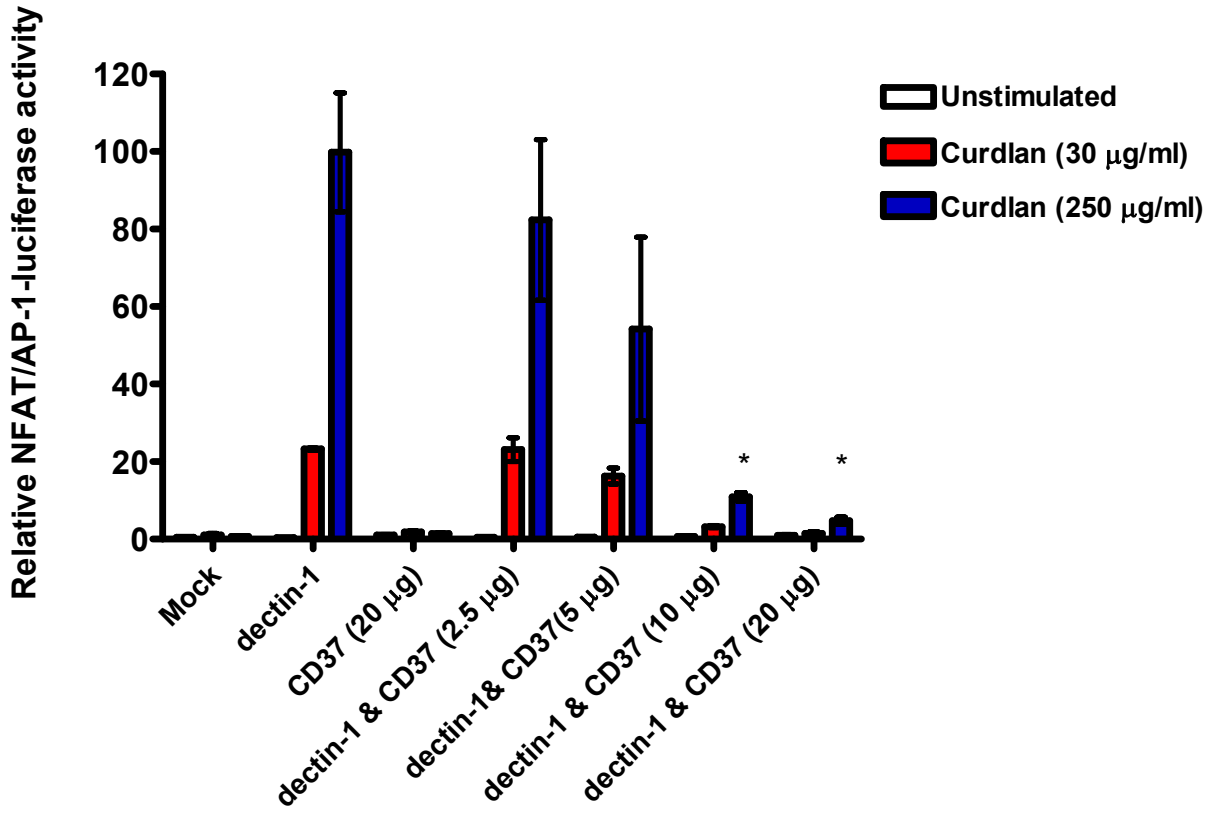
3.3.3 CD37 inhibits dectin-1 signalling in a dose-dependent manner in Jurkat

It has been shown that CD37 negatively regulates dectin-1 signalling (Meyer-Wentrup et al., 2007). Therefore, the NFAT/AP-1-luciferase reporter assay was performed to determine whether CD37 inhibits dectin-1 signalling in Jurkat. Four different amounts of a CD37 expression construct were co-transfected with dectin-1 and NFAT/AP-1 activation was measured in response to a suboptimal (30 μg/ml) and optimal (250 μg/ml) level of curdlan. CD37 inhibited dectin-1 signalling in a dose-dependent manner when stimulated with curdlan, and signalling was inhibited by over 90% when dectin-1 was co-transfected with 10 or 20 μg of CD37 (Figure 3.3A). NFAT/AP-1 activation in response to PMA and ionomycin control stimulation was found to be generally similar in all transfectants indicating that CD37-expressing cells retained the capacity to activate NFAT/AP-1 (Figure 3.3B). Cell surface expression of dectin-1 was determined by flow cytometry and ANOVA suggested that there was no difference in dectin-1 surface expression when co-transfected with all CD37 levels (Figure 3.3C). In addition, a Student's *t*-test was performed to determine whether there was a significant difference in dectin-1 cell surface expression between 0 and 20 μg of CD37. There was no significant difference (P=0.056). However, there was a general downwards trend as the amount of CD37 was increased, although this slight reduction in dectin-1 levels was not observed in later experiments in this Chapter, and cannot account for the substantial loss of signalling, as shown in the previous dectin-1 titration (Figure 3.2A). This allowed one to show that any effect on dectin-1 signalling was due to CD37, not as a result of reduced dectin-1 surface expression. Together these data suggest that CD37 potently inhibits dectin-1 signalling.

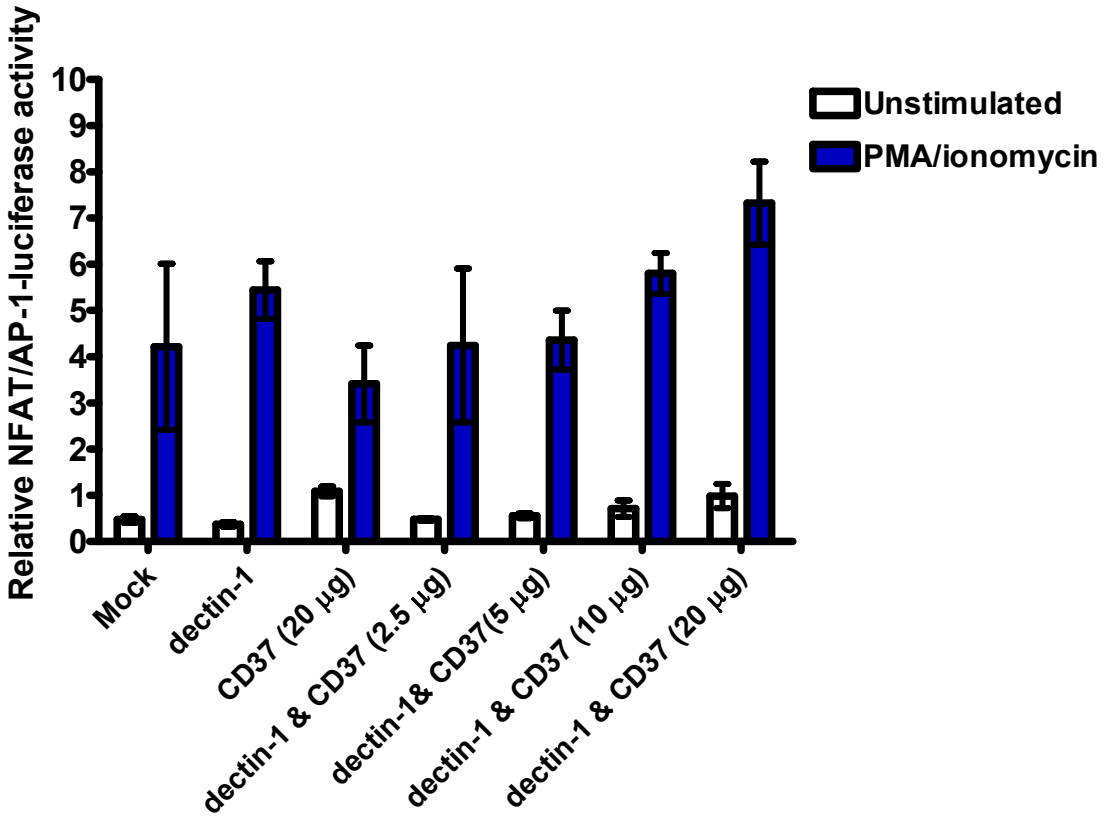
3.3.4 CD82 and Tspan31 also inhibit dectin-1 signalling in Jurkat

The role of CD82 and Tspan31 in dectin-1 signalling was investigated to determine whether CD37 inhibition of dectin-1 was specific to this tetraspanin using the NFAT/AP-1-luciferase assay. These tetraspanins were selected on the basis that CD82 is the most related (36% amino acid identity) and Tspan31 the least related (14% amino acid identity) to CD37, using the ClustalW alignment programme (www.ebi.ac.uk/clustalw). CD82 and Tspan31 had, in common with CD37, a potent inhibitory effect upon dectin-1 signalling following stimulation with 250 μg/ml curdlan (Figure 3.4A). Moreover, all transfection conditions responded following stimulation with the PMA and ionomycin positive control (Figure 3.4B). In some cases, for example dectin-1 plus Tspan31, these control stimulations yielded NFAT/AP-1 activation that was only 50% of that with dectin-1. The reason for this is not clear, but is not due to different transfection efficiencies because a β-galactosidase construct was co-transfected in all experiments, and a β-galactosidase assay performed to normalise the data for transfection efficiency. Nevertheless, it cannot account for the 90% inhibition of dectin-1-induced NFAT/AP-1 activation. Cell surface expression of dectin-1 was determined by flow cytometry and was similar between transfectants suggesting that signal inhibition was not a result of reduced dectin-1 expression when co-transfected with tetraspanins (Figure 3.4C). These data suggests that dectin-1 inhibition is not specific to CD37 and that over-expression of any tetraspanin may inhibit dectin-1 signalling.

A.


Relative NFAT/AP-1-luciferase activity
120
100
80
60
40
20
0
Unstimulated
Curdlan (30 μg/ml)
Curdlan (250 μg/ml)
*
*
Mock
dectin-1
CD37 (20 μg)
dectin-1 & CD37 (2.5 μg)
dectin-1& CD37(5 μg)
dectin-1 & CD37 (10 μg)
dectin-1 & CD37 (20 μg)

B.


Relative NFAT/AP-1-luciferase activity
10
9
8
7
6
5
4
3
2
1
0
Unstimulated
PMA/ionomycin
Mock
dectin-1
CD37 (20 μg)
dectin-1 & CD37 (2.5 μg)
dectin-1& CD37(5 μg)
dectin-1 & CD37 (10 μg)
dectin-1 & CD37 (20 μg)

C.

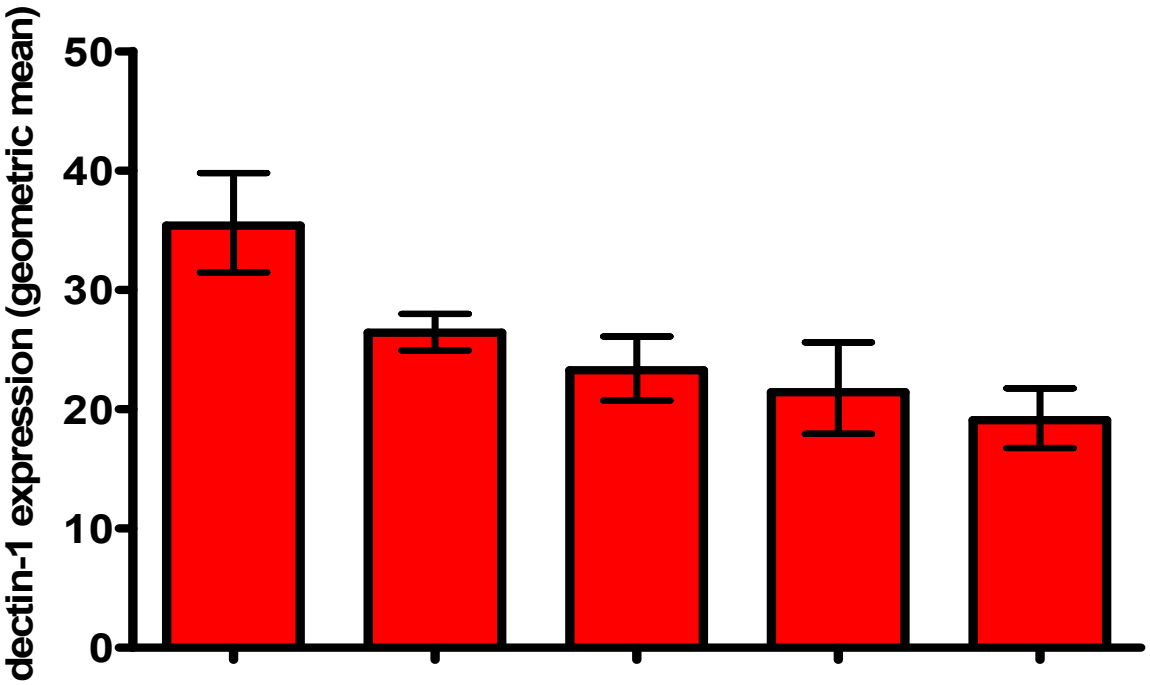
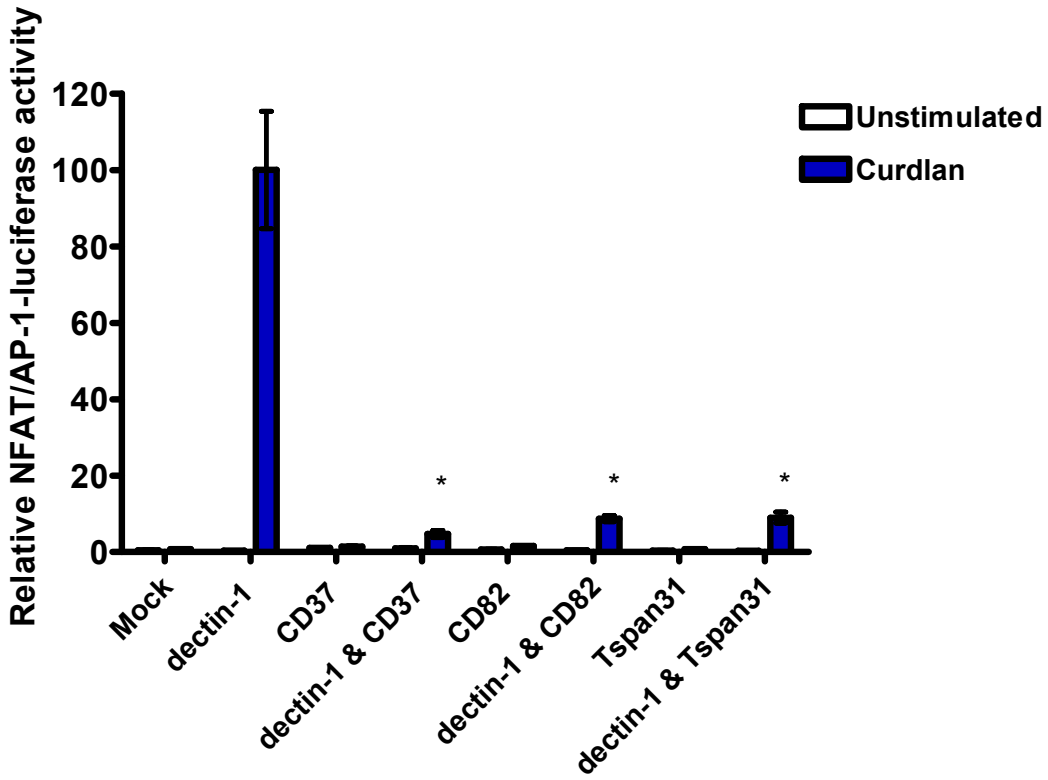


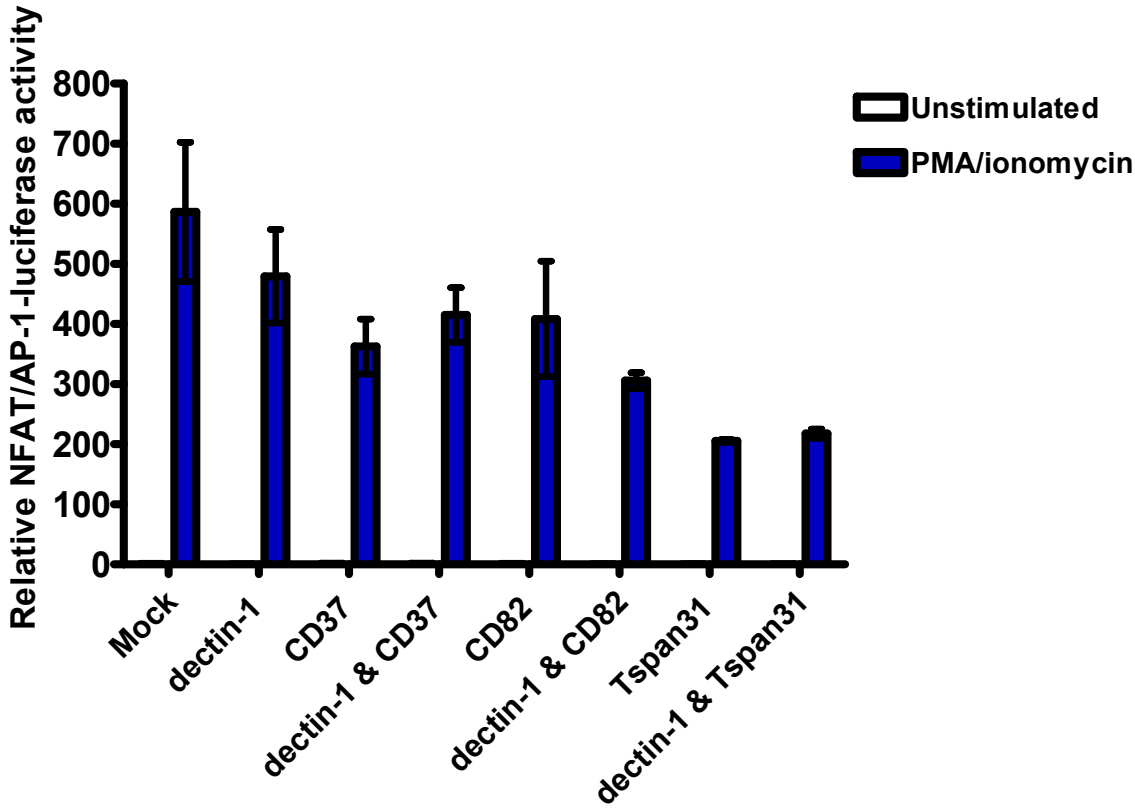
Figure 3.3 CD37 inhibits dectin-1 signalling

Jurkat T-cells were transiently transfected with an NFAT/AP-1-luciferase reporter construct, β-galactosidase construct, pEF6-MYC-dectin-1 (10 μg) and pEF6-CD37 or empty pEF6 vector (mock). Cells were stimulated with media alone (unstimulated), curdlan (250 μg/ml) (A) and PMA/ionomycin (B) for six hours as indicated. The PMA/ionomycin stimulation was weak due to a suboptimal batch of these agonists. Luciferase activity was measured following stimulation and transfection efficiency was corrected to β-galactosidase activity in individual experiments. The dectin-1 with 250 μg/ml curdlan stimulation value was set to 100% and other conditions presented were calculated relative to this. An unpaired Student's *t*-test indicated a statistically significant difference ($P<0.05$) between dectin-1 transfected alone compared to dectin-1 co-transfected with relatively high levels of CD37 as indicated *. Error bars represent the standard error of the mean. Experiments were performed three times. (C) Cells were stained and analysed by flow cytometry as described previously. Flow cytometry plots were analysed minus mock background fluorescence and data shown indicates geometric mean ± standard error. Analysis of variance (ANOVA) showed that there was no significant difference between groups. A Student's *t*-test was performed to determine whether there was a significant difference in dectin-1 cell surface expression between 0 and 20 μg of CD37, and this failed to reach significance ($P=0.056$) (n=3). Experiments were performed three times.

A.


Relative NFAT/AP-1-luciferase activity
120
100
80
60
40
20
0
*
*
*
Unstimulated
Curdlan
Mock
dectin-1
CD37
dectin-1 & CD37
CD82
dectin-1 & CD82
Tspan31
dectin-1 & Tspan31

B.


Relative NFAT/AP-1-luciferase activity
800
700
600
500
400
300
200
100
0
Unstimulated
PMA/ionomycin
Mock
dectin-1
CD37
dectin-1 & CD37
CD82
dectin-1 & CD82
Tspan31
dectin-1 & Tspan31

C.

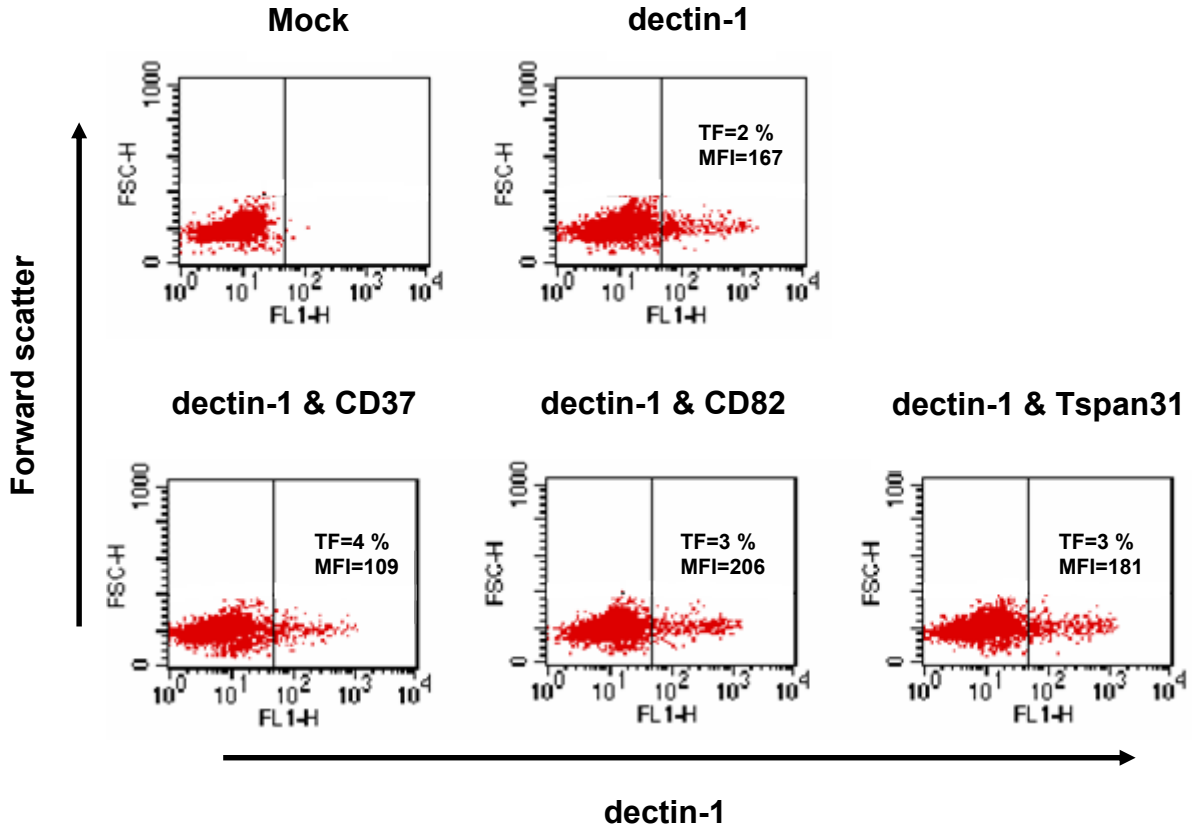


Figure 3.4 CD37, CD82 and Tspan31 inhibit dectin-1 signalling in Jurkat T-cells

Jurkat T-cells were transiently transfected with an NFAT/AP-1-luciferase reporter construct, β-galactosidase construct, pEF6-MYC-dectin-1 (10 μg) and pEF6-CD37, CD82, Tspan31 (20 μg each) or empty pEF6 vector (mock). Cells were stimulated with media alone (unstimulated), curdlan (250 μg/ml) (A) and PMA/ionomycin (B) for six hours as indicated. Luciferase activity was measured following stimulation and transfection efficiency was corrected to β-galactosidase activity in individual experiments. The dectin-1 with curdlan stimulation value was set to 100% and other conditions presented were calculated relative to this. An unpaired Student's *t*-test indicated a statistically significant difference ($P<0.05$) between dectin-1 transfected alone compared to dectin-1 co-transfected with CD37, CD82 or Tspan31 as indicated *. Error bars represent the standard error of the mean. Experiments were performed three times. (C) Cells were stained with MYC primary antibody and then anti-mouse FITC-conjugated secondary antibody. Dead cells were gated out using propidium iodide. Antibody binding was visualized by flow cytometry. Dot plots were analysed and values indicate the average transfection efficiency (TF) and mean fluorescence intensity (MFI) of protein on the cell surface. Data is representative of three independent experiments.

3.3.5 Dectin-1 signal inhibition by tetraspanin over-expression is not epitope tag-dependent in Jurkat

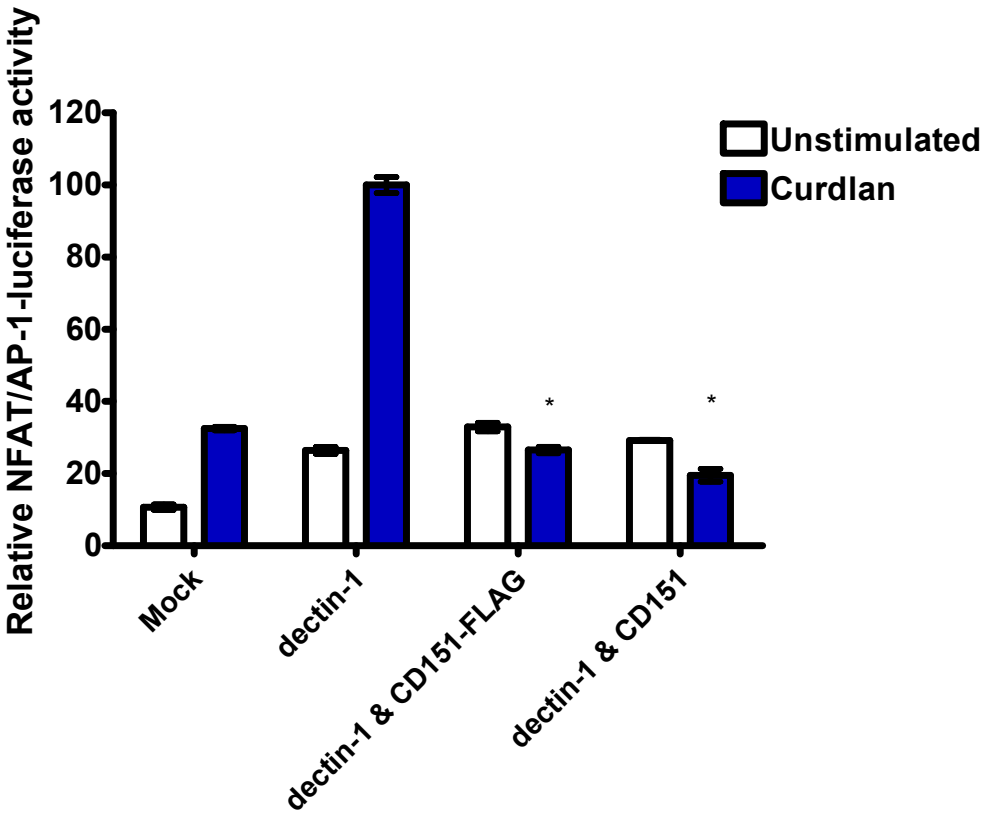
Although the FLAG tag is only eight amino acids long, it is possible that such tagging renders tetraspanins dysfunctional and that dectin-1 inhibition is an artefact. Therefore, Jurkat were transfected with dectin-1 and CD151 with or without a FLAG tag. CD151 was chosen because of the availability of non-tagged and tagged forms. In common with CD37, CD82 and Tspan31, CD151 inhibited dectin-1-induced NFAT/AP-1 activation, and this was independent of the presence of the tag (Figure 3.5A). However, the degree of dectin-1-induced NFAT/AP-1 activation following curdlan stimulation was particulary weak, suggesting that the agonist preparation was not optimal in this case. In addition, all transfection conditions responded similarly to positive control stimulations (Figure 3.5B). Indeed, the PMA and ionomycin response with dectin-1 and CD151 transfectants was relatively higher than that of dectin-1 transfectant. Cell surface expression of dectin-1 and CD151 was confirmed by flow cytometry and was similar between transfectants (Figure 3.5C-D). These data suggest that a tag on the tetraspanin is not the cause of dectin-1 signal inhibition by tetraspanin over-expression.

3.3.6 Tetraspanins do not inhibit the signalling of the collagen receptor GPVI in Jurkat

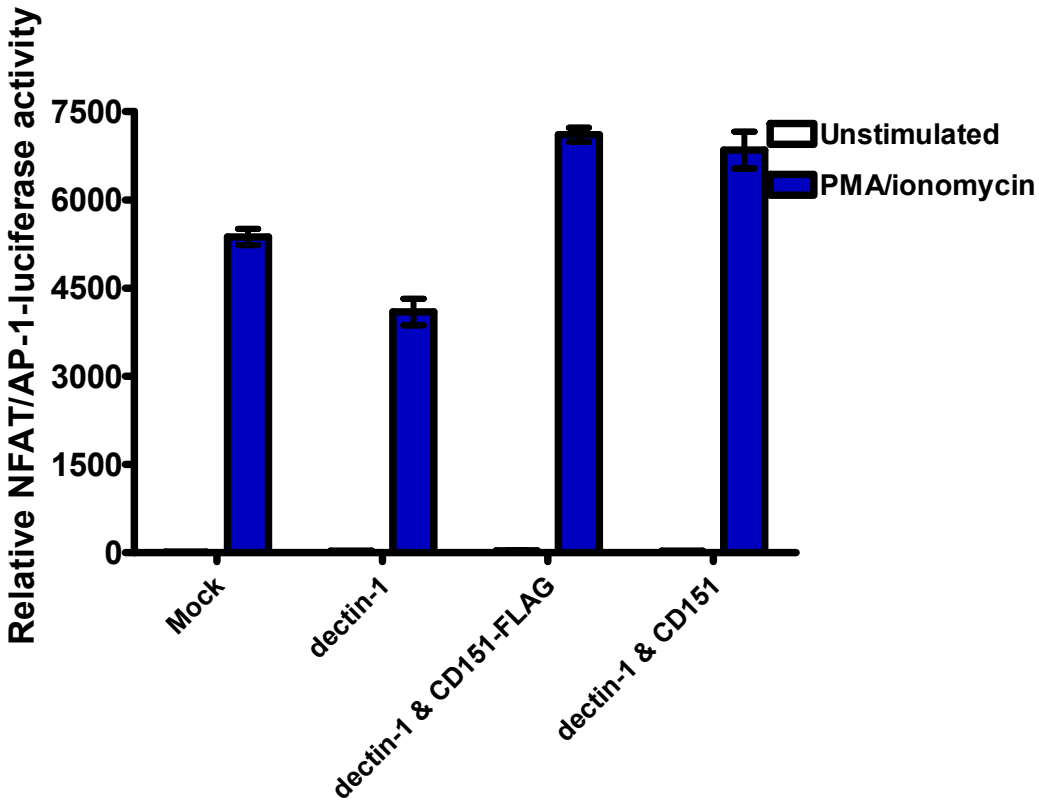
As all tetraspanins studied so far have had an inhibitory effect on dectin-1 signalling, it was important to determine whether tetraspanin over-expression was having a general inhibitory effect on receptor signalling. Consequently, the collagen receptor, GPVI/FcRγ, was studied to determine whether tetraspanins had an inhibitory effect on this receptor. GPVI and FcRγ (5 μg each) expression constructs were co-transfected with the tetraspanins CD37, CD81, CD82 and Tspan31 and stimulated with 5 μg/ml collagen. Expression of CD37, CD81, CD82 and Tspan31 had no significant effect on

GPVI signalling as suggested by ANOVA (Figure 3.6A), although NFAT/AP-1 activation in the presence of Tspan31 was only 50% of GPVI/FcRγ, for a reason that is not clear. All transfection conditions responded to positive control stimulations, albeit with some differences in magnitude (Figure 3.6B). The mock transfectant when stimulated with PMA and ionomcyin was relatively higher than all other transfectants. This data suggests that GPVI/FcRγ alone or when co-transfected with tetraspanins had a general inhibitory effect on the experiment. However, as the PMA and ionomycin response was similar, this does not detract from the conclusion presented that tetraspanins do not inhibit GPVI/FcRγ signalling following collagen stimulation. GPVI cell surface expression was determined by flow cytometry and was similar between transfectants (Figure 3.6C). These data demonstrate that tetraspanin over-expression does not globally inhibit receptor signalling.

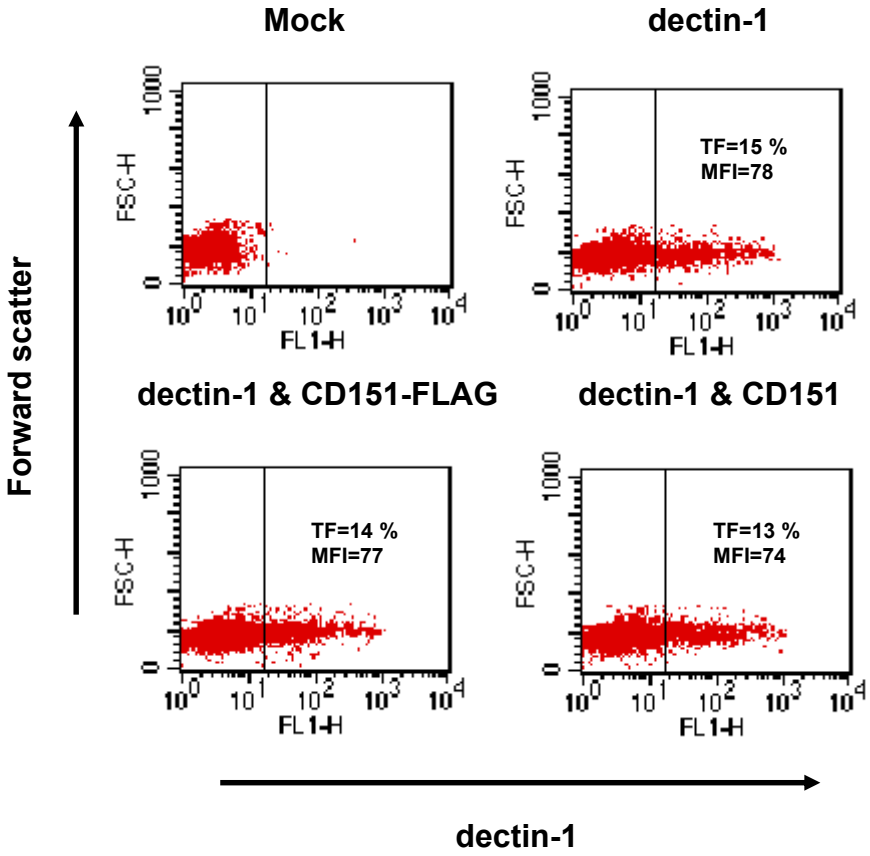
A.


Relative NFAT/AP-1-luciferase activity
120
100
80
60
40
20
0
Unstimulated
Curdlan
*
*
Mock
dectin-1
dectin-1 & CD151-FLAG
dectin-1 & CD151

B.


Relative NFAT/AP-1-luciferase activity
7500
6000
4500
3000
1500
0
Unstimulated
PMA/ionomycin
Mock
dectin-1
dectin-1 & CD151-FLAG
dectin-1 & CD151

C.


Mock
dectin-1
dectin-1 & CD151-FLAG
dectin-1 & CD151
TF=15 %
MFI=78
TF=14 %
MFI=77
TF=13 %
MFI=74
FSC-H
FL1-H
Forward scatter
dectin-1

D.

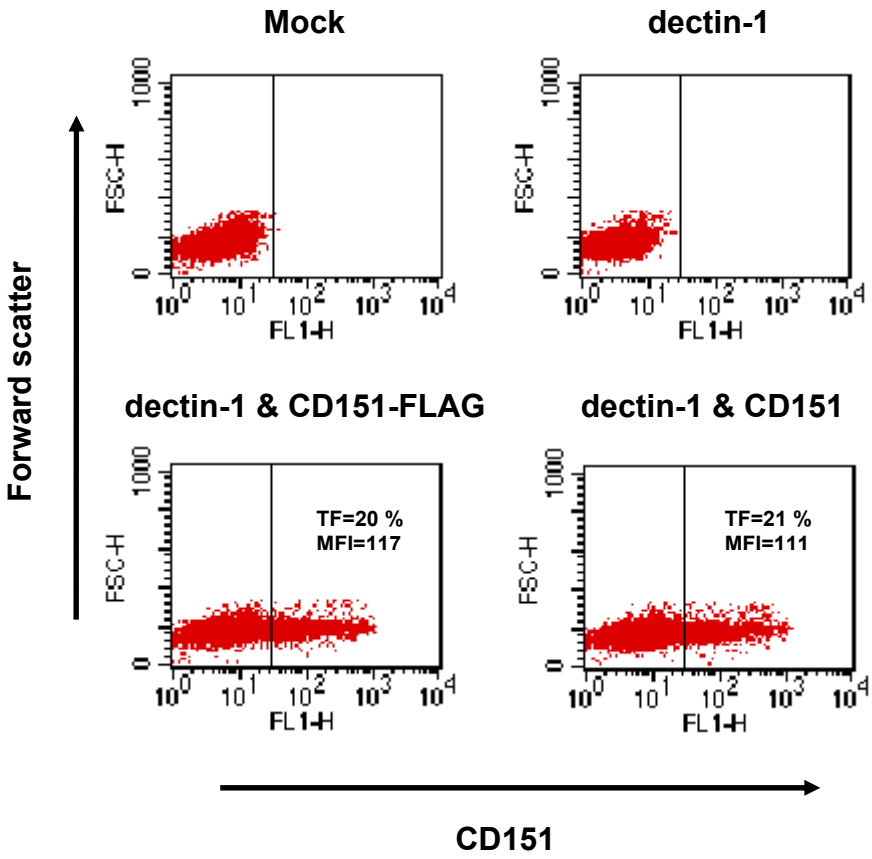
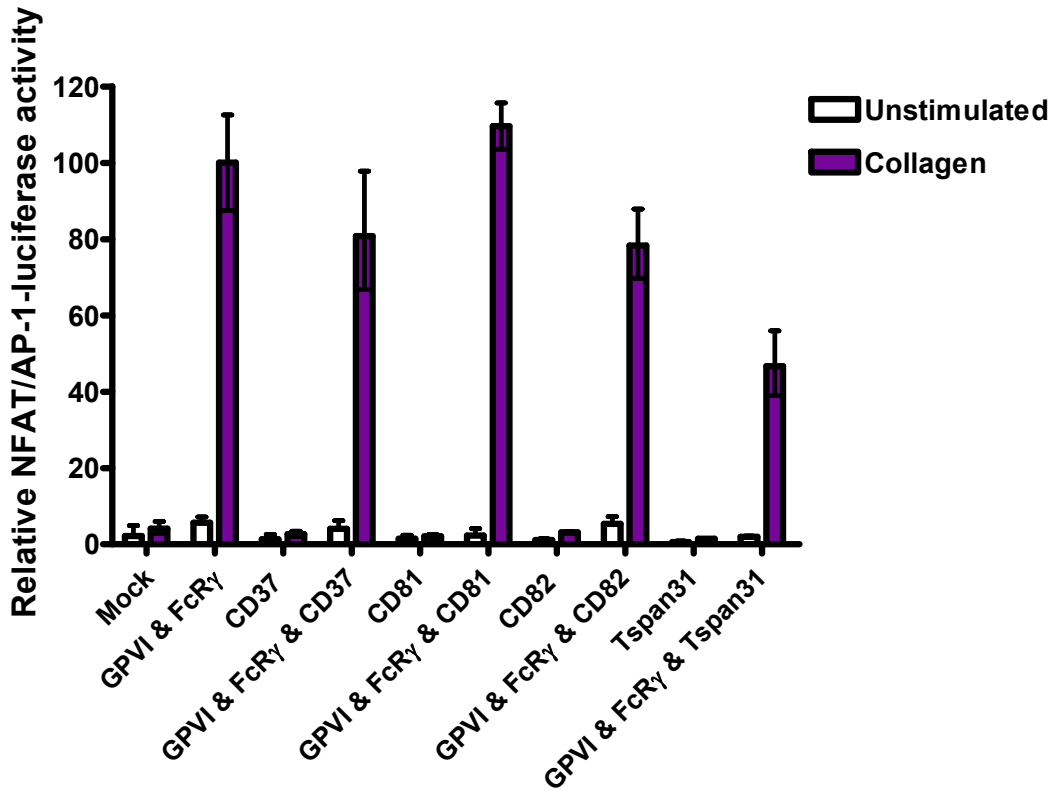

Mock
dectin-1
dectin-1 & CD151-FLAG
dectin-1 & CD151
TF=20 %
MFI=117
TF=21 %
MFI=111
FSC-H
FL1-H
Forward scatter
CD151

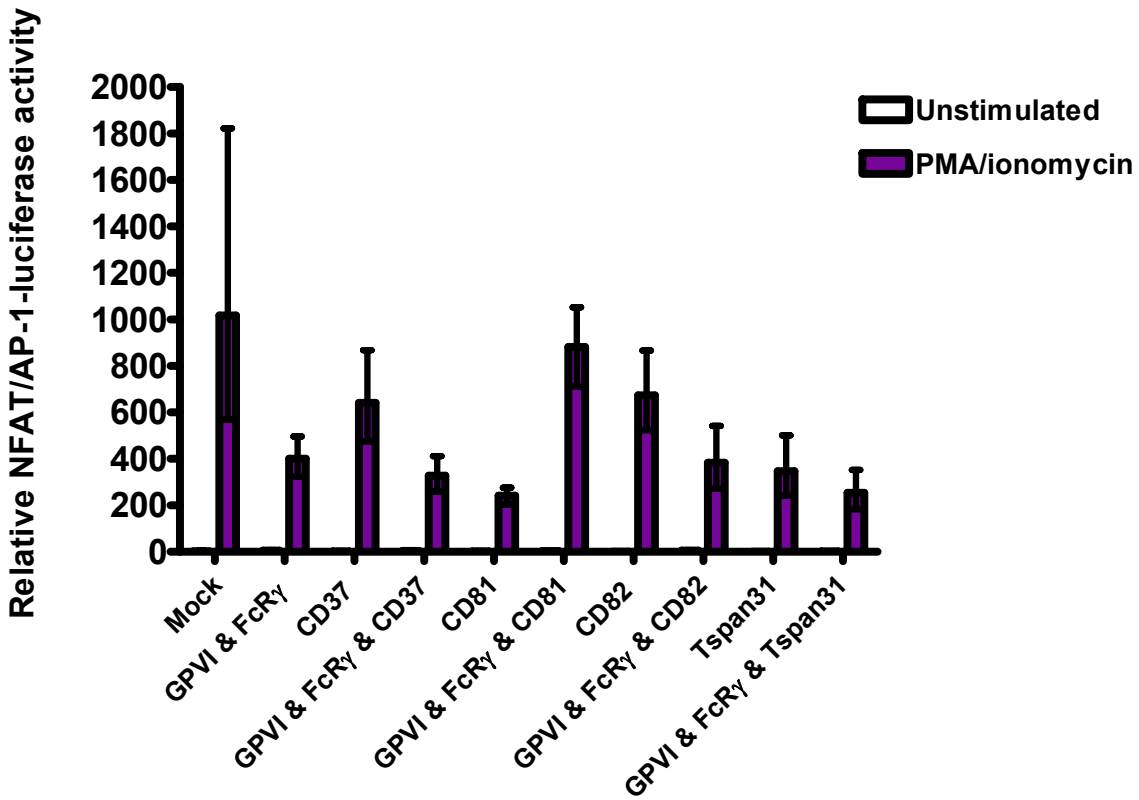
Figure 3.5 Dectin-1 signal inhibition by tetraspanin over-expression is not tag-dependent

Jurkat T-cells were transiently transfected with an NFAT/AP-1-luciferase reporter construct, β-galactosidase construct, pEF6-MYC-dectin-1 (10 μg) and pCDNA3-FLAG-CD151 (20 μg) or pCDNA3-CD151 (20 μg) or empty pEF6/pCDNA3 vector (mock). Cells were stimulated with media alone (unstimulated), curdlan (250 μg/ml) (A) and PMA/ionomycin (B) for six hours as indicated. Luciferase activity was measured following stimulation and transfection efficiency was corrected to β-galactosidase activity in individual experiments. The dectin-1 with curdlan stimulation value was set to 100% and other conditions presented were calculated relative to this. An unpaired Student's *t*-test indicated a statistically significant difference ($P<0.05$) between dectin-1 transfected alone compared to dectin-1 co-transfected with CD151-FLAG and CD151 as indicated *. Error bars represent the standard error of the mean. Experiments were performed twice. (C-D) Cells were stained with MYC or CD151 primary antibodies and then anti-mouse FITC-conjugated secondary antibody. Dead cells were gated out using propidium iodide. Antibody binding was visualized by flow cytometry. Dot plots were analysed and values indicate the average transfection efficiency (TF) and mean fluorescence intensity (MFI) of protein on the cell surface. Data is representative of two independent experiments.

A.


Relative NFAT/AP-1-luciferase activity
120
100
80
60
40
20
0
Unstimulated
Collagen
Mock
GPVI & FcRγ
CD37
GPVI & FcRγ & CD37
CD81
GPVI & FcRγ & CD81
CD82
GPVI & FcRγ & CD82
Tspan31
GPVI & FcRγ & Tspan31

B.


Relative NFAT/AP-1-luciferase activity
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Unstimulated
PMA/ionomycin
Mock
GPVI & FcRγ
CD37
GPVI & FcRγ & CD37
CD81
GPVI & FcRγ & CD81
CD82
GPVI & FcRγ & CD82
Tspan31
GPVI & FcRγ & Tspan31

C.

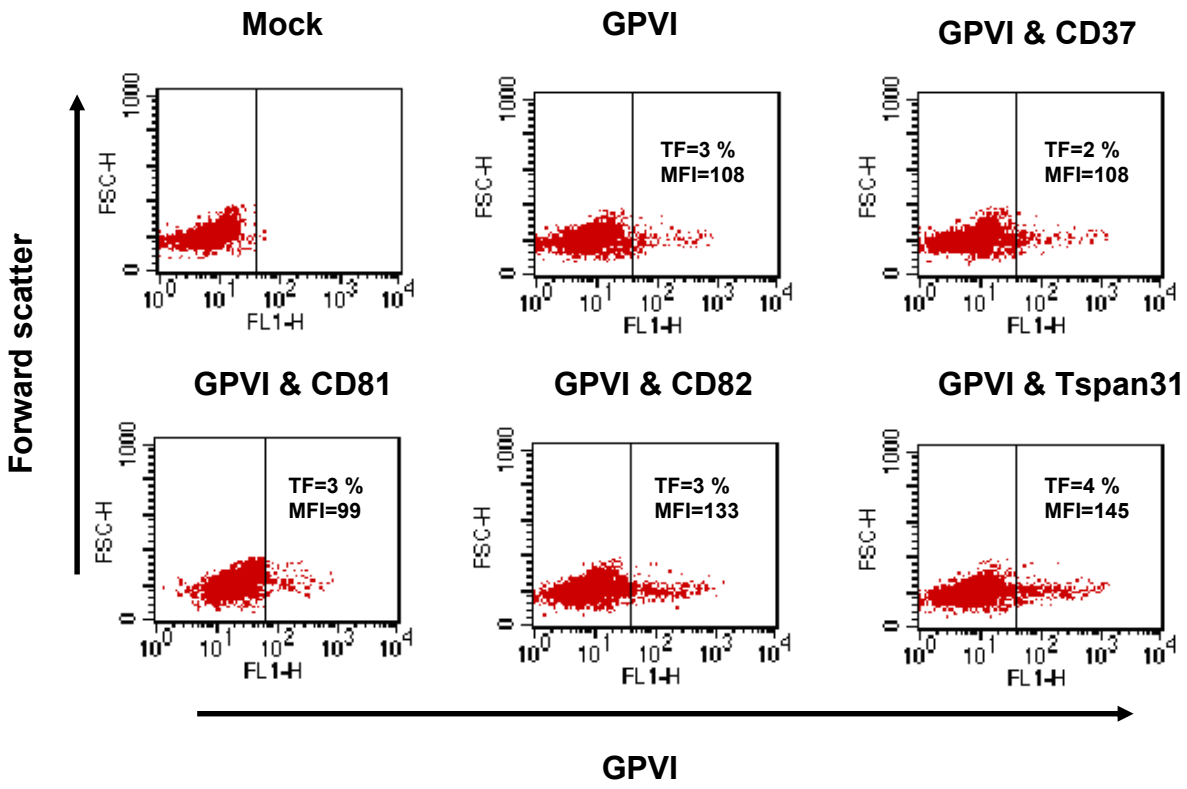


Figure 3.6 CD37, CD81, CD82 and Tspan31 do not inhibit GPVI/FcRγ signalling in Jurkat T-cells

Jurkat T-cells were transiently transfected with an NFAT/AP-1-luciferase reporter construct, β-galactosidase construct, pCDNA3-MYC-GPVI/pEF6-FcRγ (5 µg each) and pEF6-CD37, CD81, CD82, Tspan31 (20 µg each) or empty pEF6 vector (mock). Cells were stimulated with media alone (unstimulated), collagen (5 µg/ml) (A) and PMA/ionomycin (B) for six hours as indicated. Luciferase activity was measured following stimulation and transfection efficiency was corrected to β-galactosidase activity in individual experiments. The GPVI/FcRγ with collagen stimulation value was set to 100% and other conditions presented were calculated relative to this. Analysis of variance (ANOVA) showed there was no significant difference between groups. Error bars represent the standard error of the mean. Experiments were performed three times. (C) Cells were stained with anti-human GPVI and then anti-mouse FITC-conjugated secondary antibody. Dead cells were gated out using propidium iodide. Antibody binding was visualized by flow cytometry. Dot plots were analysed and values indicate the average transfection efficiency (TF) and mean fluorescence intensity (MFI) of protein on the cell surface. Data is representative of three independent experiments.

3.3.7 The four-transmembrane L6 antigen does not inhibit dectin-1 signalling in Jurkat

Since all tested tetraspanins inhibit dectin-1 signalling, it was important to investigate the possibility that any transmembrane protein has the ability to inhibit dectin-1 signalling when over-expressed. Therefore, the L6 antigen was selected because it shares the four-transmembrane topology of the tetraspanin superfamily. Jurkat were transfected with dectin-1 and L6 expression constructs and NFAT/AP-1 activation was measured in response to 250 μg/ml curdlan. Unlike the previous data for tetraspanins, dectin-1 could still signal when co-transfected with L6 (Figure 3.7A). In addition, all transfection conditions responded similarily to positive control stimulations, apart from the mock transfectant which responsed weakly (Figure 3.7B). Dectin-1 and L6 cell surface expression was confirmed by flow cytometry (Figure 3.7C-D). Together, these data suggest that over-expression of any four-transmembrane protein does not inhibit dectin-1 signalling and that the inhibitory effect may be specific to tetraspanins.

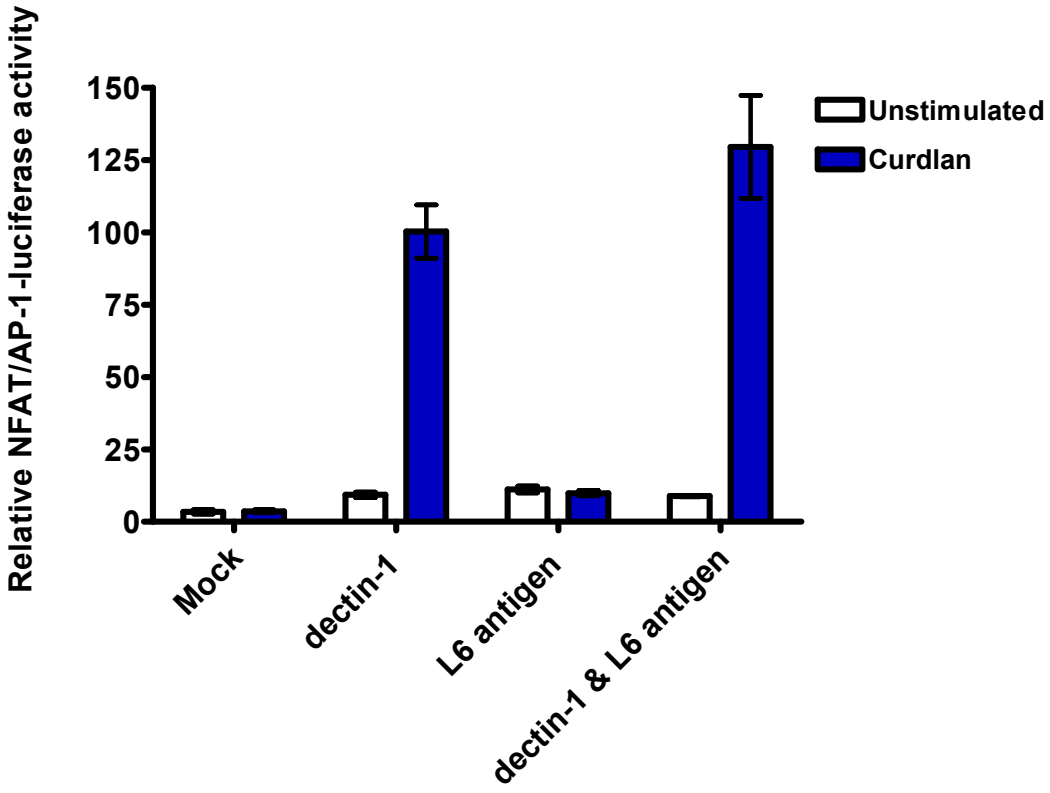
3.3.8 Evaluation of the level of tetraspanin over-expression and the role of palmitoylation in dectin-1 signal inhibition

In the experiments conducted so far, it is not clear to what extent the tetraspanins were over-expressed compared to endogenous levels. Indeed, the previous experiments used mouse tetraspanins for which no antibodies were available. In order to determine the level of tetraspanin over-expression that is achieved in these experiments, human CD81 was selected because of the availability of antibodies and the strong endogenous expression on Jurkat, at about 10-fold over negative control staining as measured by flow cytometry (Figure 3.1).

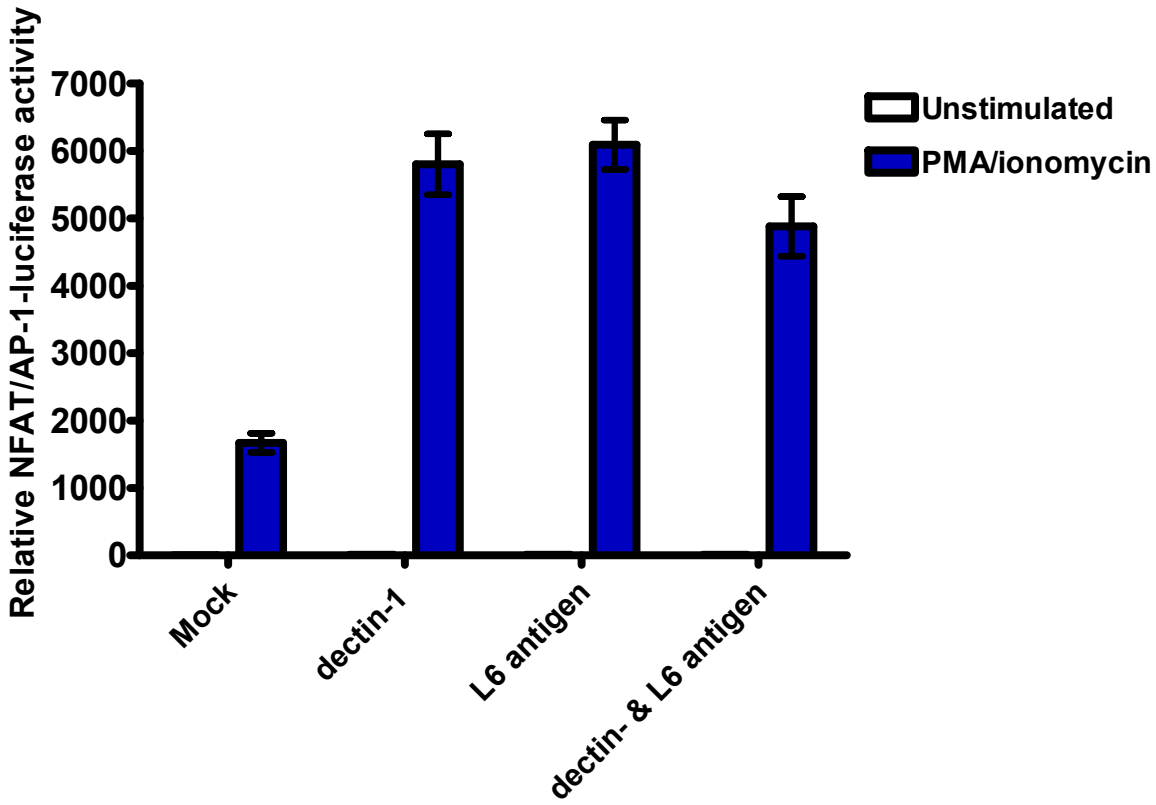
Cell surface expression of transfected CD81 was determined by flow cytometry (Figure 3.8A). Using mean fluorescence intensity (MFI) values it was possible to determine that there was approximately 10-fold over-expression of CD81 compared to mock-transfected cells. CD81 significantly inhibited dectin-1 signalling when stimulated with 250 μg/ml curdlan (Figure 3.8B). In addition, all transfection conditions responded similarly to positive control stimulations (Figure 3.8C) and dectin-1 was expressed at the cell surface as determined by flow cytomety (Figure 3.8D). These data suggest 10-fold over-expression of a tetraspanin is sufficient to inhibit dectin-1 signalling.

The role of palmitoylation was investigated, as it is thought palmitoylation is important for protein stability, localization, trafficking and protein-protein interactions (Berditchevski et al., 2002). Therefore, the utilisation of a CD81 palmitoylation mutant may affect dectin-1 signalling by tetraspanin over-expression. The CD81 palmitoylation mutant was expressed at the cell surface as detemined by flow cytometry (Figure 3.8A). Despite this, dectin-1 signalling was still significantly inhibited by over-expression of the CD81 palmitoylation mutant (Figure 3.8B). These data suggest that palmitoylation is not required for dectin-1 signal inhibition by tetraspanin over-expression.

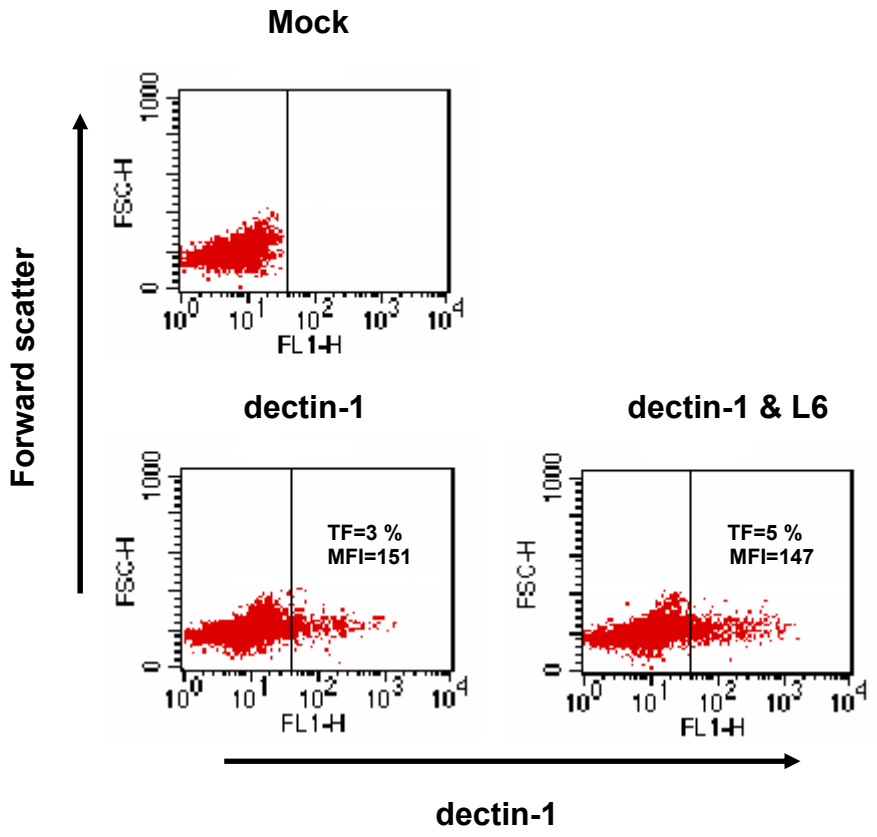
A.


Relative NFAT/AP-1-luciferase activity
150
125
100
75
50
25
0
Unstimulated
Curdlan
Mock
dectin-1
L6 antigen
dectin-1 & L6 antigen

B.


Relative NFAT/AP-1-luciferase activity
7000
6000
5000
4000
3000
2000
1000
0
Unstimulated
PMA/ionomycin
Mock
dectin-1
L6 antigen
dectin- & L6 antigen

C.


Mock
dectin-1
dectin-1 & L6
TF=3 %
MFI=151
TF=5 %
MFI=147
FSC-H
FL1-H
Forward scatter
dectin-1

D.

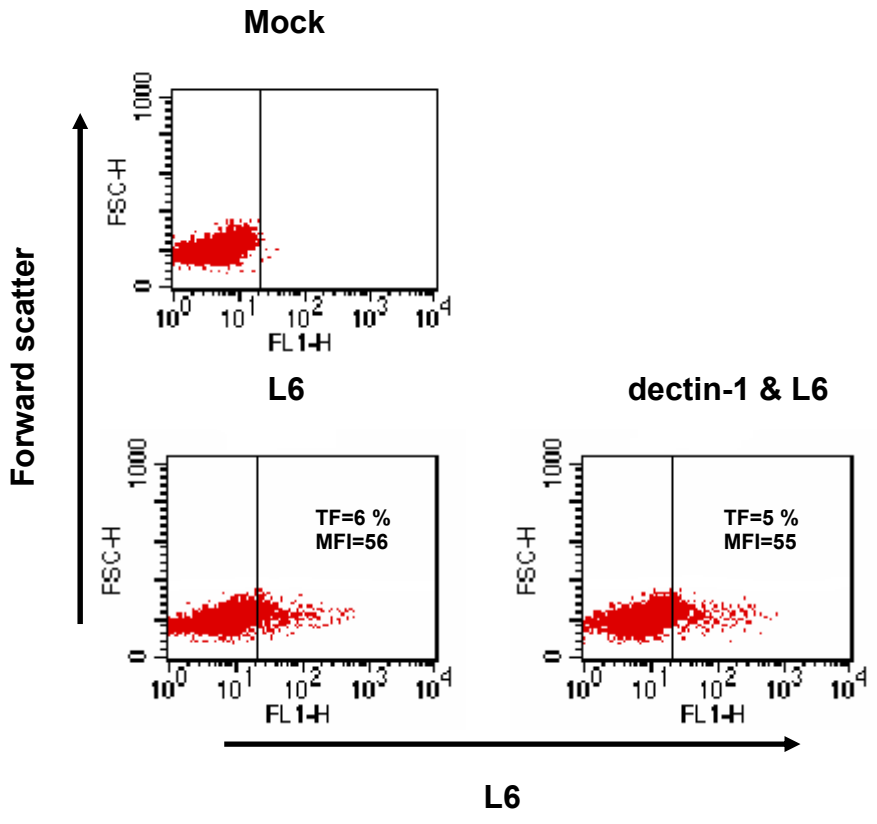
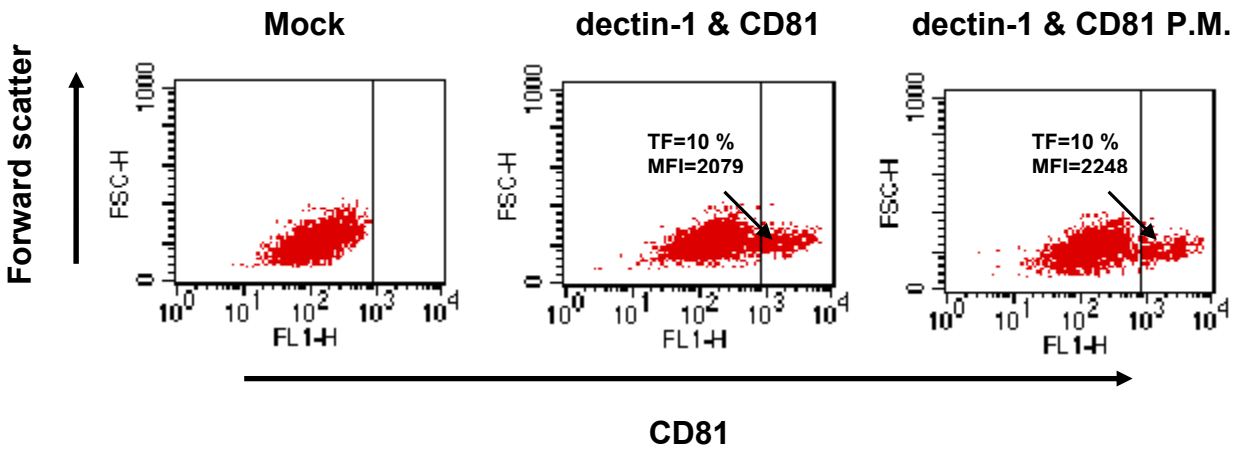

Mock
L6
dectin-1 & L6
TF=6 %
MFI=56
TF=5 %
MFI=55
FSC-H
FL1-H
Forward scatter
L6

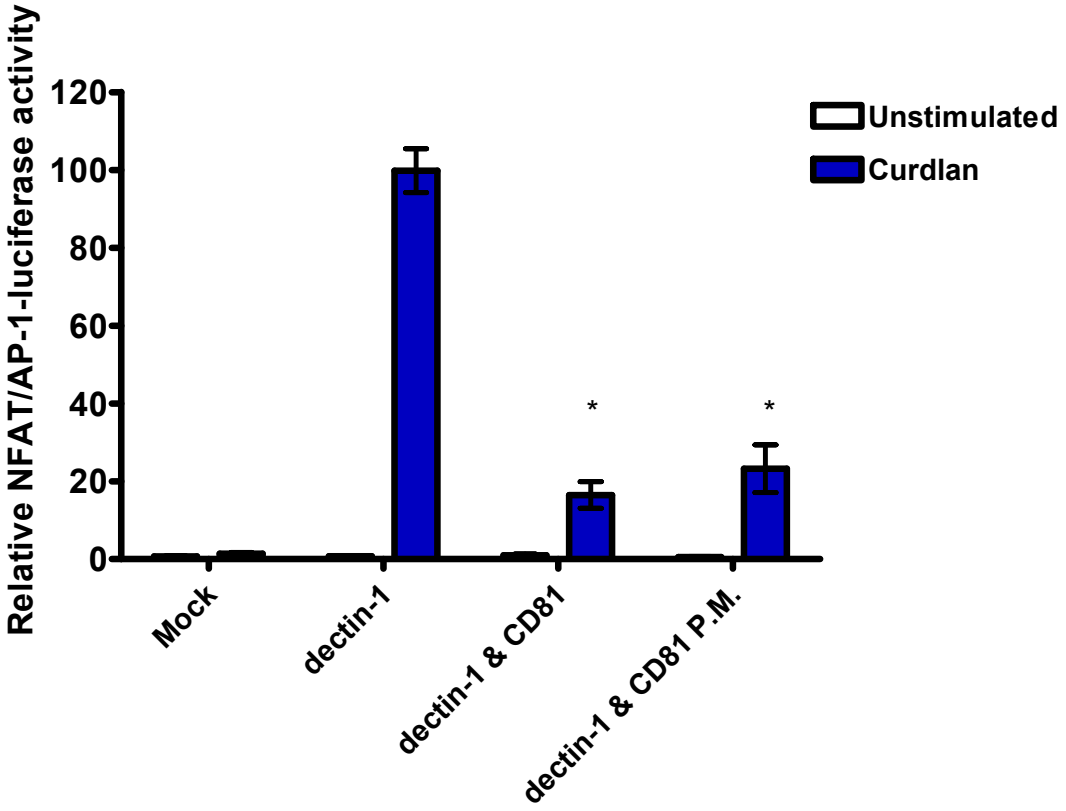
Figure 3.7 The L6 antigen does not affect dectin-1 signalling in Jurkat T-cells

Jurkat T-cells were transiently transfected with an NFAT/AP-1-luciferase reporter construct, β-galactosidase construct, pEF6-MYC-dectin-1 (10 μg) and pCDNA3-L6 (20 μg) or empty pEF6/pCDNA3 vector (mock). Cells were stimulated with media alone (unstimulated), curdlan (250 μg/ml) (A) and PMA/ionomycin (B) for six hours as indicated. Luciferase activity was measured following stimulation and transfection efficiency was corrected to β-galactosidase activity in individual experiments. The dectin-1 with curdlan stimulation value was set to 100% and other conditions presented were calculated relative to this. Analysis of variance (ANOVA) showed that there was no significant difference between groups. Error bars represent the standard error of the mean. Experiments were performed three times. (C-D) Cells were stained with either MYC or L6 antigen primary antibody and then anti-mouse FITC-conjugated secondary antibody. Dead cells were gated out using propidium iodide. Antibody binding was visualized by flow cytometry. Dot plots were analysed and values indicate the average transfection efficiency (TF) and mean fluorescence intensity (MFI) of protein on the cell surface. Data is representative of three independent experiments.

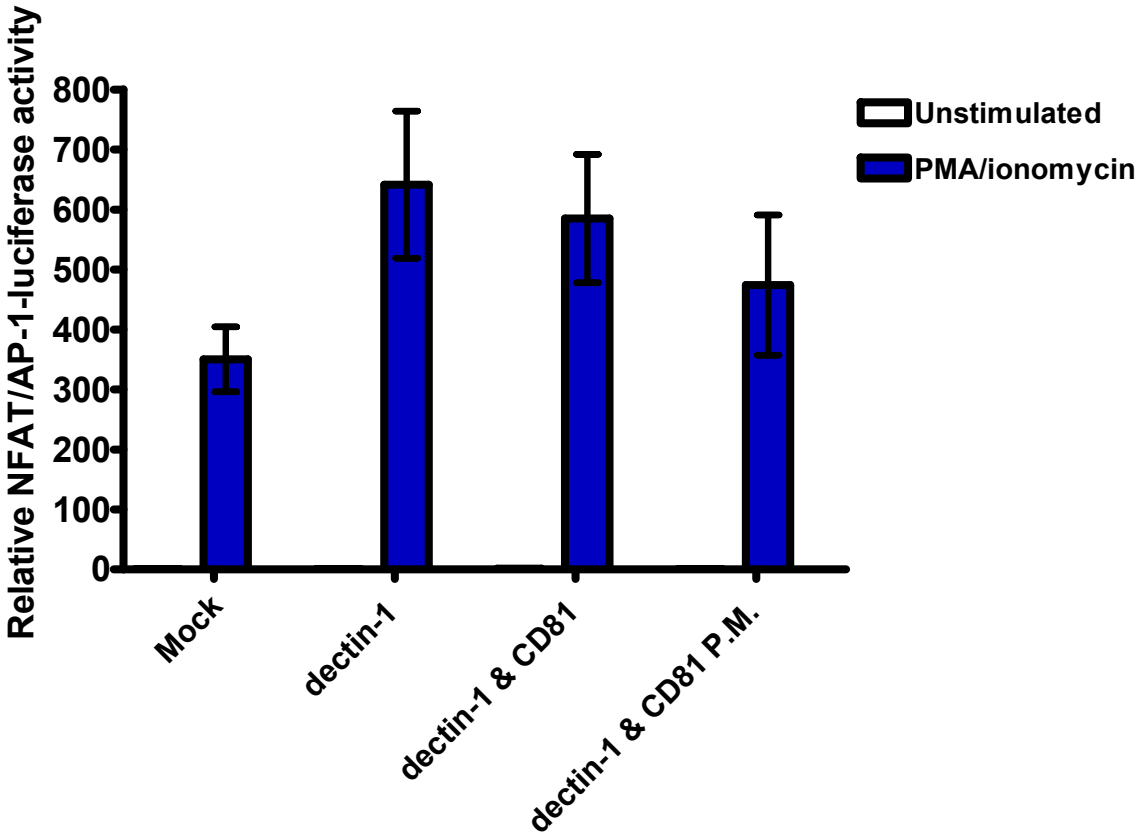
A.


Mock
dectin-1 & CD81
dectin-1 & CD81 P.M.
Forward scatter
FSC-H
FL1-H
TF=10 %
MFI=2079
TF=10 %
MFI=2248
CD81

B.


Relative NFAT/AP-1-luciferase activity
120
100
80
60
40
20
0
Unstimulated
Curdlan
*
*
Mock
dectin-1
dectin-1 & CD81
dectin-1 & CD81 P.M.

C.


Relative NFAT/AP-1-luciferase activity
800
700
600
500
400
300
200
100
0
Unstimulated
PMA/ionomycin
Mock
dectin-1
dectin-1 & CD81
dectin-1 & CD81 P.M.

D.

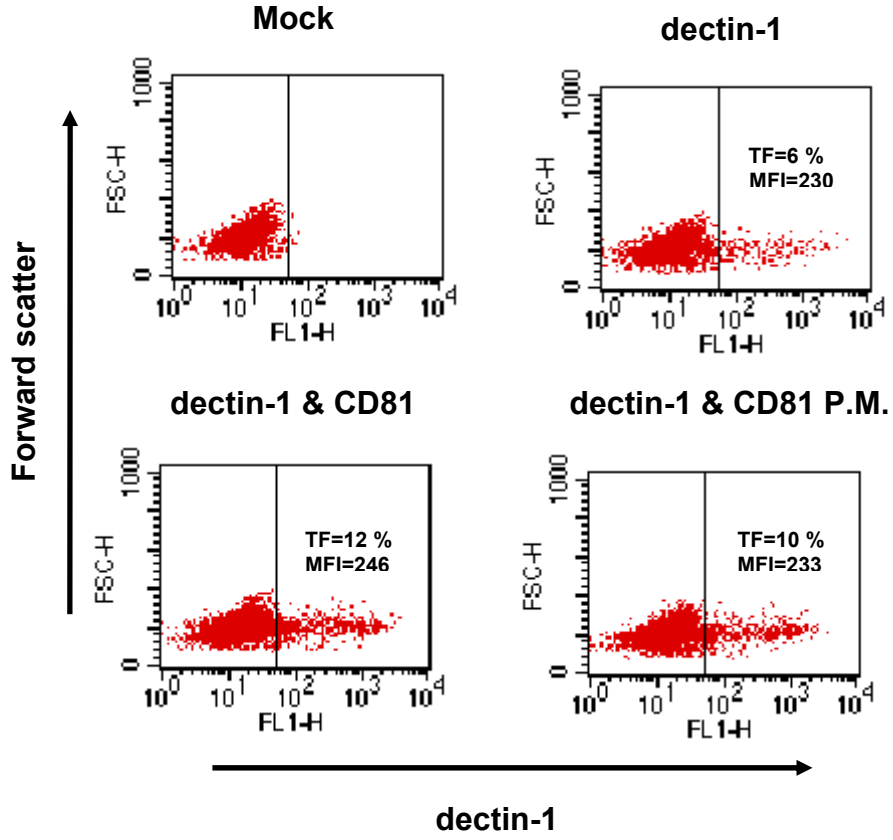

Mock
dectin-1
dectin-1 & CD81
dectin-1 & CD81 P.M.
TF=6 %
MFI=230
TF=12 %
MFI=246
TF=10 %
MFI=233
FSC-H
FL1-H
Forward scatter
dectin-1

Figure 3.8 Tetraspanin palmitoylation is not required for inhibition of dectin-1 signalling

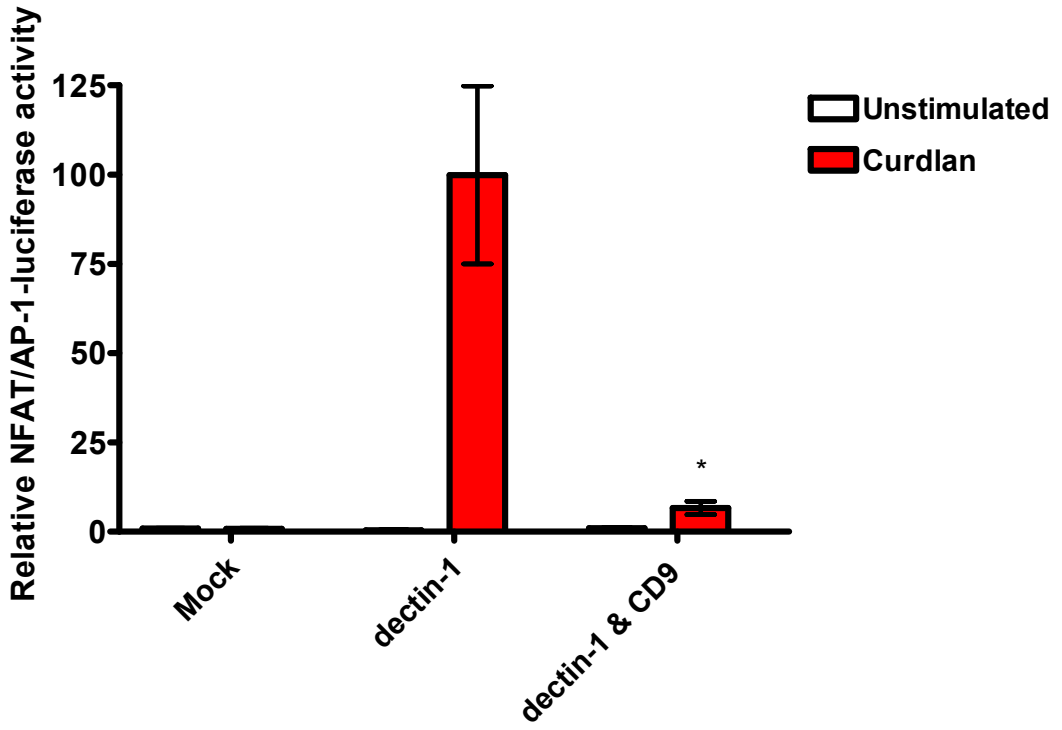
Jurkat T-cells were transiently transfected with an NFAT/AP-1-luciferase reporter construct, β-galactosidase construct, pEF6-MYC-dectin-1 (10 μg) and pEF6-CD81 (20 μg) or pEF6-CD81 palmitoylation mutant (P.M.) (20 μg) or empty pEF6 vector (mock). Cells were stimulated with media alone (unstimulated), curdlan (250 μg/ml) (B) and PMA/ionomycin (C) for six hours as indicated. Luciferase activity was measured following stimulation and transfection efficiency was corrected to β-galactosidase activity in individual experiments. The dectin-1 with curdlan stimulation value was set to 100% and other conditions presented were calculated relative to this. An unpaired Student's *t*-test indicated a statistically significant difference ($P<0.05$) between dectin-1 transfected alone compared to dectin-1 co-transfected with CD81 and CD81 P.M. as indicated *. Error bars represent the standard error of the mean. Experiments were performed three times. (A & D) Cells were stained with either MYC or CD81 primary antibody and then anti-mouse FITC-conjugated secondary antibody. Dead cells were gated out using propidium iodide. Antibody binding was visualized by flow cytometry. Dot plots were analysed and values indicate the average transfection efficiency (TF) and mean fluorescence intensity (MFI) of protein on the cell surface. Data is representative of three independent experiments.

3.3.9 Dectin-1 signalling is not inhibited by stable over-expression of the tetraspanin CD9 in Jurkat

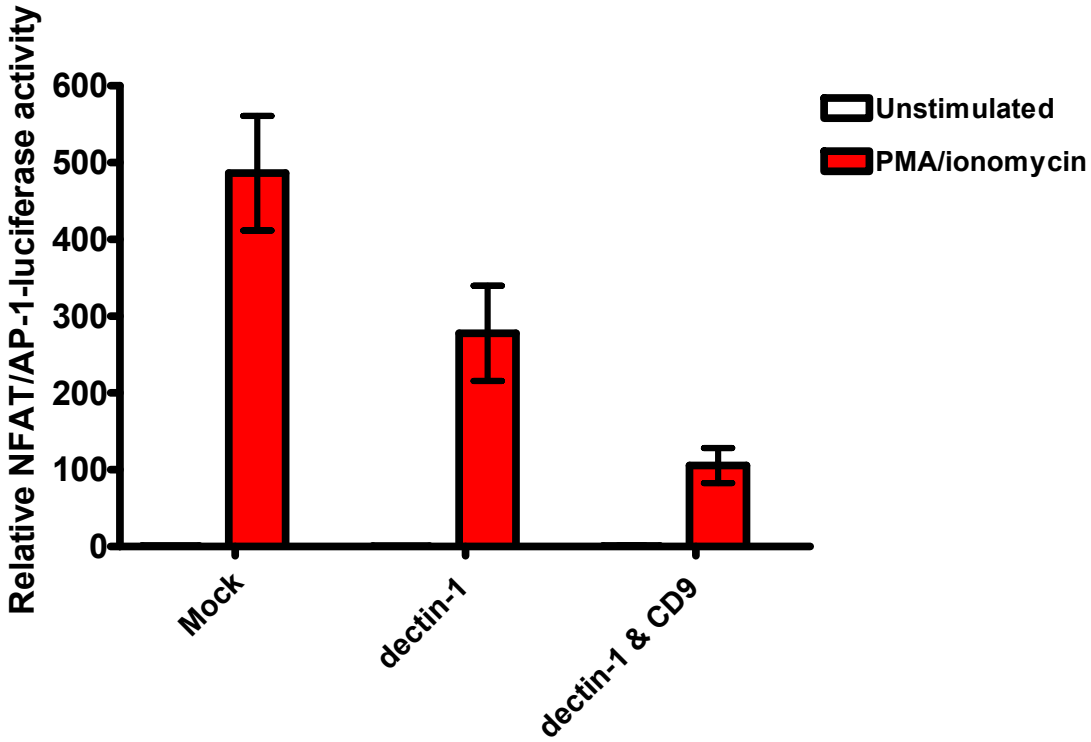
All experiments performed so far have used transient transfectants. It is possible that any tetraspanin that is transiently over-expressed might become the major component of tetraspanin microdomains on the cell, so disrupting microdomain function by displacing endogenous components. If this is true, one might predict that stable over-expression of a tetraspanin would have less of an inhibitory effect because the cell might adapt to the presence of the stably over-expressed tetraspanin. However, this was not determined in this study, and full analysis of adaptation, possibly through upregulation of other tetraspanins, could be assessed when antibodies are available for all tetraspanins. In order to investigate whether a stably over-expressed tetraspanin could inhibit dectin-1-mediated signalling, CD9 was selected because of the availability of stably CD9-transfected Jurkat (generated by M.B. Protty). Firstly, to determine whether transiently over-expressed CD9, like other tetraspanins could inhibit dectin-1 signalling, dectin-1 and CD9 were transiently transfected into Jurkat. CD9 potently inhibited dectin-1 signalling by over 90% following stimulation with 250 μg/ml curdlan (Figure 3.9A). All transfection conditions responded to control PMA and ionomycin stimulation, although a 4-fold decrease was observed upon dectin-1 and CD9 expression when compared to the mock-transfected cells, suggesting that the combination of dectin-1 and CD9 had a general inhibitory effect on this experiment (Figure 3.9B). However, even if data was expressed as a percentage of the PMA and ionomycin control, the data would still suggest that over-expression of CD9 inhibits dectin-1 signalling. In addition, flow cytometry studies indicated expression of both dectin-1 and CD9 (Figure 3.9C-D).

Dectin-1 signalling was next measured in stably CD9-expressing Jurkat cells. Stable CD9 over-expression in two transfected cell lines (AE9 & AE12) was first confirmed by flow cytometry, along with a human platelet control (Figure 3.9E); the latter is expressed at 49,000 copies per platelet (Higashihara et al., 1990; Protty et al., 2009). The CD9 expression levels were comparable to those in platelets and to those in the transient transfection experiment (Figure 3.9D & E). Dectin-1 was transiently transfected into Jurkat AE9 and AE12 and stimulated with 250 μg/ml curdlan. In contrast to the transient experiments, where dectin-1 signalling was inhibited by over 90% (Figure 3.9A), dectin-1 signalling was only inhibited by about 40 to 50% in the two stable CD9 transfectants (Figure 3.9F). Moreover, ANOVA of the data suggested there was no significant difference between groups. As a control, all transfection conditions responded to PMA and ionomycin stimulation (Figure 3.9G). The PMA and ionomycin response for both mock transfected clones was a little higher than that of other transfectants. However, this does not detract from the conclusion of the experiment, as the PMA and ionomycin response for parental Jurkat transfected with dectin-1 compared to the stable CD9 clones following dectin-1 transfection was similar. Dectin-1 cell surface expression was not affected by the presence of CD9 (Figure 3.9H). In summary, these data suggest that dectin-1 signalling is almost completely inhibited by transient tetraspanin over-expression, but is not significantly inhibited by stable over-expression.

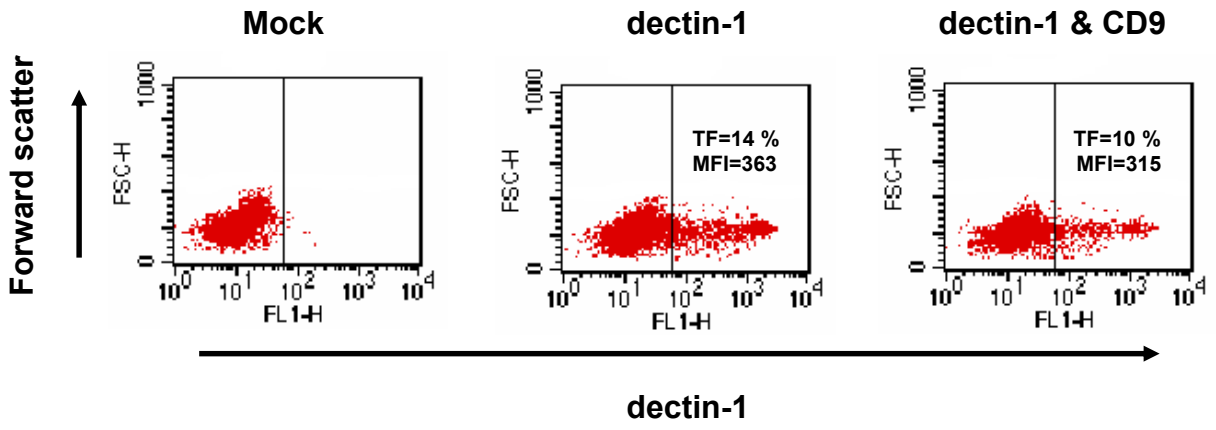
A.


Relative NFAT/AP-1-luciferase activity
125
100
75
50
25
0
*
Unstimulated
Curdlan
Mock
dectin-1
dectin-1 & CD9

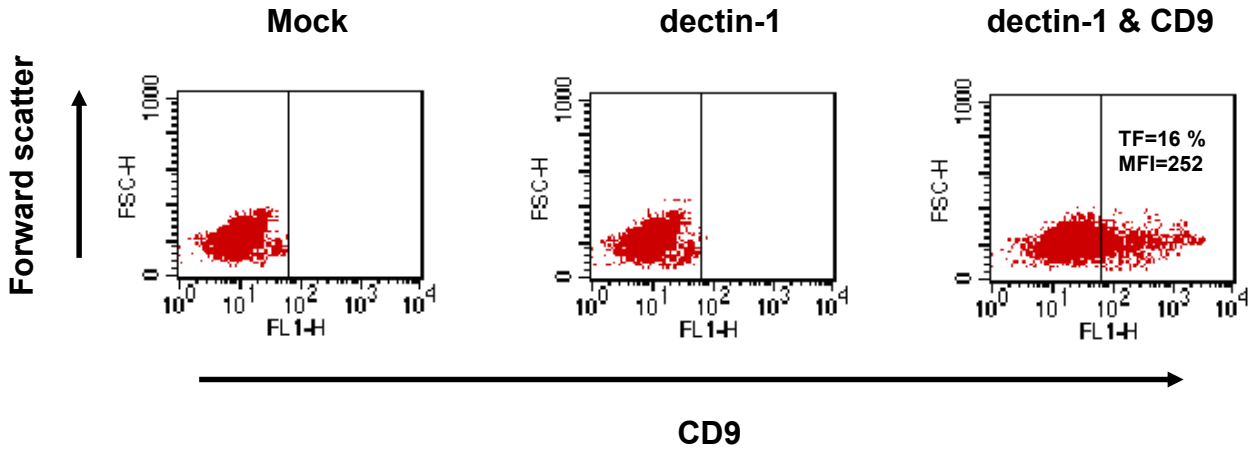
B.


Relative NFAT/AP-1-luciferase activity
600
500
400
300
200
100
0
Unstimulated
PMA/ionomycin
Mock
dectin-1
dectin-1 & CD9

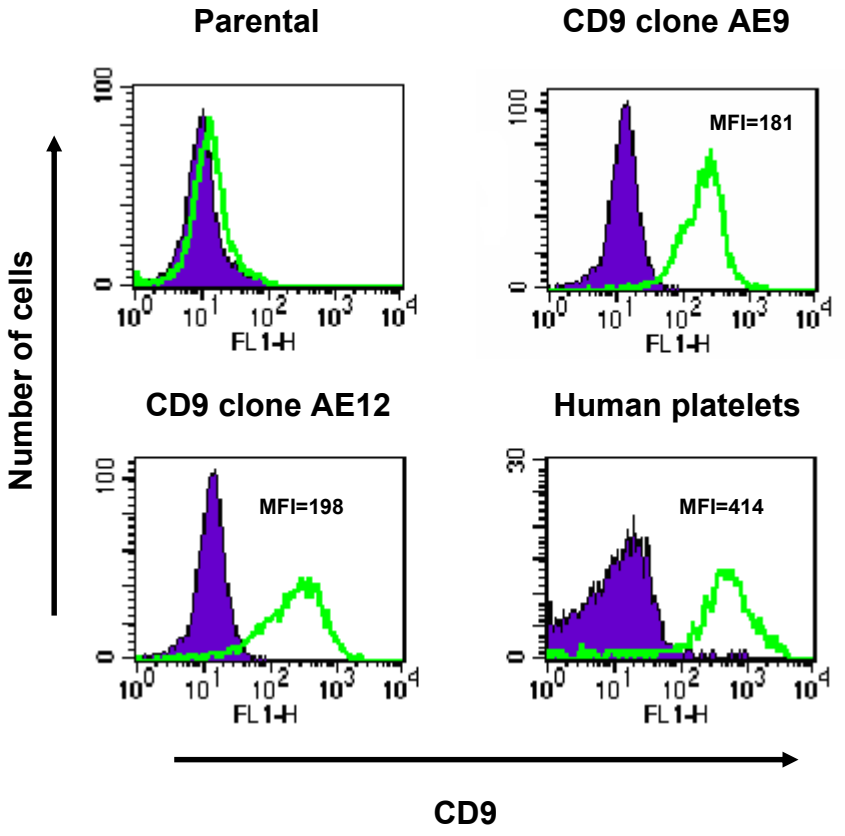
C.


Mock
dectin-1
dectin-1 & CD9
Forward scatter
FSC-H
FL1-H
TF=14 %
MFI=363
TF=10 %
MFI=315
dectin-1

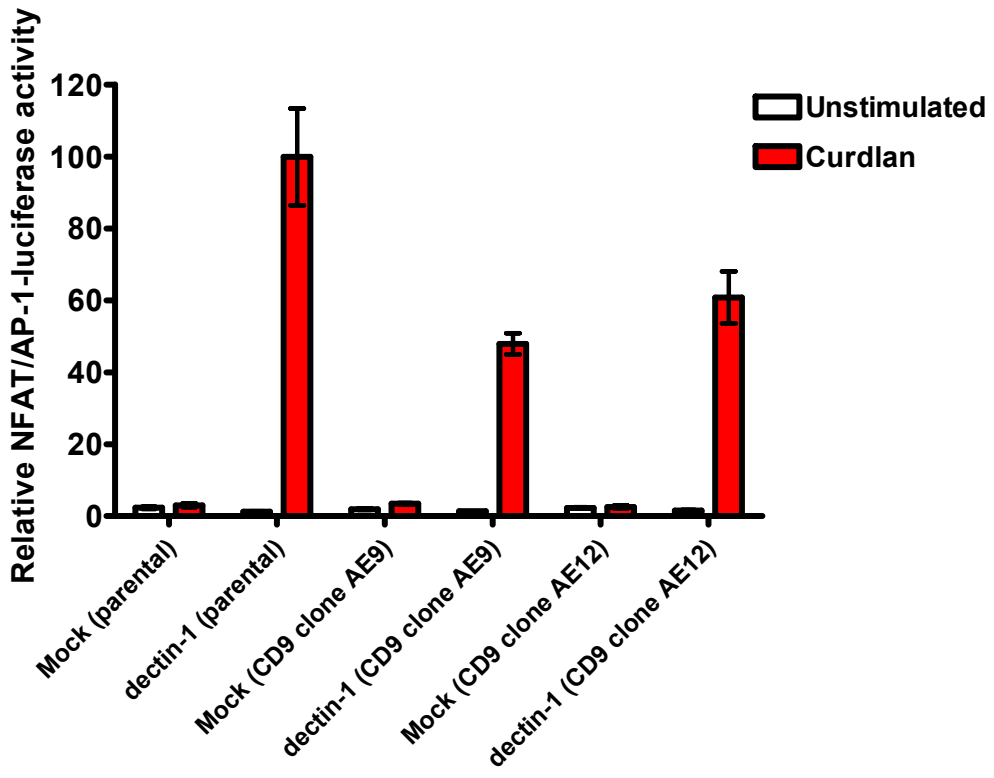
D.


Mock
dectin-1
dectin-1 & CD9
Forward scatter
FSC-H
FL1-H
TF=16 %
MFI=252
CD9

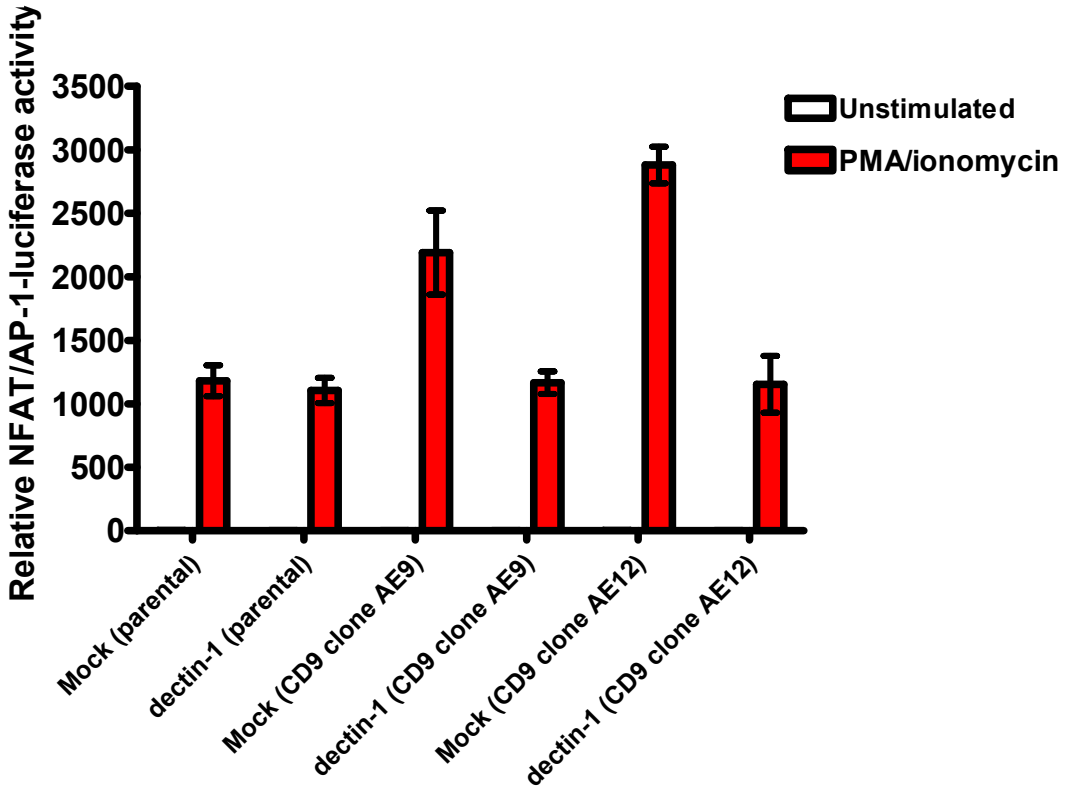
E.


Parental
CD9 clone AE9
CD9 clone AE12
Human platelets
MFI=181
MFI=198
MFI=414
FL1-H
Number of cells
CD9

F.


Relative NFAT/AP-1-luciferase activity
120
100
80
60
40
20
0
Unstimulated
Curdlan
Mock (parental)
dectin-1 (parental)
Mock (CD9 clone AE9)
dectin-1 (CD9 clone AE9)
Mock (CD9 clone AE12)
dectin-1 (CD9 clone AE12)

G.


Relative NFAT/AP-1-luciferase activity
3500
3000
2500
2000
1500
1000
500
0
Unstimulated
PMA/ionomycin
Mock (parental)
dectin-1 (parental)
Mock (CD9 clone AE9)
dectin-1 (CD9 clone AE9)
Mock (CD9 clone AE12)
dectin-1 (CD9 clone AE12)

H.

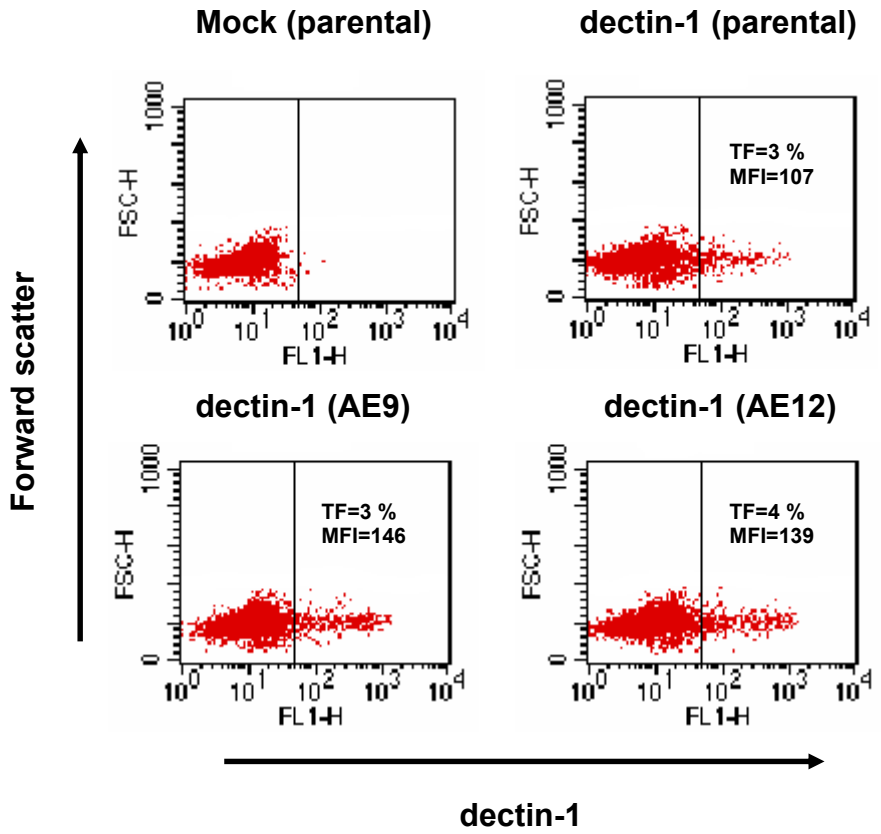

Mock (parental)
dectin-1 (parental)
dectin-1 (AE9)
dectin-1 (AE12)
TF=3 %
MFI=107
TF=3 %
MFI=146
TF=4 %
MFI=139
FSC-H
FL1-H
1000
0
Forward scatter
dectin-1

Figure 3.9 Transiently, but not stably, over-expressed CD9 significantly inhibits dectin-1 signalling in Jurkat T-cells

Jurkat T-cells were transiently transfected with an NFAT/AP-1-luciferase reporter construct, β-galactosidase construct, pEF6-MYC-dectin-1 (10 μg) and pEF6-CD9 (20 μg) or empty pEF6 vector (mock). Cells were stimulated with media alone (unstimulated), curdlan (250 μg/ml) (A) and PMA/ionomycin (B) for six hours as indicated. Luciferase activity was measured following stimulation and transfection efficiency was corrected to β-galactosidase activity in individual experiments. The dectin-1 with curdlan stimulation value was set to 100% and other conditions presented were calculated relative to this. An unpaired Student's *t*-test indicated a statistically significant difference ($P<0.05$) between dectin-1 transfected alone compared to dectin-1 co-transfected with CD9 as indicated *. Error bars represent the standard error of the mean. Experiments were performed three times. (C & D) Cells were stained with either MYC or CD9 primary antibody and then anti-mouse FITC-conjugated secondary antibody. Dead cells were gated out using propidium iodide. Antibody binding was visualized by flow cytometry. Dot plots were analysed and values indicate the average transfection efficiency (TF) and mean fluorescence intensity (MFI) of protein on the cell surface. Data is representative of three independent experiments. (E) Jurkat T-cells, those stably-expressing CD9 (AE9 & AE12) and human platelets were stained with either mouse negative (closed histograms) or CD9 (open histograms) primary antibody and then anti-mouse FITC-conjugated secondary antibody. Dead cells were gated out using propidium iodide. Antibody binding was visualized by flow cytometry. Data is representative of three independent experiments. Jurkat T-cells stably over-expressing CD9 were transiently transfected with an NFAT/AP-1-luciferase reporter construct, β-galactosidase construct, pEF6-MYC-dectin-1 (10 μg) or empty pEF6 vector (mock). Cells were stimulated with media alone (unstimulated), curdlan (250 μg/ml) (F) and PMA/ionomycin (G) for six hours as indicated. Luciferase activity was measured following stimulation and transfection efficiency was corrected to β-galactosidase activity in individual experiments. The dectin-1 (parental) with curdlan stimulation value was set to 100% and other conditions presented were calculated relative to this. Analysis of variance (ANOVA) showed that there was no significant difference between groups. Experiments were performed three times. (H) Cells were stained with MYC primary antibody and then anti-mouse FITC-conjugated secondary antibody. Dead cells were gated out using propidium iodide. Antibody binding was visualized by flow cytometry. Dot plots were analysed and values indicate the average transfection efficiency (TF) and mean fluorescence intensity (MFI) of protein on the cell surface. Data is representative of three independent experiments.

3.4 DISCUSSION

In this Chapter we have shown that over-expression of CD37, and the other tetraspanins CD9, CD81, CD82 and Tspan31 potently inhibit dectin-1-induced NFAT/AP-1 by over 90% in the Jurkat T-cell line. The observation was not dependent upon the tag attached to the tetraspanin, which has been suggested to interfere with certain types of protein-protein interaction (Wahl, Zoller & Claas; 2nd European Mini-Symposium on Tetraspanins, Madrid, 2007). The data in this Chapter suggests that more than one tetraspanin has the ability to negatively regulate dectin-1 signalling in Jurkat, and in particular that dectin-1 regulation is not unique to CD37, as suggested previously (Meyer-Wentrup et al., 2007).

In order to determine specificity, over-expression of the L6 antigen, which shares the four-transmembrane topology of the tetraspanin superfamily, did not inhibit dectin-1 signalling, despite its potential for recruitment into tetraspanin-enriched microdomains (Lekishvili et al., 2008). This suggests that the effects observed may be specific to the tetraspanins, and that over-expression of any four-transmembrane protein will not inhibit dectin-1 signalling. Moreover, it was imperative to determine if tetraspanin over-expression would inhibit the signalling of any receptor that signals via a tyrosine kinase-linked mechanism in a similar manner to dectin-1. Therefore, the major platelet collagen receptor, GPVI, that signals in conjunction with the FcRγ chain via an ITAM was utilised. In contrast to dectin-1, over-expression of CD37, CD81, CD82 and Tspan31 did not significantly inhibit the signalling of GPVI.

In addition, the role of palmitoylation in dectin-1 signal inhibition by tetraspanin over-expression was investigated by utilising a CD81 palmitoylation mutant. It is thought that palmitoylation is important for protein stability, localization, trafficking

and protein-protein interactions (Berditchevski et al., 2002). However, data in this Chapter suggests that palmitoylation is not required for the inhibitory effect.

The present results show that tetraspanin over-expression inhibits dectin-1 signalling in the Jurkat T-cell line. All initial experiments were transient in nature, and so Jurkat T-cells stably over-expressing CD9 were generated. In line with previous data, transient over-expression of CD9 inhibited dectin-1 signalling by over 90%. However, those Jurkat stably over-expressing CD9 did not exhibit significantly reduced dectin-1 signalling, although interpretation of this data is complicated by the partial (40-50%) inhibition that was observed. One possibility is that transient over-expression of tetraspanins may disrupt the normal tetraspanin-enriched microdomain organisation, possibly by displacing endogenous proteins normally present within the microdomains, which may be essential for dectin-1-mediated signalling. The stable transfectants may have partially adapted to the presence of CD9, perhaps by modulating the expression of other tetraspanins or associated proteins, hence the partial reduction in dectin-1 signalling.

The PMA and ionomycin stimulation used in this Chapter, and elsewhere in this thesis, is an important control that complements the transfection control that is included in each experiment, namely the transfection of a constitutively-expressed β-galactosidase construct followed by a β-galactosidase assay, to which all data are normalised. The primary aim of PMA and ionomycin is to induce a relatively strong MAPK activation and calcium influx, respectively, that bypasses receptor-proximal events. This therefore determines whether the cells are still responsive to stimulation and have not become signalling incompetent following transfection. This is particulary important as tetraspanin over-expression has been shown to almost

completely inhibit dectin-1 signalling in this Chapter. Interestingly, although a number of studies that have used NFAT/AP-1 luciferase reporter assays have presented their data in an identical manner to this thesis (Fuller et al., 2007; Tomlinson et al., 2004), most others have not. For example, many studies have not utilised the β-galactosidase control, instead presenting their data as a percentage of the PMA and ionomycin response (Seet et al., 2007; Tomlinson et al., 2007; Yablonski et al., 2001). Other studies have used β-galactosidase to normalise for transfection efficiency but have not shown PMA and ionomycin responses (Shan et al., 2000; Williams et al., 1997). The best way to normalise such data is thus debatable and different investigators clearly have their own preferences. However, if the product of transfection were to affect β-galactosidase expression or activity, this method of normalisation would be misleading. Similarly, if the transfection product affected signalling downstream of PMA and ionomycin, this would not be an ideal normalisation method. For these reasons, both β-galactosidase normalisation and PMA and ionomycin were presented in this thesis.

There is clearly some variability in the degree of PMA and ionomycin response between transfections in this and subsequent Chapters. In this Chapter, between two- and five-fold variation in PMA and ionomycin responses were observed in four of the eight figures that contained luciferase data (3.4B, 3.6B, 3.7B and 3.9B & G). The reasons for these differences are not clear, but could be due to an effect of the transfection product on PMA and ionomycin signalling. For example, if over-expression of the functionally uncharacterised tetraspanin Tspan31 could inhibit PMA and/or ionomycin responses, this could explain the 60% reductions observed in Figures 3.4B and 3.6B. Importantly, however, even if all of the data were expressed as ‘percentage of the PMA and ionomycin response,’ all conclusions drawn would

have been entirely unchanged. Similar unexplained variations in PMA and ionomycin responses have been observed previously (Tomlinson et al., 2004). For example, constutively active Tec kinase constructs resulted in a dose dependent reduction in PMA and ionomycin signalling, perhaps due to the prior activation of negative feedback responses (Tomlinson et al., 2004). Mutant Tec constructs also caused up to a 60% reduction in PMA and ionomycin signalling, although in this case it did not correlate with constitutive signalling (Tomlinson et al., 2004). Finally, a fifteen-fold variation in PMA and ionomycin signalling was observed between wild-type and five different DT40 mutant cell lines, each mutation of which was upstream of PMA and ionomycin signalling (Tomlinson et al., 2004). In conclusion, PMA and ionomycin signalling, though relatively well understood, is likely to be affected by positive and negative feedback pathways that we do not understand. It is therefore important not to rely on PMA and ionomycin for normalisation of NFAT/AP-1 reporter assay data, but to include additional controls such as the β-galactosidase assay used throughout this thesis.

3.5 SUMMARY

In this Chapter it has been shown that transient over-expression of CD37 and other tetraspanins potently inhibit dectin-1-mediated signalling in an NFAT/AP-1-luciferase reporter assay in Jurkat. This model system will be useful for future mechanistic studies and to test for an inhibitory effect of tetraspanins on other family members such as CLEC-2.

CHAPTER 4 –

INVESTIGATION OF THE MECHANISM BY WHICH TETRASPANINS INHIBIT DECTIN-1 SIGNALLING

4.1 INTRODUCTION

4.1.1 The mechanism of dectin-1 signal regulation by tetraspanins

The tetraspanin CD37 has been shown to interact with and to negatively regulate dectin-1 signalling by an as yet unknown mechanism (Meyer-Wentrup et al., 2007). The authors hypothesise that CD37 may associate with a molecule that may dephosphorylate or sequester a key molecule involved in dectin-1 signalling. Indeed, the tetraspanin CD53 has been shown to associate with an unknown phosphatase (Carmo and Wright, 1995). As discussed in Chapter Three, the van Spriel group focus on primary cells, and as they are not readily transfected and thus not amenable to genetic manipulation, the mechanism of dectin-1 signal regulation by CD37 is not explored. In addition to CD37, the tetraspanin CD63 has been shown to co-immunoprecipiate with dectin-1 in dendritic cells, using the relatively mild detergent CHAPS, suggesting that dectin-1 may be regulated within tetraspanin microdomains (Mantegazza et al., 2004).

In Chapter Three, it has been shown that over-expression of multiple tetraspanins can inhibit dectin-1 signalling in the Jurkat T-cell line using an NFAT/AP-1-luciferase reporter assay. Therefore, the aim of this Chapter was to explore the mechanism of dectin-1 signal inhibition by tetraspanin over-expression by utilising several methods. These include using different leukocyte cell lines to determine whether the effects observed in Jurkat are specific to this cell type, RNAi-knockdown of tetraspanins and co-immunoprecipitation studies to determine whether tetraspanins interact with dectin-1.

4.2 AIMS

- To determine whether tetraspanins inhibit the capacity of dectin-1 to activate the AP-1 promoter.
- To determine whether tetraspanins inhibit dectin-1 signalling in other leukocyte cell lines.
- To determine the effect of Syk/Zap-70 family tyrosine kinases upon dectin-1 signalling by tetraspanins.
- To investigate the effect of tetraspanin knockdown on dectin-1 signalling.
- To determine whether tetraspanins co-immunoprecipitate with dectin-1.

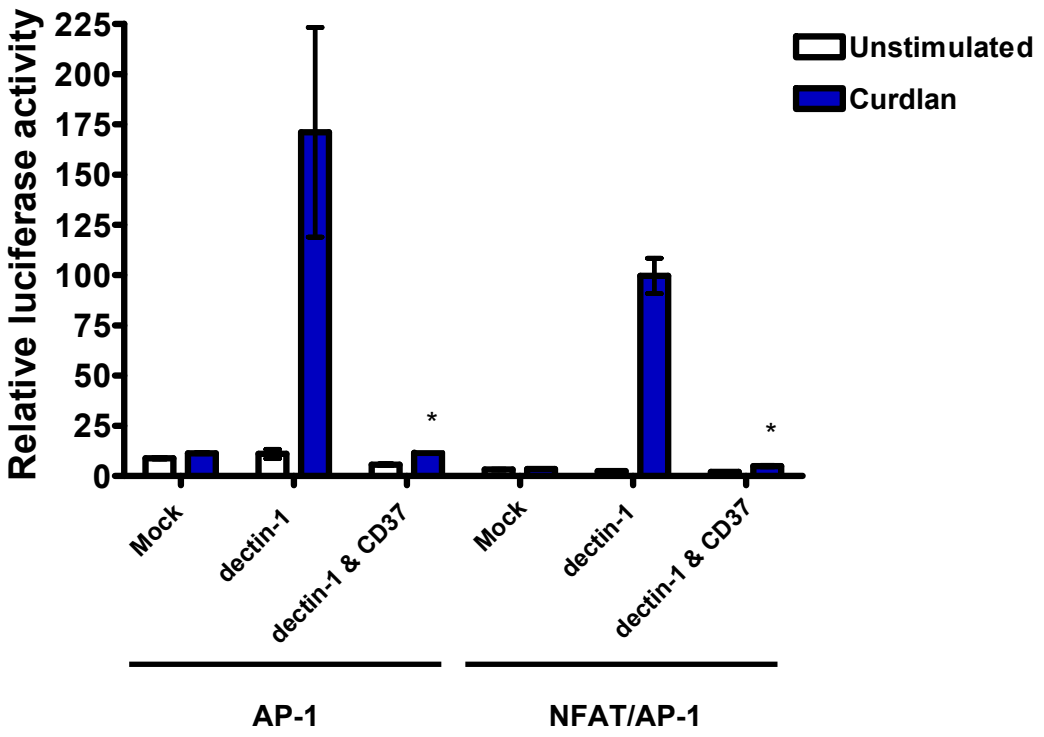
4.3 RESULTS

4.3.1 CD37 inhibits AP-1 activation by dectin-1 in Jurkat

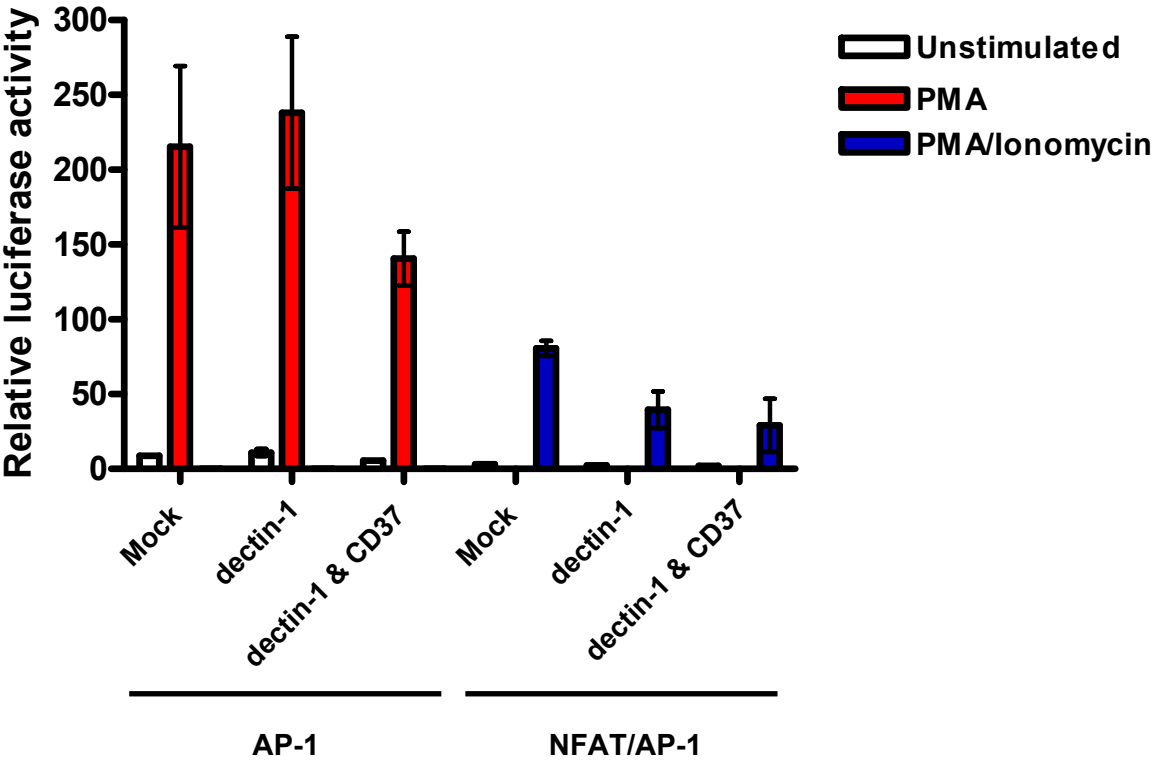
The data in Chapter Three showed that tetraspanins inhibit dectin-1-induced NFAT/AP-1 activation in Jurkat, which requires both calcium and MAPK signalling respectively. Therefore, there are three distinct mechanisms by which tetraspanins might inhibit the NFAT/AP-1 response: firstly by inhibiting dectin-1 from sending any signal; secondly by specifically inhibiting the calcium arm of the pathway; or thirdly by specifically inhibiting the MAPK pathway. To begin to address this issue, a MAPK-responsive reporter construct was used in which luciferase is driven by the AP-1 promoter only.

Jurkat were transfected with NFAT/AP-1 or AP-1 luciferase reporters, dectin-1 and/or CD37 and stimulated with 250 μg/ml curdlan. Dectin-1 signalling was inhibited by over 90% when co-transfected with CD37 when either NFAT/AP-1 or AP-1 elements were used (Figure 4.1A). However, the degree of NFAT/AP-1 activation was relatively lower compared to AP-1. Consistent with this, NFAT/AP-1 activation following PMA and ionomycin stimulation was lower than that of AP-1 (Figure 4.1B). Cell surface expression of dectin-1 was determined by flow cytometry and was similar between transfectants (Figure 4.1C). This data suggests that tetraspanins either specifically inhibit dectin-1-induced MAPK activation or inhibit dectin-1 signalling at a more receptor-proximal level.

A.


Relative luciferase activity
225
200
175
150
125
100
75
50
25
0
Unstimulated
Curdlan
*
*
Mock
dectin-1
dectin-1 & CD37
Mock
dectin-1
dectin-1 & CD37
AP-1
NFAT/AP-1

B.


Relative luciferase activity
300
250
200
150
100
50
0
Unstimulated
PMA
PMA/Ionomycin
Mock
dectin-1
dectin-1 & CD37
Mock
dectin-1
dectin-1 & CD37
AP-1
NFAT/AP-1

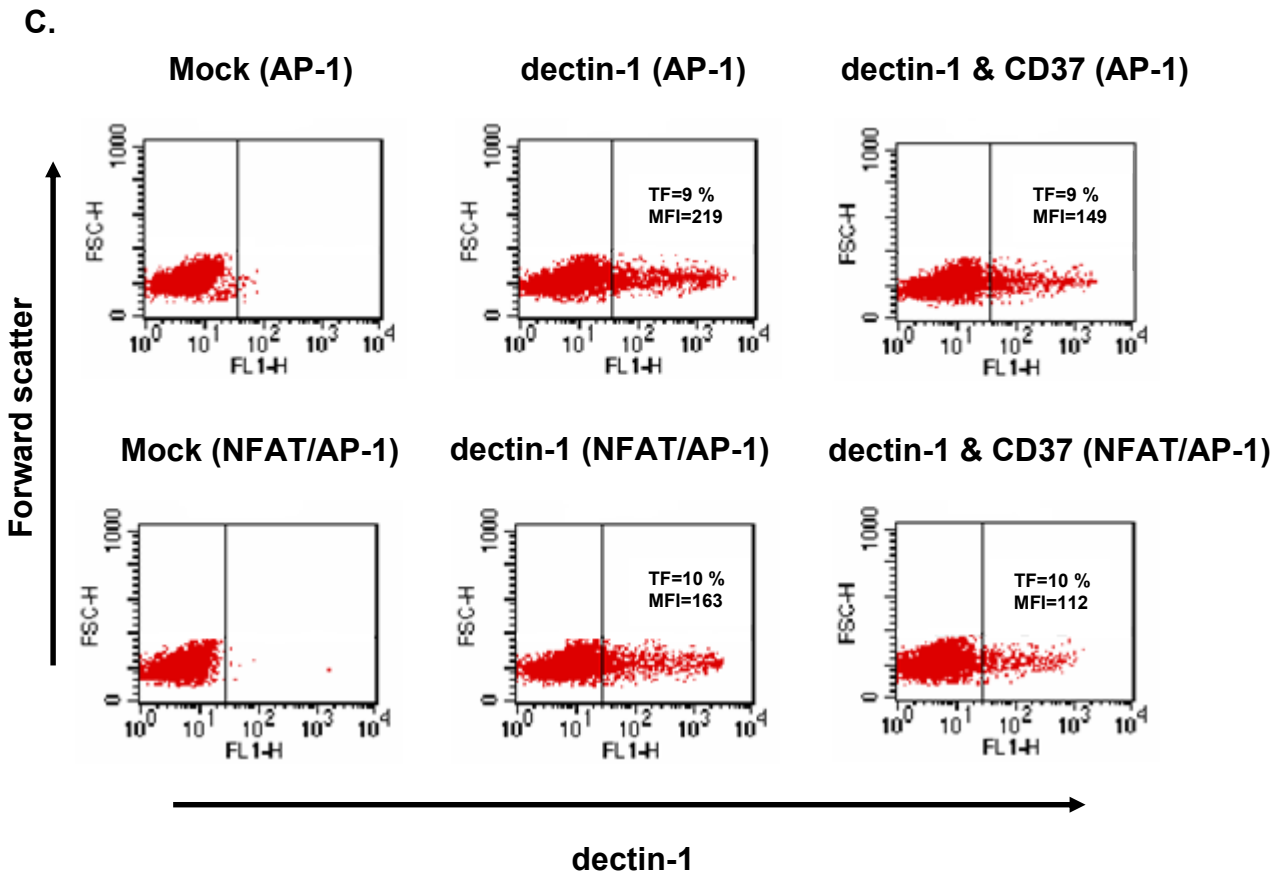


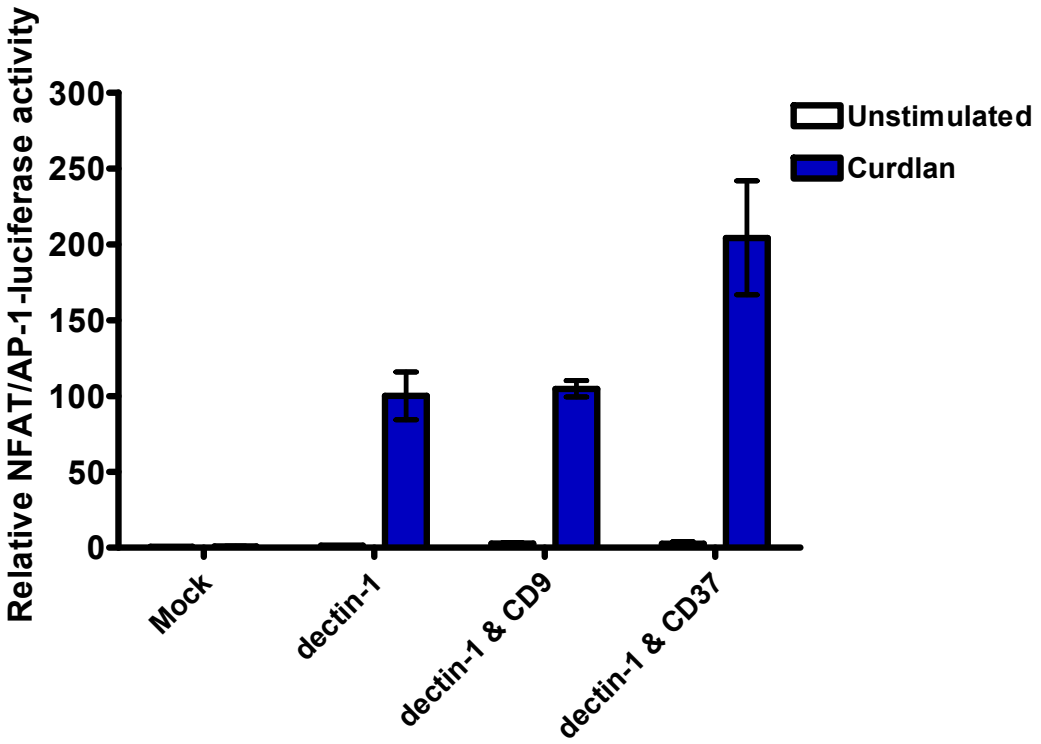
Figure 4.1 CD37 inhibits AP-1 and NFAT/AP-1 activation by dectin-1 in Jurkat T-cells

Jurkat T-cells were transiently transfected with an NFAT/AP-1- or AP-1-luciferase reporter construct, β-galactosidase construct, pEF6-MYC-dectin-1 (10 μg) and pEF6-CD37 (20 μg) or empty pEF6 vector (mock). Cells were stimulated with media alone (unstimulated), curdlan (250 μg/ml) (A) and PMA or PMA plus ionomcyin (B) for six hours as indicated. Luciferase activity was measured following stimulation and transfection efficiency was corrected to β-galactosidase activity in individual experiments. The dectin-1 (NFAT/AP-1) with curdlan stimulation value was set to 100% and other conditions presented were calculated relative to this. An unpaired Student's *t*-test indicated a statistically significant difference ($P<0.05$) between dectin-1 transfected alone compared to dectin-1 co-transfected with CD37 as indicated *. Error bars represent the standard error of the mean. Experiments were performed twice. (C) Cells were stained with MYC primary antibody and then anti-mouse FITC-conjugated secondary antibody. Dead cells were gated out using propidium iodide. Antibody binding was visualized by flow cytometry. Dot plots were analysed and values indicate the average transfection efficiency (TF) and mean fluorescence intensity (MFI) of protein on the cell surface. Data is representative of two independent experiments.

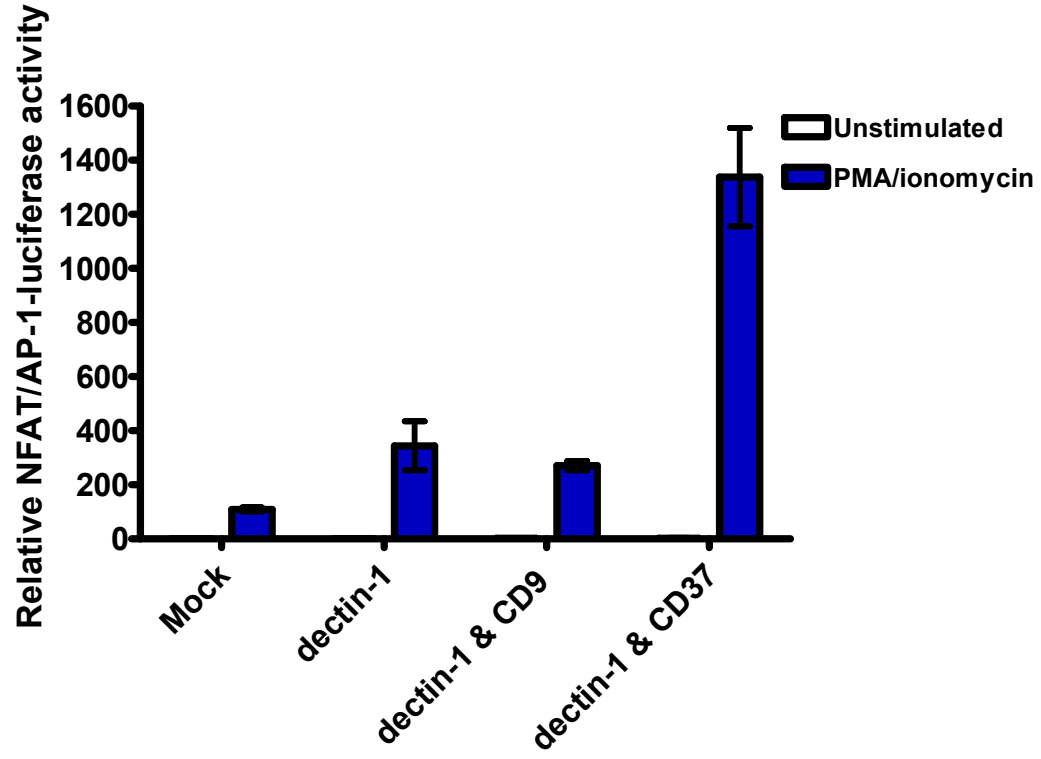
4.3.2 The tetraspanins CD9 and CD37 do not inhibit dectin-1 signalling in DT40 B- and RBL-2H3 cells

In order to determine whether the capacity of tetraspanins to inhibit dectin-1-induced NFAT/AP-1 activation is cell type dependent, chicken DT40 B- and rat RBL-2H3 mast-cell lines were utilised to investigate the effect of tetraspanin over-expression upon dectin-1 signalling. In contrast to the Jurkat data, dectin-1 signalling in DT40 cells was not inhibited in response to curdlan when co-transfected with CD9 and CD37 as determined by an NFAT/AP-1 reporter assay (Figure 4.2A). Antibodies were not available for mouse CD37, so human CD9 was selected for study, as antibodies are available to determine cell surface expression. All transfection conditions responded to the PMA and ionomycin control stimulation, although the combination of dectin-1 and CD37 transfection substantially increased NFAT/AP-1 activation (Figure 4.2B). Nevertheless, even if the data was expressed as a percentage of the PMA and ionomycin control, it would still suggest that CD37 over-expression does not inhibit dectin-1 signalling in DT40. Cell surface expression of both dectin-1 and CD9 was determined by flow cytometry and was similar between transfectants (Figures 4.2C & D). Similar data was gathered using the RBL-2H3 mast-cell line (Figure 4.3A-C), although in this experiment only CD37 was used and its expression was not confirmed because of the relatively low numbers of RBL-2H3 cells that were harvested following transfection. This demonstrates that the inhibition of dectin-1 signalling by tetraspanin over-expression is dependent on the cell type in which these proteins are expressed.

A.


Relative NFAT/AP-1-luciferase activity
300
250
200
150
100
50
0
Unstimulated
Curdlan
Mock
dectin-1
dectin-1 & CD9
dectin-1 & CD37

B.


Relative NFAT/AP-1-luciferase activity
1600
1400
1200
1000
800
600
400
200
0
Unstimulated
PMA/ionomycin
Mock
dectin-1
dectin-1 & CD9
dectin-1 & CD37

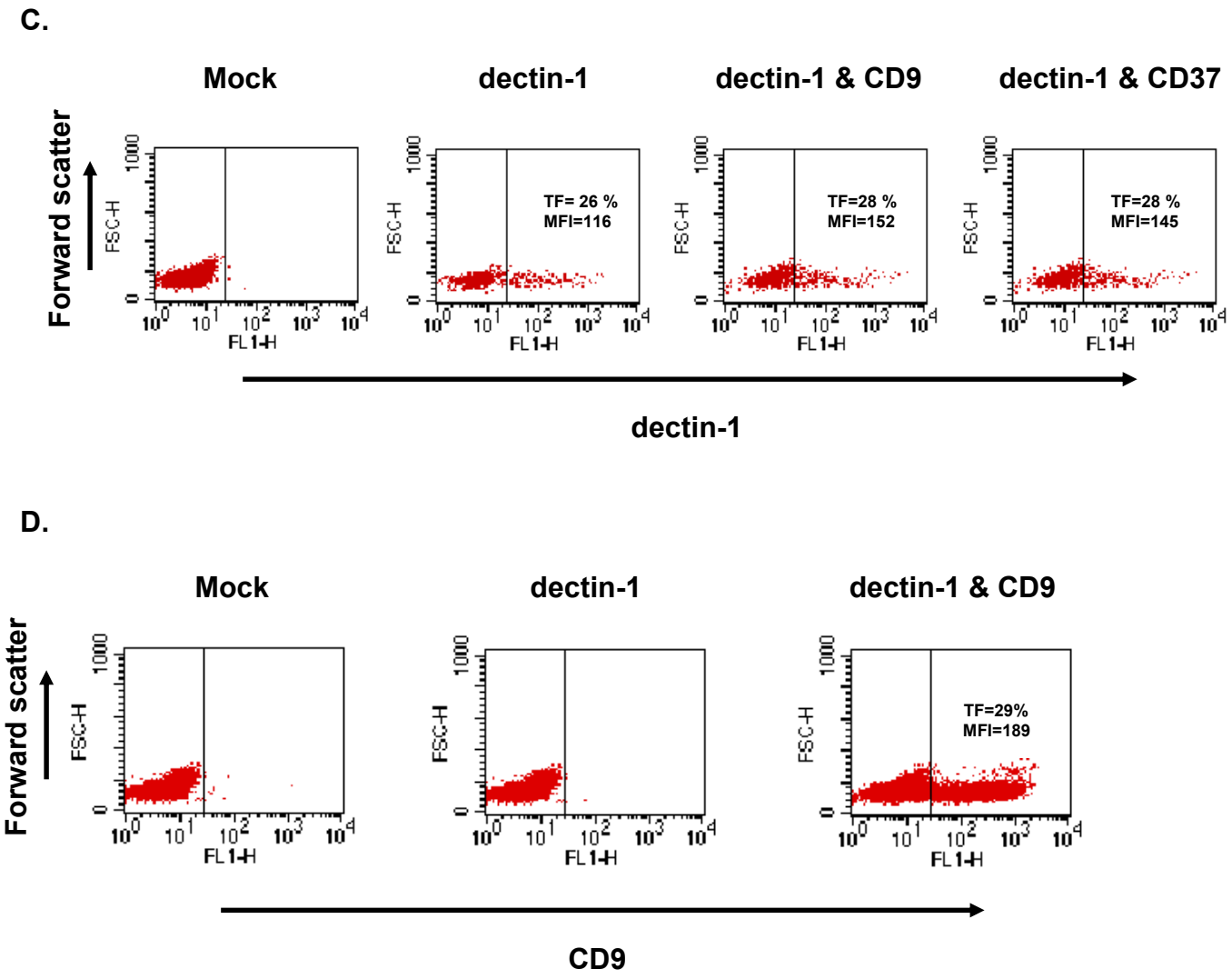
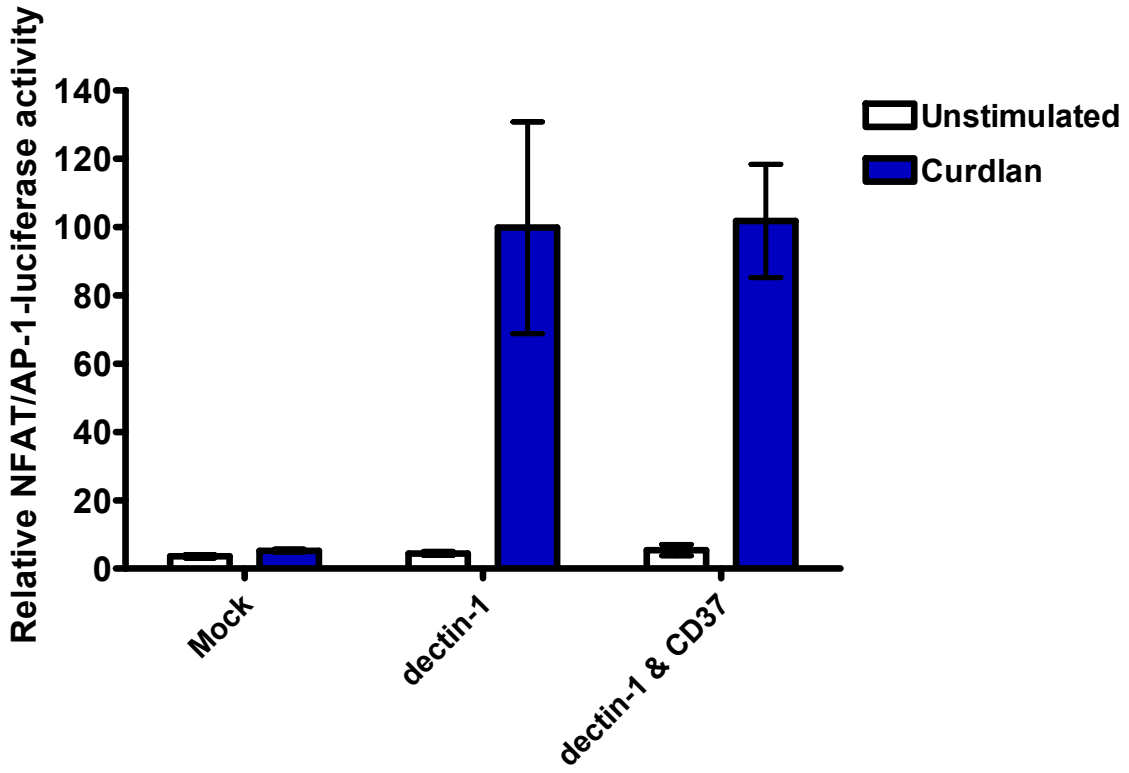


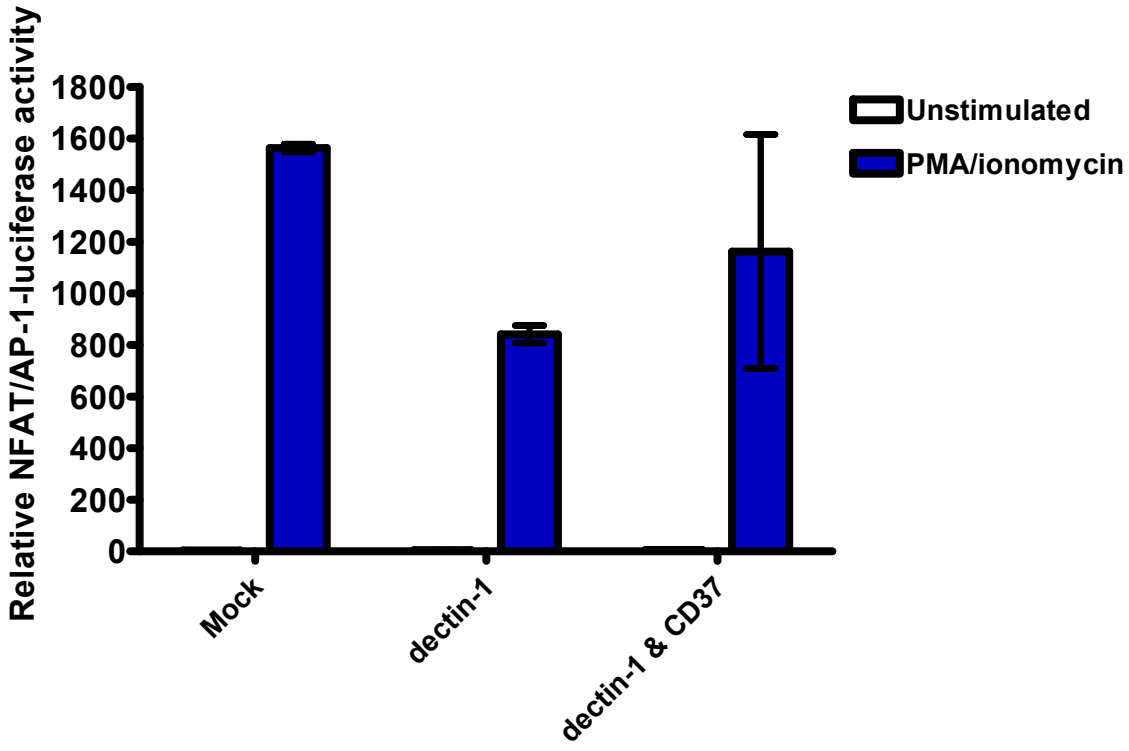
Figure 4.2 Dectin-1 signalling is not inhibited when co-transfected with CD9 and CD37 in DT40 B-cells

DT40 B-cells were transiently transfected with an NFAT/AP-1-luciferase reporter construct, β-galactosidase construct, pEF6-MYC-dectin-1 (5 µg), pEF6-CD9, CD37 (2.5 µg) or empty pEF6 vector (mock). Cells were stimulated with media alone (unstimulated), curdlan (100 µg/ml) (A) and PMA/ionomycin (B) for six hours as indicated. Luciferase activity was measured following stimulation and transfection efficiency was corrected to β-galactosidase activity in individual experiments. The dectin-1 with curdlan stimulation value was set to 100% and other conditions presented were calculated relative to this. Analysis of variance (ANOVA) of the data showed that there was no significant difference between dectin-1 and dectin-1 co-transfected with either CD9 or CD37. Error bars represent the standard error of the mean. Experiments were performed twice. (C & D) DT40 B-cells were stained with MYC or CD9 primary antibody and then anti-mouse-FITC conjugated secondary antibody. Dead cells were gated out using propidium iodide. Antibody binding was visualized by flow cytometry. Dot plots were analysed and values indicate the average transfection efficiency (TF) and mean fluorescence intensity (MFI) of protein on the cell surface. Data is representative of two independent experiments.

A.


Relative NFAT/AP-1-luciferase activity
140
120
100
80
60
40
20
0
Mock
dectin-1
dectin-1 & CD37
Unstimulated
Curdlan

B.


Relative NFAT/AP-1-luciferase activity
1800
1600
1400
1200
1000
800
600
400
200
0
Mock
dectin-1
dectin-1 & CD37
Unstimulated
PMA/ionomycin

C.

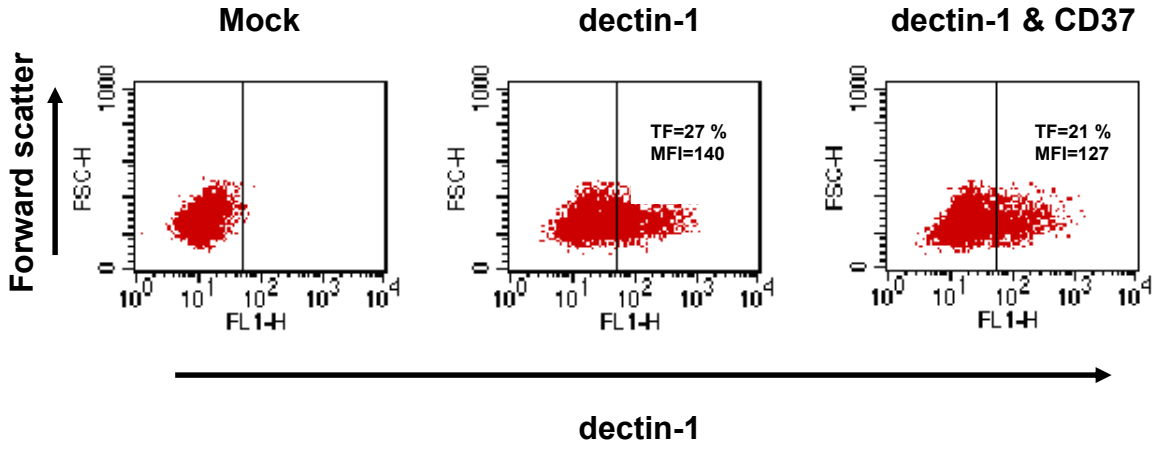


Figure 4.3 Dectin-1 signalling is not inhibited when co-transfected with CD37 in RBL-2H3 cells

RBL-2H3 cells were transiently transfected with an NFAT/AP-1-luciferase reporter construct, β-galactosidase construct, pEF6-MYC-dectin-1 (10 μg), pEF6-CD37 (20 μg) or empty pEF6 vector (mock). Cells were stimulated with media alone (unstimulated), curdlan (250 μg/ml) (A) and PMA/ionomycin (B) for six hours as indicated. Luciferase activity was measured following stimulation and transfection efficiency was corrected to β-galactosidase activity in individual experiments. The dectin-1 with curdlan stimulation value was set to 100% and other conditions presented were calculated relative to this. Analysis of variance (ANOVA) of the data suggests no difference between dectin-1 and dectin-1 co-transfected with CD37. Error bars represent the standard error of the mean. Experiments were performed three times. (C) Cells were stained with MYC primary antibody and then anti-mouse-FITC conjugated secondary antibody. Dead cells were gated out using propidium iodide. Antibody binding was visualized by flow cytometry. Dot plots were analysed and values indicate the average transfection efficiency (TF) and mean fluorescence intensity (MFI) of protein on the cell surface. CD37 flow cytometry was not performed to confirm expression in this experiment. Data is representative of two independent experiments.

4.3.3 CD37 does not inhibit AP-1 activation by dectin-1 in DT40

The failure of tetraspanins to inhibit dectin-1-induced NFAT/AP-1 activation in DT40 might be due to the subtle difference between these cells and Jurkat in their response to ionomycin. For a reason that is not clear, ionomycin alone can induce weak NFAT/AP-1 activation in DT40 but not in Jurkat (Figure 4.4A & B). Therefore, if tetraspanins specifically inhibit dectin-1-induced MAPK activation, leaving the calcium arm of the pathway intact, this could partially explain why NFAT/AP-1 is not inhibited in DT40, as these cells have less of a requirement for AP-1 activation. If true, one would predict that tetraspanins would inhibit dectin-1-induced AP-1 activation in DT40.

To address this hypothesis, DT40 were transfected with the AP-1 luciferase reporter, or NFAT/AP-1 as a control, plus dectin-1 and/or CD37 and stimulated with 100 μg/ml curdlan. As found previously for NFAT/AP-1, dectin-1-induced AP-1 activation was not inhibited by CD37 in DT40 cells (Figure 4.5A). In positive control stimulations, all transfection conditions responded in a similar manner (Figure 4.5B) and cell surface expression of dectin-1 was similar between transfectants (Figure 4.5C). These data show that dectin-1 signalling, as detected by either AP-1 or NFAT/AP-1 reporters, is not inhibited by tetraspanins in DT40. Moreover, the striking inhibitory effect of tetraspanins in Jurkat cannot be explained by a specific effect on the MAPK-driven AP-1 pathway.

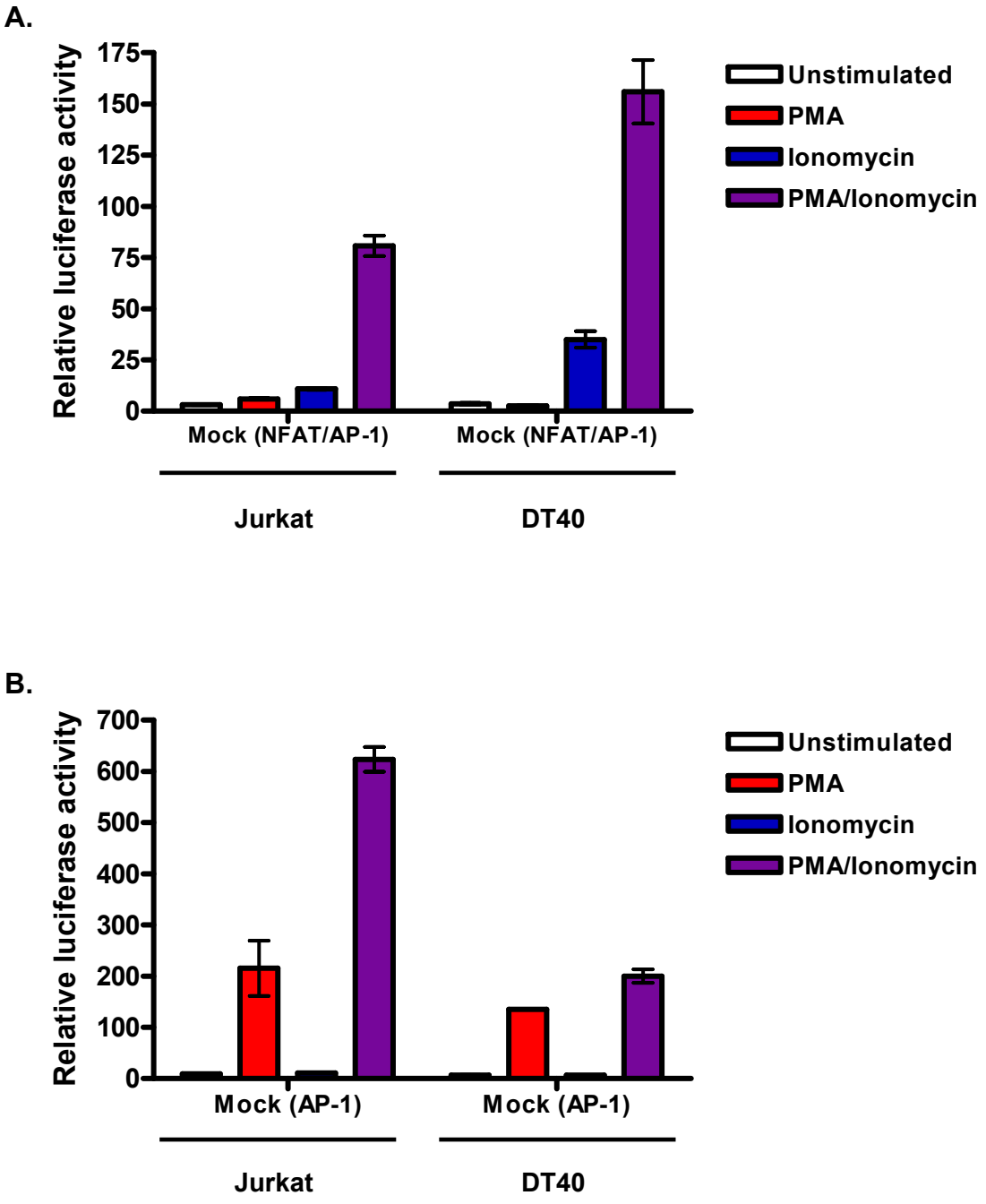
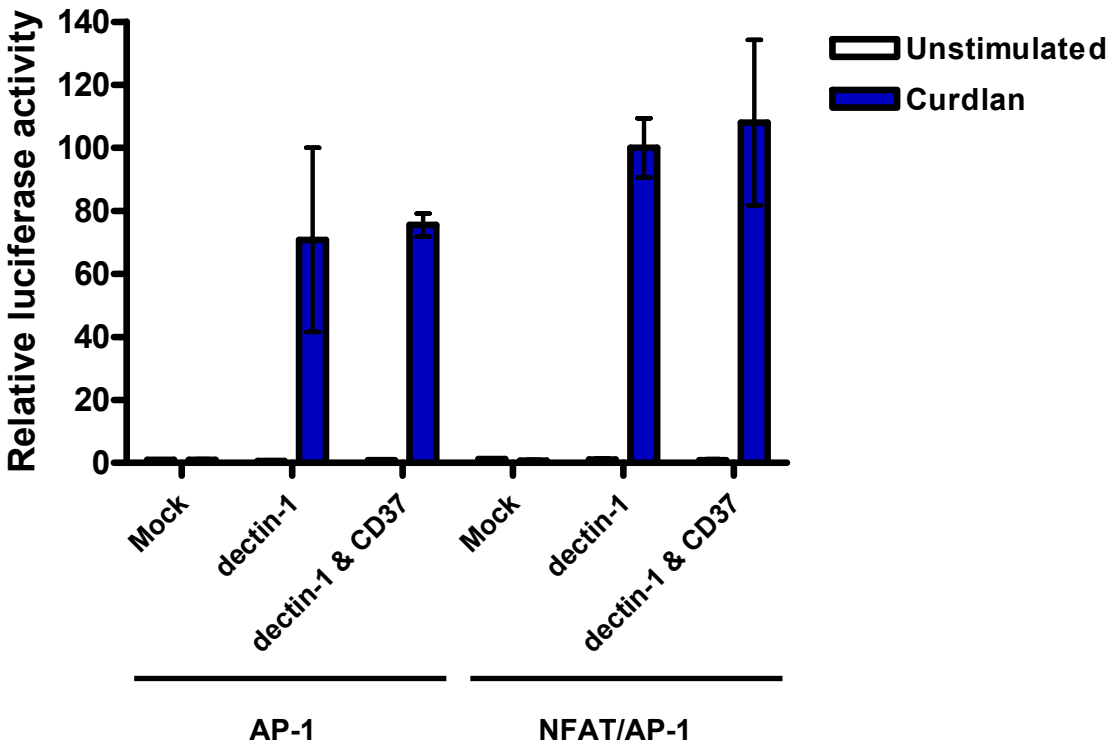


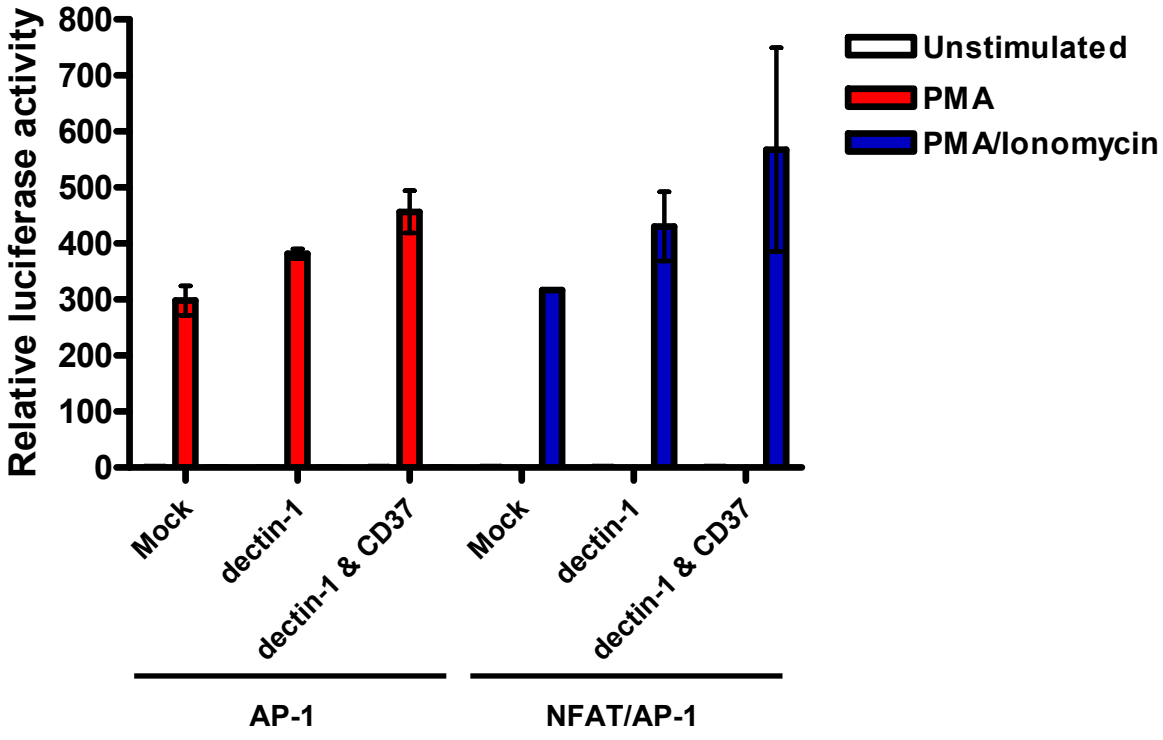
Figure 4.4 Ionomcyin alone can induce weak NFAT/AP-1 activation in DT40 but not in Jurkat

DT40 B-cells were transiently transfected with an NFAT/AP-1- (A) or AP-1-luciferase reporter construct (B) and the β-galactosidase construct. Cells were stimulated with media alone (unstimulated), PMA, ionomycin or PMA plus ionomycin for six hours as indicated. Luciferase activity was measured following stimulation and transfection efficiency was corrected to β-galactosidase activity in individual experiments. Error bars represent the standard error of the mean. Data is representative of three independent experiments.

A.


Relative luciferase activity
140
120
100
80
60
40
20
0
Unstimulated
Curdlan
Mock
dectin-1
dectin-1 & CD37
Mock
dectin-1
dectin-1 & CD37
AP-1
NFAT/AP-1

B.


Relative luciferase activity
800
700
600
500
400
300
200
100
0
Unstimulated
PMA
PMA/Ionomycin
Mock
dectin-1
dectin-1 & CD37
Mock
dectin-1
dectin-1 & CD37
AP-1
NFAT/AP-1

C.

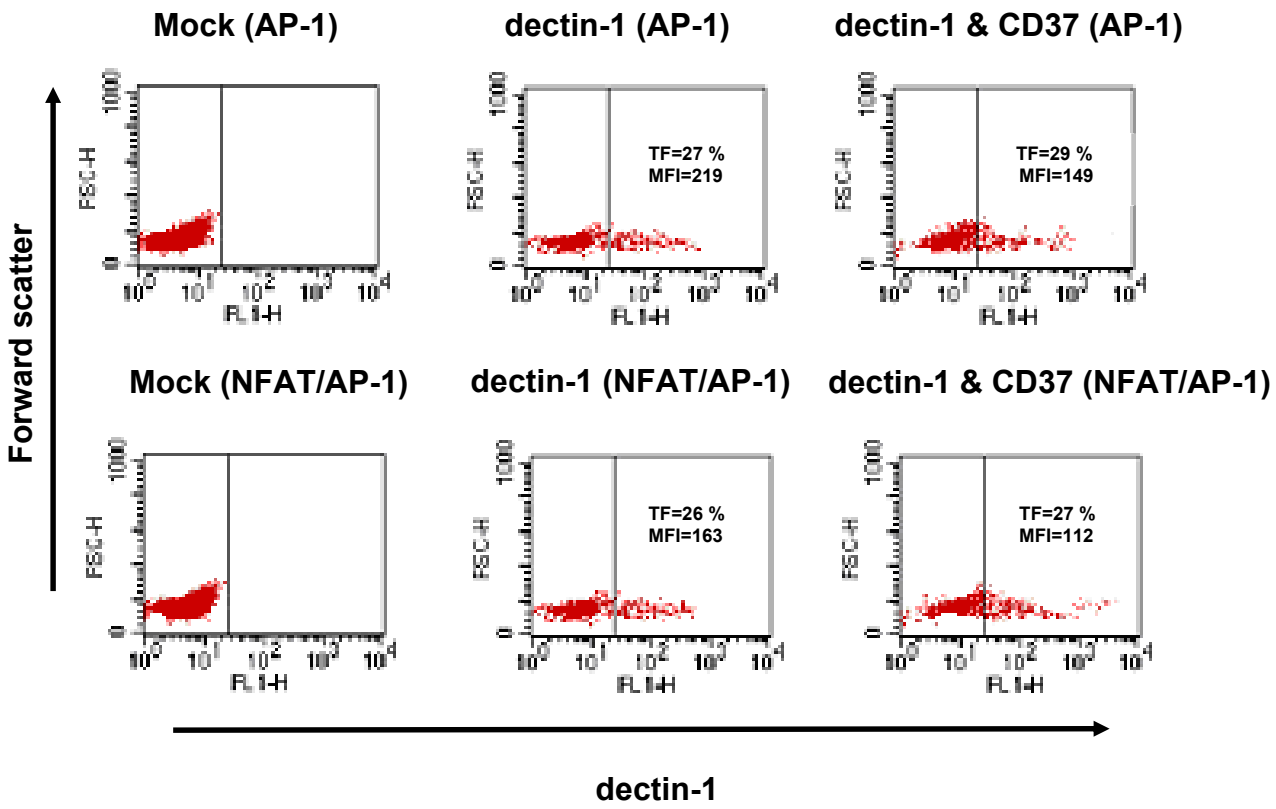


Figure 4.5 CD37 does not inhibit AP-1 activation by dectin-1 in DT40 B-cells

DT40 B-cells were transiently transfected with an NFAT/AP-1- or AP-1-luciferase reporter construct, β-galactosidase construct, pEF6-MYC-dectin-1 (5 µg) and pEF6-CD37 (2.5 µg) or empty pEF6 vector (mock). Cells were stimulated with media alone (unstimulated), curdlan (100 µg/ml) (A) and PMA or PMA plus ionomycin (B) for six hours as indicated. Luciferase activity was measured following stimulation and transfection efficiency was corrected to β-galactosidase activity in individual experiments. The dectin-1 with curdlan stimulation value was set to 100% and other conditions presented were calculated relative to this. Analysis of variance (ANOVA) of the data suggests no difference between dectin-1 and dectin-1 co-transfected with CD37. Error bars represent the standard error of the mean. Experiments were performed twice. (C) Cells were stained with MYC primary antibody and then anti-mouse FITC-conjugated secondary antibody. Dead cells were gated out using propidium iodide. Antibody binding was visualized by flow cytometry. Dot plots were analysed and values indicate the average transfection efficiency (TF) and mean fluorescence intensity (MFI) of protein on the cell surface. Data is representative of two independent experiments.

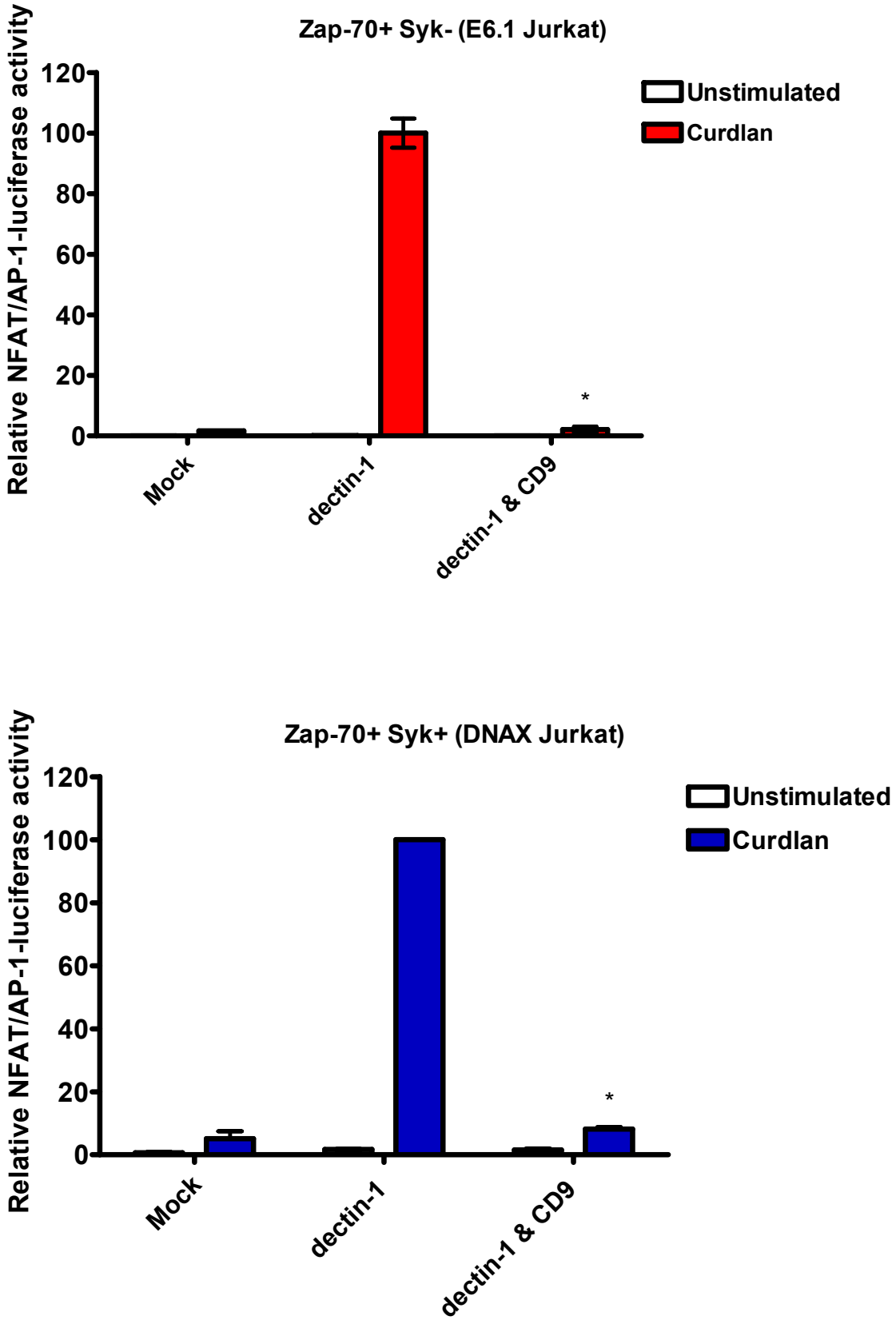
4.3.4 Tetraspanins inhibit dectin-1 signalling in Zap-70$^+$/Syk$^-$ and Zap-70$^+$/Syk$^+$ Jurkat cell lines

The previous sections in this Chapter suggest that tetraspanins may negatively regulate a component of the dectin-1 signalling pathway that is present in Jurkat but not in DT40 or RBL-2H3 cells. The list of possibilities includes the tyrosine kinases Lck, Itk and Zap-70, the adapter SLP-76 and the phospholipase PLCγ1. This is because for each of these proteins, a different family member is expressed in DT40 and RBL-2H3 cells, namely Lyn, Btk, Syk, BLNK and PLCγ2 (Figure 1.11). A particularly promising candidate is Zap-70, since dectin-1 is known to signal by interacting with and activating Syk/Zap-70 family tyrosine kinases. Could tetraspanin over-expression result in inhibition of Zap-70 but not Syk? Interestingly, there are two major Jurkat clones that are commonly used, the E6.1 clone, used in earlier sections, which expresses Zap-70 but not Syk, and the DNAX Jurkat clone that expresses both Zap-70 and Syk (Fargnoli et al., 1995). If tetraspanin over-expression does inhibit Zap-70 but not Syk, the latter would be expected to compensate Zap-70 and abrogate the inhibitory effect of tetraspanin over-expression, thus similar to the lack of inhibition observed previously in Syk-expressing DT40 and RBL-2H3 cells.

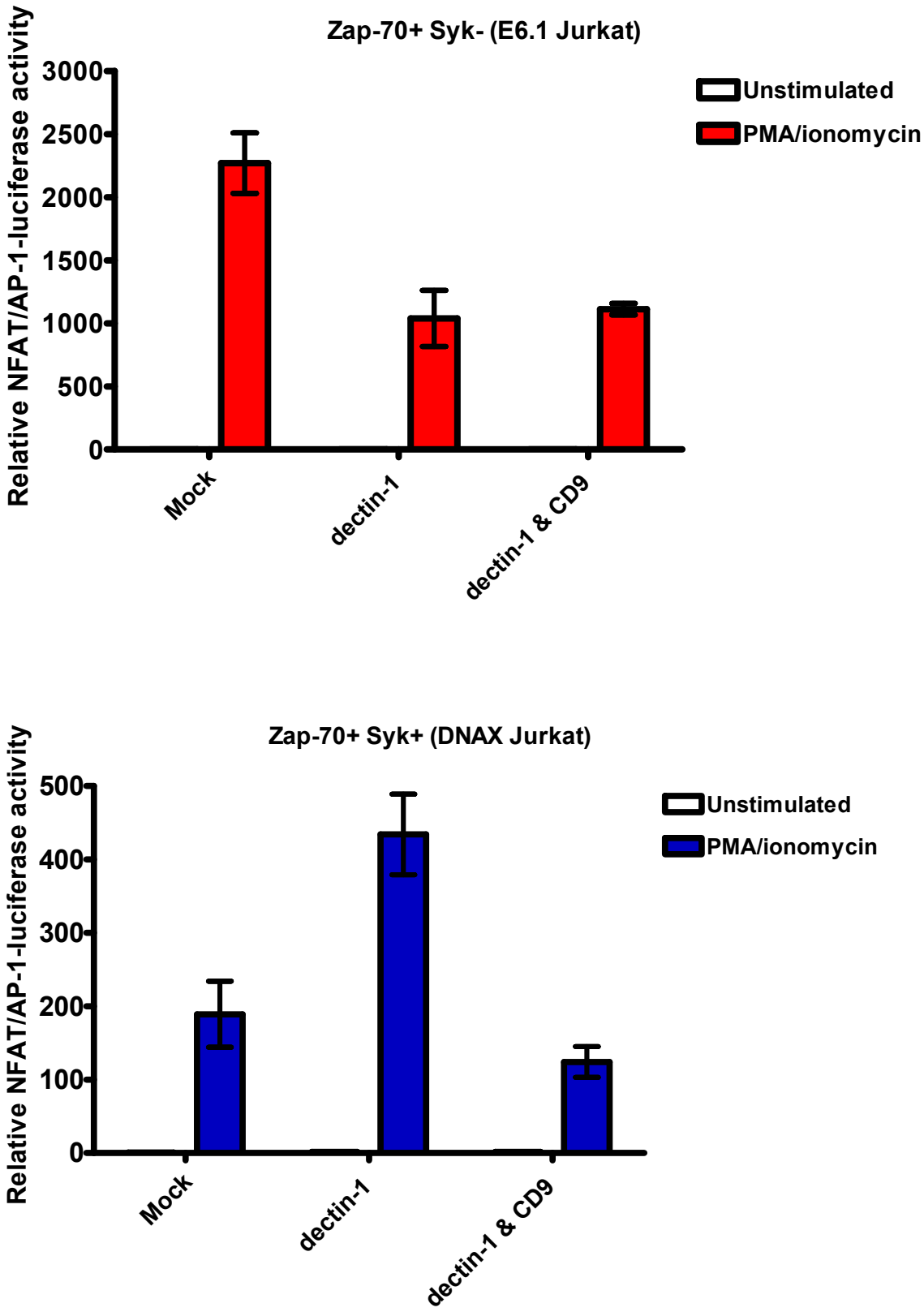
An NFAT/AP-1-luciferase reporter assay was therefore performed to compare the effect of CD9 on dectin-1-induced NFAT/AP-1 activation in Zap-70$^+$/Syk$^-$ (E6.1 Jurkat clone) versus Zap-70$^+$/Syk$^+$ (DNAX Jurkat clone). CD9 was selected due to the availability of antibodies to determine cell surface expression. In both Jurkat clones, dectin-1 signalling was inhibited by over 90% when co-transfected with CD9 following stimulation with curdlan (Figure 4.6A). This strongly suggests that tetraspanin over-expression does not inhibit Zap-70 while leaving Syk function intact. Positive control stimulations induced NFAT/AP-1 activation in all cases, with some

variation observed between transfections (Figure 4.6B). However, in the Jurkat E6.1 clone, the PMA and ionomycin response in both the dectin-1 and dectin-1/CD9 transfectants were almost identical, although 50% that of the mock. Moreover, in the DNAX Jurkat clone the PMA and ionomycin response, following transfection of dectin-1, alone was two- to three-fold higher than mock and dectin-1/CD9 transfected cells (Figure 4.6B). This suggests a partial (three-fold) inhibitory effect of CD9 over-expression on maximal NFAT/AP-1 activation. But this cannot explain the almost complete inhibition of dectin-1-induced NFAT/AP-1 activation, as seen by comparing the similar 'mock curdlan' with 'dectin-1 & CD9 curdlan' data (Figure 4.6A). In addition, expression of both dectin-1 and CD9 was similar between the two Jurkat clones (Figure 4.6C & D). Together these data suggest that tetraspanin over-expression does not inhibit dectin-1 signalling by a mechanism which involves specific inhibition of Zap-70, with Syk function remaining intact.

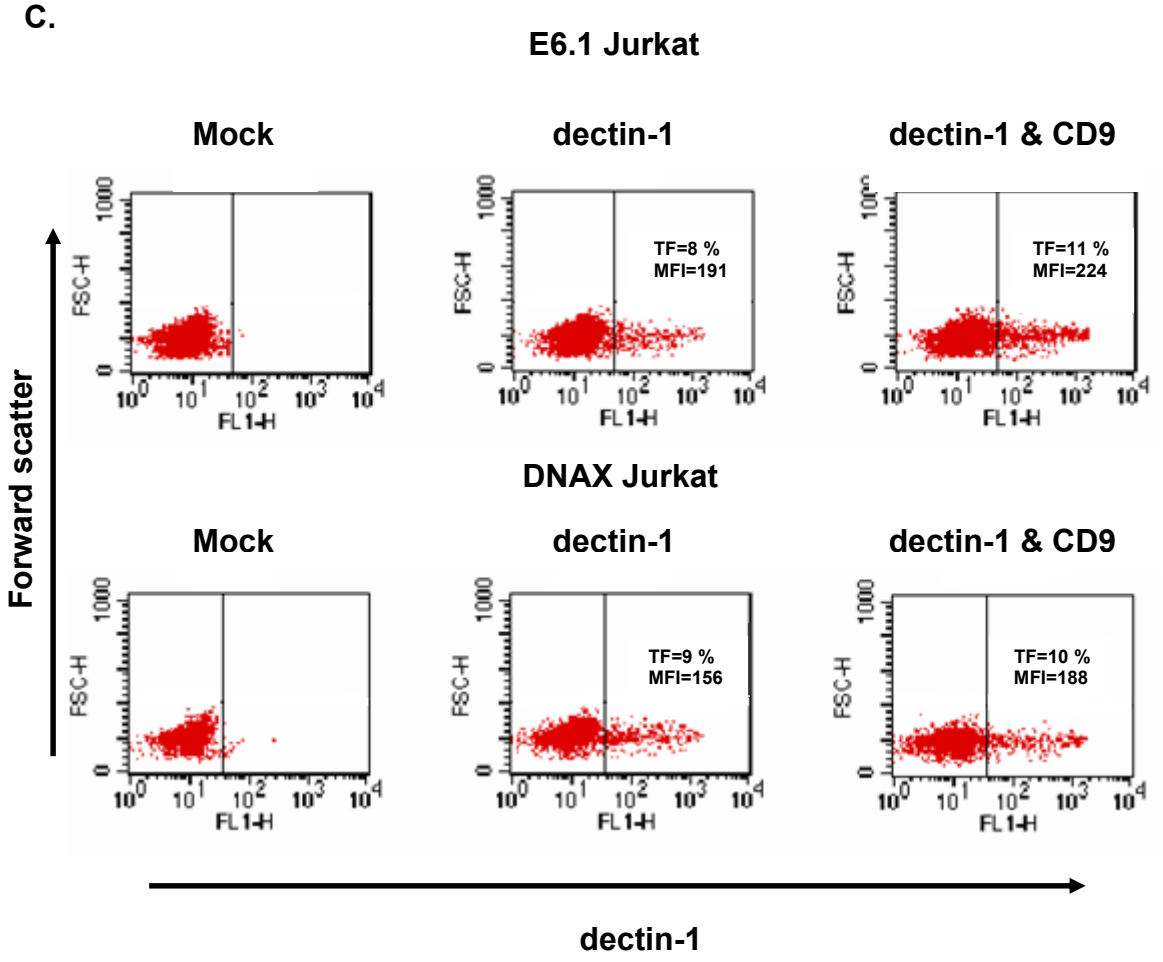
A.


Zap-70+ Syk- (E6.1 Jurkat)
Relative NFAT/AP-1-luciferase activity
120
100
80
60
40
20
0
Unstimulated
Curdlan
*
Mock
dectin-1
dectin-1 & CD9
Zap-70+ Syk+ (DNAX Jurkat)
Relative NFAT/AP-1-luciferase activity
120
100
80
60
40
20
0
Unstimulated
Curdlan
*
Mock
dectin-1
dectin-1 & CD9

B.


Zap-70+ Syk- (E6.1 Jurkat)
Relative NFAT/AP-1-luciferase activity
3000
2500
2000
1500
1000
500
0
Unstimulated
PMA/ionomycin
Mock
dectin-1
dectin-1 & CD9
Zap-70+ Syk+ (DNAX Jurkat)
Relative NFAT/AP-1-luciferase activity
500
400
300
200
100
0
Unstimulated
PMA/ionomycin
Mock
dectin-1
dectin-1 & CD9

C.


E6.1 Jurkat
Mock
dectin-1
dectin-1 & CD9
TF=8 %
MFI=191
TF=11 %
MFI=224
DNAX Jurkat
Mock
dectin-1
dectin-1 & CD9
TF=9 %
MFI=156
TF=10 %
MFI=188
FSC-H
FL1-H
Forward scatter
dectin-1

D.

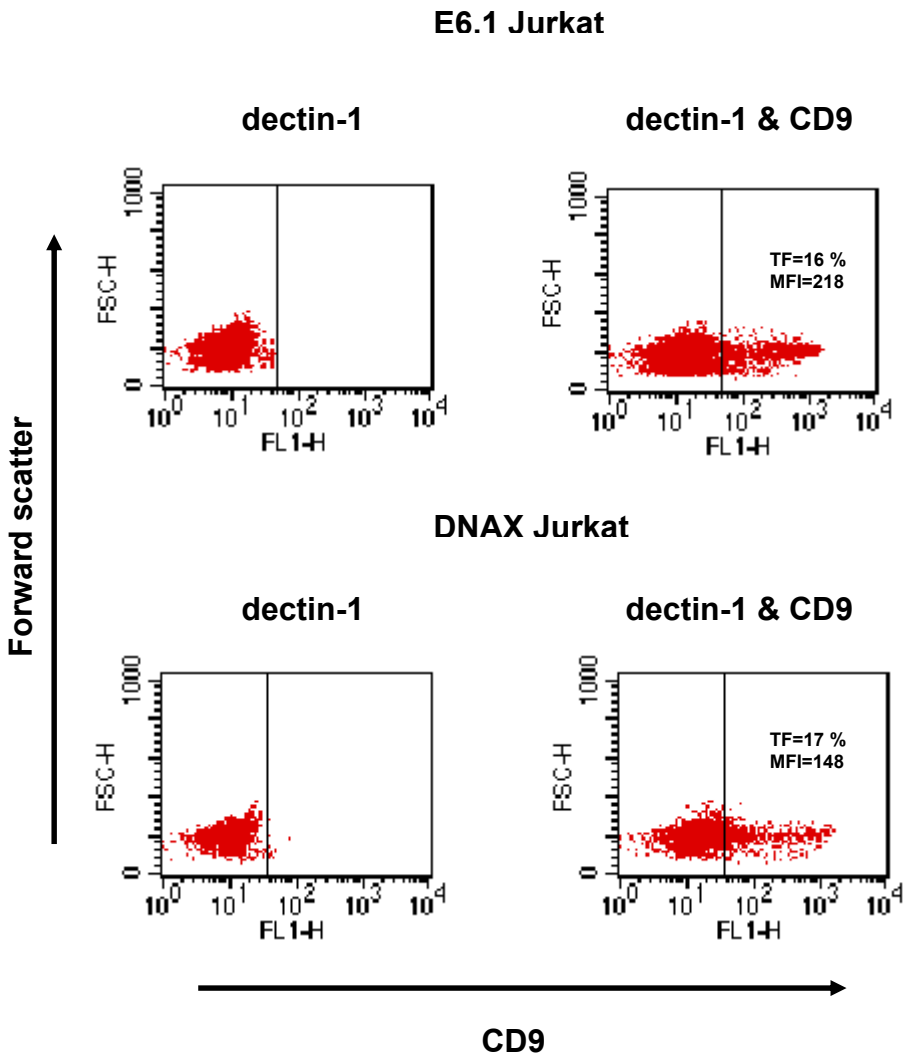

E6.1 Jurkat
dectin-1
dectin-1 & CD9
TF=16 %
MFI=218
DNAX Jurkat
dectin-1
dectin-1 & CD9
TF=17 %
MFI=148
FSC-H
FL1-H
Forward scatter
CD9

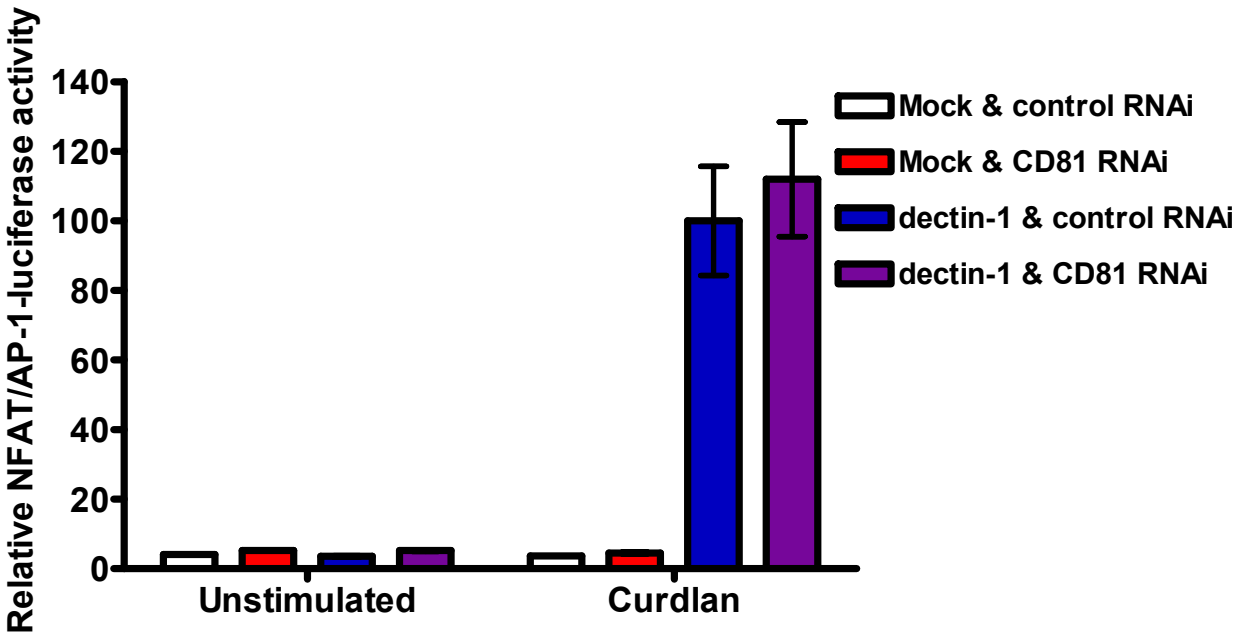
Figure 4.6 CD9 significantly inhibits dectin-1 signalling in E6.1 or DNAX Jurkat T-cell clones

Jurkat T-cells, either E6.1 or DNAX clones, were transiently transfected with an NFAT/AP-1-luciferase reporter construct, β-galactosidase construct, pEF6-MYC-dectin-1 (10 μg), pEF6-CD9 (20 μg) or empty pEF6 vector (mock). Cells were stimulated with media alone (unstimulated), curdlan (250 μg/ml) (A) and PMA/ionomycin (B) for six hours as indicated. Luciferase activity was measured following stimulation and transfection efficiency was corrected to β-galactosidase activity in individual experiments. The dectin-1 with curdlan stimulation value (E6.1 & DNAX Jurkat) was set to 100% and other conditions presented were calculated relative to this. An unpaired Student's *t*-test indicated a statistically significant difference ($P<0.05$) between dectin-1 transfected alone compared to dectin-1 co-transfected with CD9 as indicated *. Error bars represent the standard error of the mean. Experiments were performed twice. (C & D) Jurkat T-cells, either E6.1 or DNAX clones, were stained with either MYC or CD9 primary antibody, followed by anti-mouse-FITC-conjugated secondary antibody. Dead cells were gated out using propidium iodide. Antibody binding was visualized by flow cytometry. Dot plots were analysed and values indicate the average transfection efficiency (TF) and mean fluorescence intensity (MFI) of protein on the cell surface. Data is representative of two independent experiments.

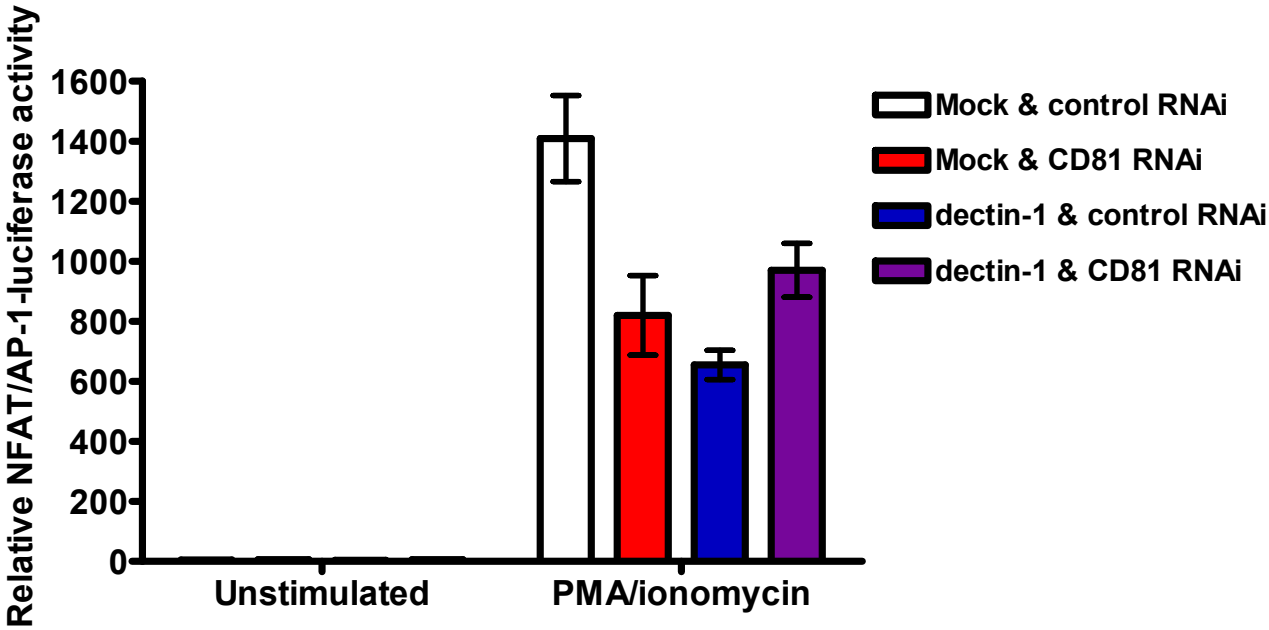
4.3.5 CD81 knockdown does not affect dectin-1-induced NFAT/AP-1

An RNAi approach was performed on CD81 to determine the effect of knockdown of this protein upon dectin-1 signalling, as previous experiments have primarily involved tetraspanin over-expression. Moreover, the CD37 knockout was previously shown to result in hyper-responsive dectin-1 on primary mouse macrophages (Meyer-Wentrup et al., 2007). CD81 was selected due to its high expression on Jurkat (Figure 3.1) and the availability of CD81 siRNA duplexes, previously shown to cause 100% knockdown of CD81 on human umbilical cord endothelial cells (M.G. Tomlinson, unpublished data). Control or CD81 siRNA duplexes were transfected into Jurkat in the presence or absence of dectin-1, and two days post transfection the cells were stimulated with curdlan. Dectin-1-induced NFAT/AP-1 activation was not affected by CD81 knockdown (Figure 4.7A & B). Flow cytometry demonstrated substantial CD81 knockdown in greater than 50% of the cells and no knockdown in the remainder (Figure 4.7C). The latter probably represent non-transfected cells that also failed to take up the expression constructs and the NFAT/AP-1 reporter, and thus did not contribute to the assay. Similar dectin-1 surface expression between control and CD81 siRNA transfectants was also confirmed by flow cytometry (Figure 4.7D). Therefore, this data suggests that knockdown of CD81, unlike tetraspanin over-expression, does not affect the ability of dectin-1 to signal.

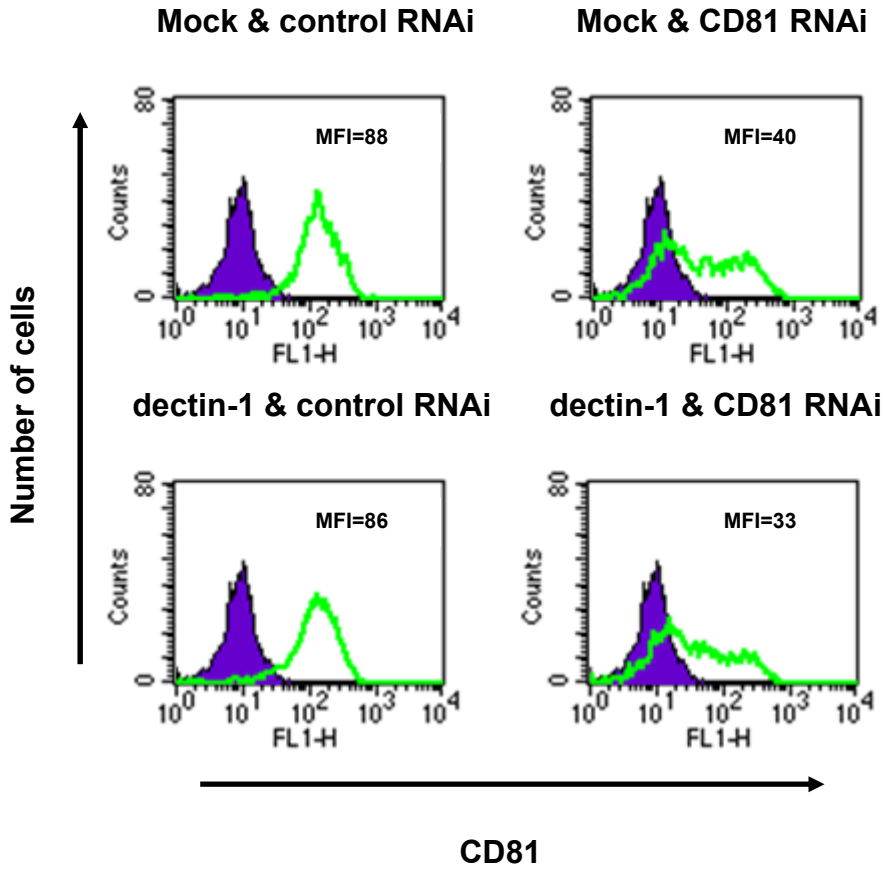
A.


Relative NFAT/AP-1-luciferase activity
140
120
100
80
60
40
20
0
Unstimulated
Curdlan
Mock & control RNAi
Mock & CD81 RNAi
dectin-1 & control RNAi
dectin-1 & CD81 RNAi

B.


Relative NFAT/AP-1-luciferase activity
1600
1400
1200
1000
800
600
400
200
0
Unstimulated
PMA/ionomycin
Mock & control RNAi
Mock & CD81 RNAi
dectin-1 & control RNAi
dectin-1 & CD81 RNAi

C.


Mock & control RNAi
Mock & CD81 RNAi
dectin-1 & control RNAi
dectin-1 & CD81 RNAi
MFI=88
MFI=40
MFI=86
MFI=33
Counts
FL1-H
Number of cells
CD81

D.

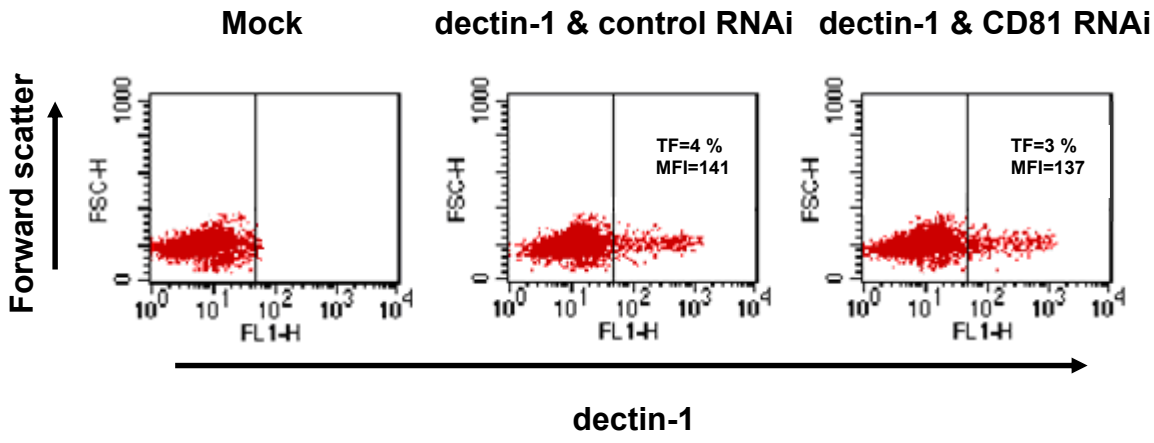

Mock
dectin-1 & control RNAi
dectin-1 & CD81 RNAi
TF=4 %
MFI=141
TF=3 %
MFI=137
FSC-H
FL1-H
Forward scatter
dectin-1

Figure 4.7 CD81 knockdown does not affect dectin-1 signalling in Jurkat T-cells

Jurkat T-cells were transiently transfected with an NFAT/AP-1-luciferase reporter construct, β-galactosidase, pEF6-MYC-dectin-1 or empty pEF6 vector (mock) and either CD81 siRNA or control siRNA. Cells were stimulated with media alone (unstimulated), curdlan (250 μg/ml) (A) and PMA/ionomycin (B) for six hours as indicated. Luciferase activity was measured following stimulation and transfection efficiency was corrected to β-galactosidase activity in individual experiments. The dectin-1 and control RNAi with curdlan stimulation value was set to 100% and other conditions presented were calculated relative to this. Error bars represent the standard error of the mean. Analysis of variance (ANOVA) of the data suggested no significant difference between groups. Experiments were performed twice. (C & D) Cells were stained with either negative (closed histograms), CD81 (open histograms) or MYC primary antibody and then anti-mouse FITC-conjugated secondary antibody. Dead cells were gated out using propidium iodide. Dot plots/histograms were analysed and values indicate the average transfection efficiency (TF) and/or mean fluorescence intensity (MFI) of protein on the cell surface. Data is representative of two independent experiments.

4.3.6 Tetraspanins do not co-immunoprecipitate with dectin-1 in Jurkat

It is possible that tetraspanins inhibit dectin-1 signalling through a direct interaction, given that the van Spriel group has shown an interaction between dectin-1 and CD37 in HEK293T cells (Meyer-Wentrup et al., 2007). To test for such an interaction in Jurkat, cells were transiently transfected with MYC-tagged dectin-1 and the FLAG-tagged tetraspanins CD37 and CD151, both of which inhibit dectin-1 signalling (Figures 3.3A & 3.5A). Cells were lysed in the relatively mild detergent Brij97 that is thought to maintain tetraspanin-tetraspanin interactions (Hemler, 2005), immunoprecipitated for tetraspanins (FLAG) and blotted for dectin-1 (MYC). Neither CD37 nor CD151 co-immunoprecipitated with dectin-1 (Figure 4.8A). However, dectin-1 was detected in whole cell lysate samples (Figure 4.8B) and both tetraspanins were detected in the immunoprecipitates, although CD37 appeared to be expressed at a lower level than CD151 (Figure 4.8C). This data suggests that tetraspanins do not interact with dectin-1 in Jurkat, although it is possible that there is a relatively low affinity interaction that is lost during cell lysis and immunoprecipitation. However, as this experiment was performed once and no positive control was used, it may be necessary to repeat with a suitable control for studies in the future.

4.3.7 Tetraspanins co-immunoprecipitate with dectin-1 in HEK293T-cells

The failure to detect a dectin-1 interaction with CD37 was surprising since the proteins were previously shown to co-immunoprecipitate from transfected HEK293T cells (Meyer-Wentrup et al., 2007). In an attempt to repeat this and to determine whether the interaction is specific to CD37, HEK293T cells were transiently transfected with MYC-tagged dectin-1 and a panel of 18 FLAG-tagged tetraspanins. Cells were lysed in the stringent detergent Triton X-100 that is thought to disrupt tetraspanin-tetraspanin interactions but maintain some stronger tetraspanin-partner

interactions (Hemler, 2005). Transfected HEK293T cell lysates were immunoprecipitated for tetraspanins with FLAG antibody and blotted for dectin-1 with MYC. Consistent with Meyer-Wentrup et al. (2007), dectin-1 was readily detected following CD37 immunoprecipitation (Figure 4.9A), the positive control in this experiment. However, all other tetraspanins, with the exception of Tspan31, were also readily detected. As a negative control, dectin-1 was not detected in FLAG immunoprecipitations from control cells that lacked tetraspanin transfection (Figure 4.9A). The efficiency of dectin-1 co-immunoprecipitation differed between tetraspanins, but interpretation is complicated by different levels of transfected dectin-1, as detected by MYC blotting of whole cell lysates (Figure 4.9B), and tetraspanins, as detected by FLAG blotting of immunoprecipitates (Figure 4.9C). Nevertheless, this data raises the possibility that dectin-1 has a general affinity for tetraspanins that might only be revealed when the proteins are co-expressed at relatively high levels in a cell such as HEK293T. However, this HEK293T data may be an artefact of the high levels of over-expression. Consistent with this idea, multiple tetraspanin interactions have also been observed between tetraspanins and the major platelet integrin $\alpha_{IIb}\beta_3$ in HEK293T cells (Figure 4.10), under conditions in which co-immunoprecipitation of endogenous levels from platelet lysates failed to identify an interaction (Protty et al., 2009). Together these data reinforce the widely-held belief that co-immunoprecipitation studies of highly over-expressed proteins are prone to artefacts, and provide no definitive evidence that dectin-1 interacts with tetraspanins.

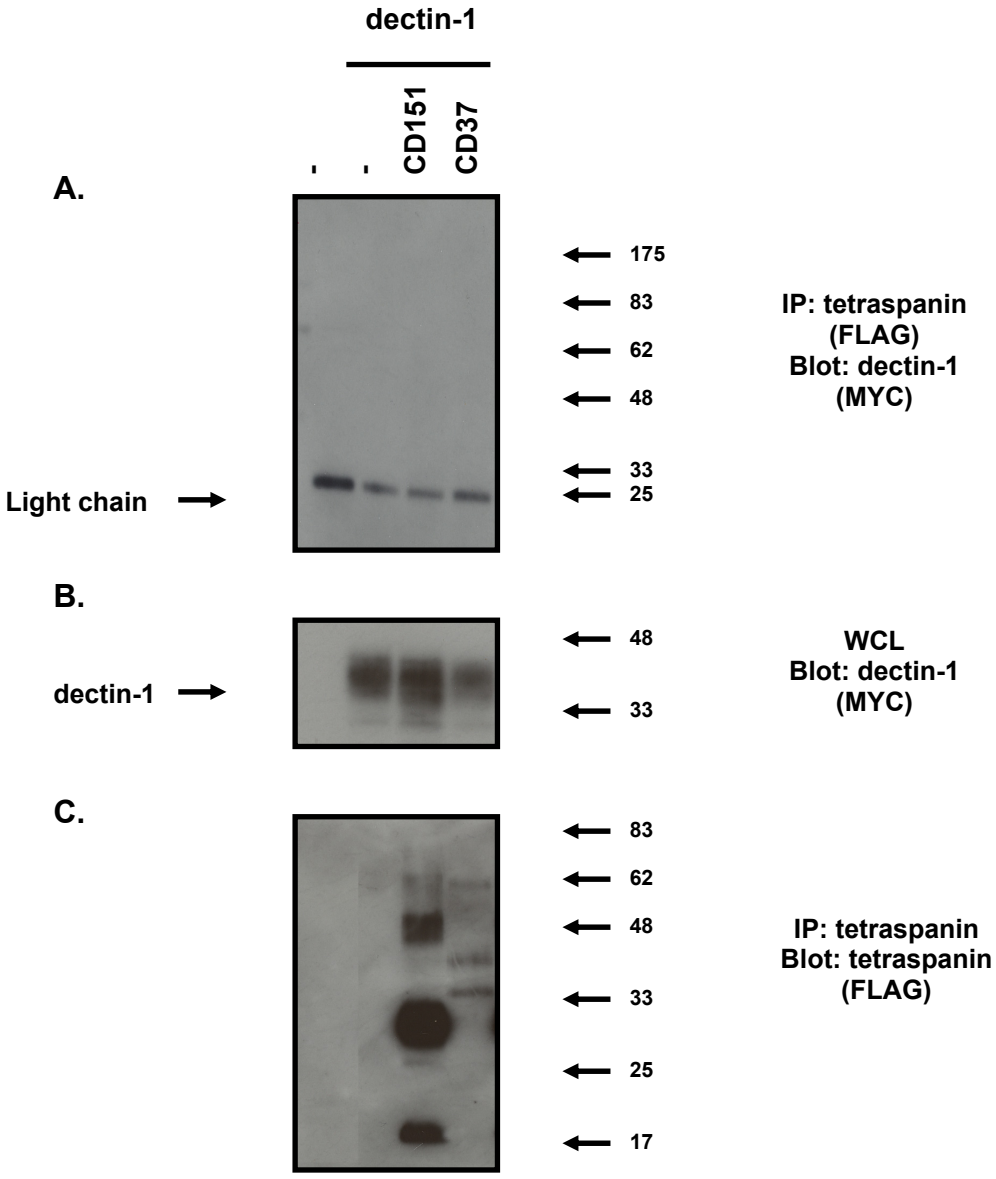


Figure 4.8 Tetraspanins do not co-immunoprecipitate with dectin-1 in Jurkat

Jurkat cells were lysed in 1% Brij97 after transient transfection with dectin-1 (20 μg) and tetraspanins (20 μg) and immunoprecipitated (IP) for tetraspanins (FLAG) for 1.5 hours. Proteins were visualised by SDS-PAGE and western blotting with indicated antibodies. In panel A, only the light chain from the immunoprecipitating antibody is detectable. This experiment was performed once.

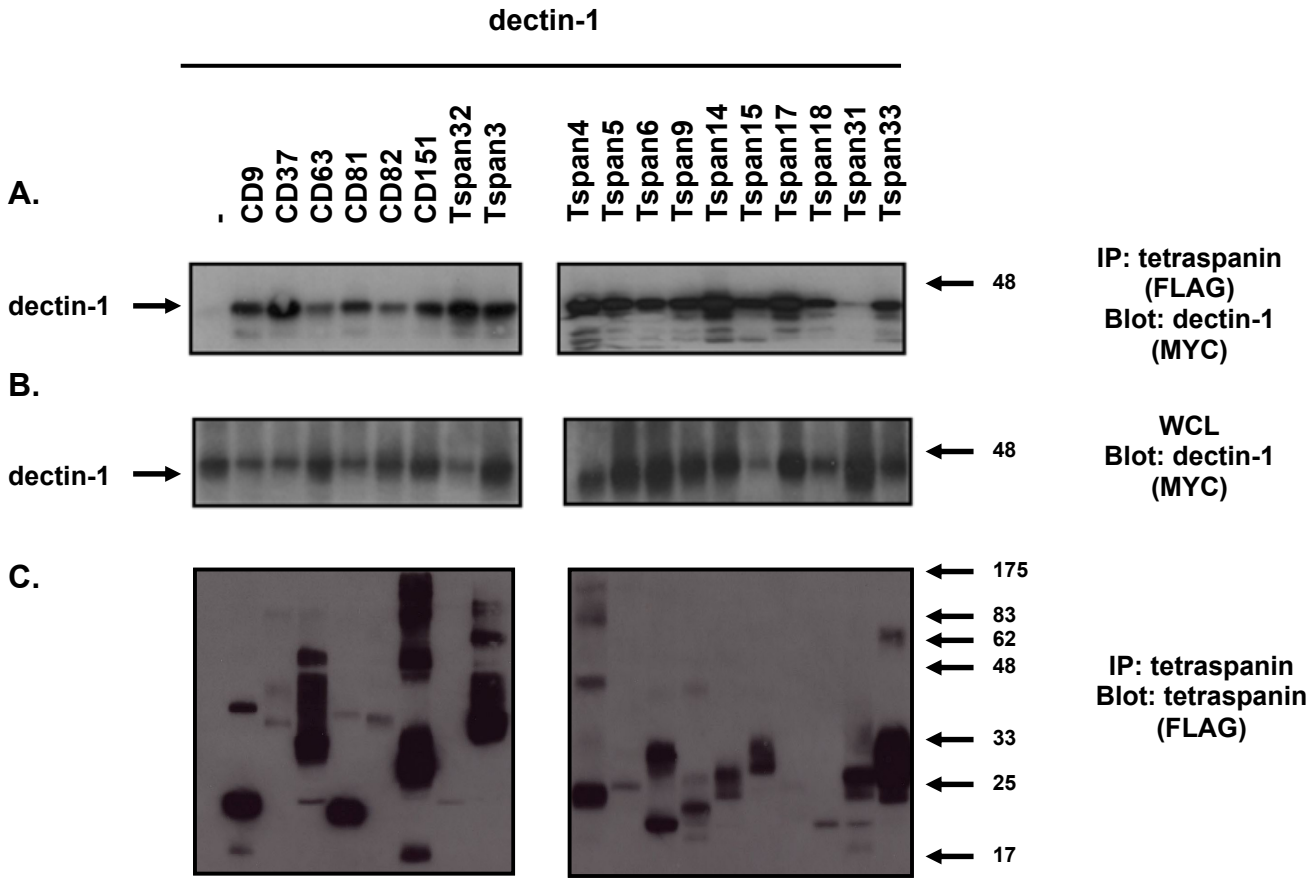


Figure 4.9 Tetraspanins co-immunoprecipitate with dectin-1 in HEK293T cells

HEK293T cells were lysed in 1% Triton X-100 two days after transient transfection with dectin-1 (5 μg) and tetraspanins (5 μg) and immunoprecipitated (IP) for tetraspanins (FLAG) for 1.5 hours. Proteins were visualised by SDS-PAGE and western blotting with indicated antibodies. CD37 has previously been shown to co-immunoprecipitate with dectin-1 in HEK293T cells (Meyer-Wentrup et al., 2007)

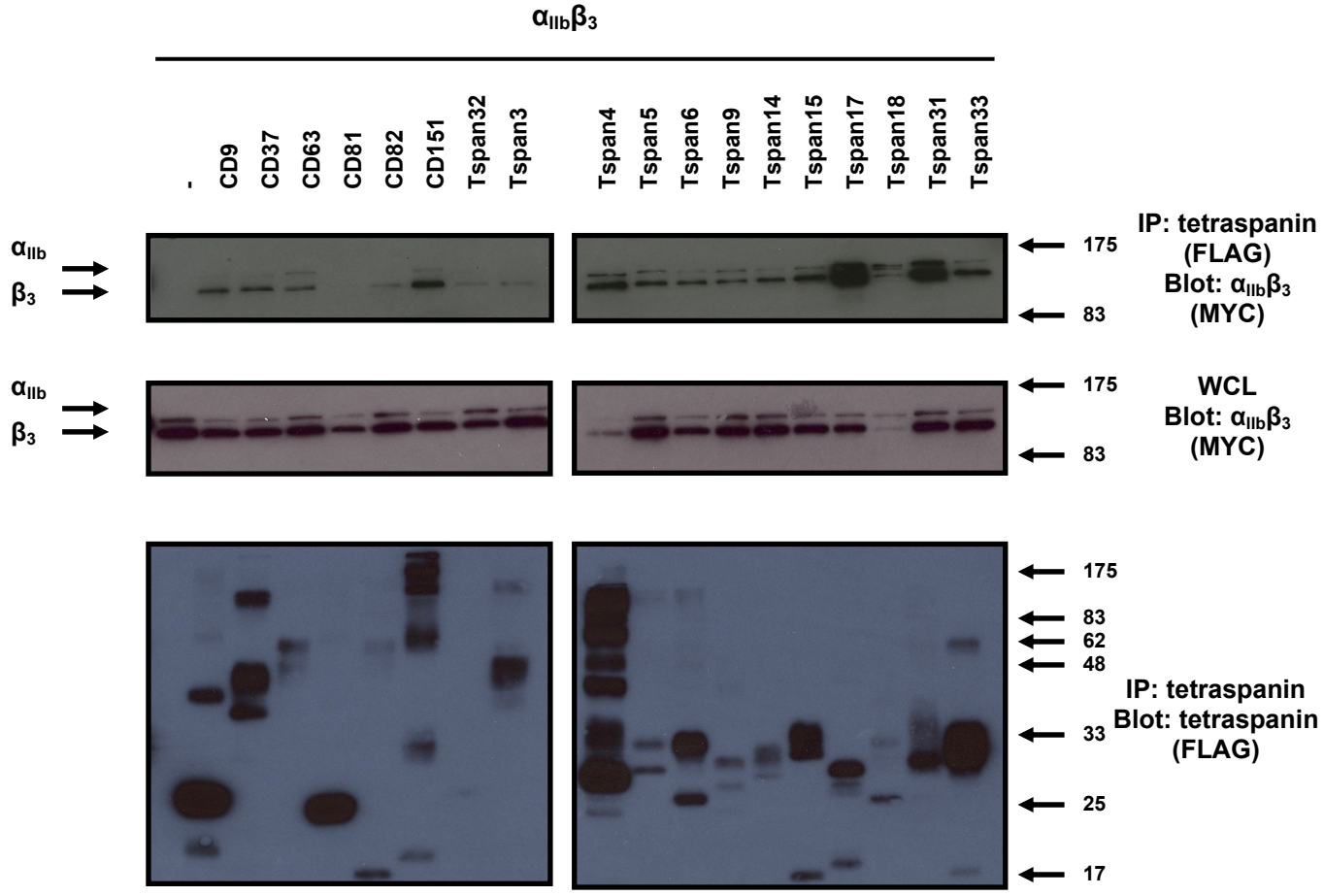


Figure 4.10 Tetraspanins co-immunoprecipitate with $\alpha_{IIb}\beta_3$ in HEK293T cells

HEK293T cells were lysed in 1% Brij97 two days after transient transfection with $\alpha_{IIb}\beta_3$ (5 μg) and tetraspanins (5 μg) and immunoprecipitated (IP) for tetraspanins (FLAG) for 1.5 hours. Proteins were visualised by SDS-PAGE and western blotting with indicated antibodies.

4.4 DISCUSSION

In this Chapter a number of methods have been utilised to investigate the mechanism of dectin-1 signal inhibition by tetraspanin over-expression in the Jurkat T-cell line. Several hypotheses have been tested and further work is necessary to fully elucidate possible mechanisms.

The data in Chapter Three showed that tetraspanin over-expression inhibits dectin-1-induced NFAT/AP-1 activation in Jurkat T-cells. Since this reporter requires both calcium, to activate NFAT, and MAPK, to activate AP-1, it is possible that tetraspanin over-expression inhibits either calcium or MAPK signalling. This was investigated using an AP-1 reporter construct because of the unavailability of a good NFAT reporter (Shapiro et al., 1996). Similar to the NFAT/AP-1 data, CD37 was found to almost completely inhibit dectin-1-induced AP-1 activation. This suggests that either tetraspanins specifically inhibit signalling at a more receptor-proximal level, before the pathway bifurcates at the level of PLCγ, or that tetraspanins specifically inhibit the calcium arm of the pathway. This calcium possibility could be investigated further using calcium imaging experiments. However, it seems more likely that there is a specific inhibition of the proximal events in dectin-1 signalling. This is due to the fact that tetraspanins do not inhibit GPVI signalling, as shown in Chapter Three: it is difficult to imagine how tetraspanins could inhibit calcium signalling downstream of dectin-1 but not GPVI, because both utilise a similar tyrosine kinase/PLCγ/calcium pathway.

A key question was to determine whether inhibition of dectin-1 signalling by tetraspanin over-expression was specific to the Jurkat T-cell line. Therefore, the DT40 B- and RBL-2H3 mast cell lines, both of which are established models to study

tyrosine kinase-linked receptor signalling (Fuller et al., 2007; Tomlinson et al., 2007), were selected to answer the question. However, unlike in Jurkat, tetraspanin over-expression did not inhibit dectin-1 signalling in DT40 B- and RBL-2H3 cell lines. Such a striking difference did not appear to be due to different tetraspanin or dectin-1 expression levels in the different cell lines, because flow cytometry proved them to be similar. Instead, the contrasting findings may be attributed to the different signalling proteins in these cell lines. One potential candidate is the tyrosine kinase Syk, which appears to be central to dectin-1 signalling through interaction with the YXXL motif in the cytoplasmic tail of the receptor. Interestingly, Syk is expressed in DT40 and RBL-2H3 but not in the Jurkat clone used in this thesis, clone E6.1, which instead expresses Zap-70 (Fargnoli et al., 1995). This was explored further by comparing dectin-1 signalling in Jurkat that express Zap-70 (clone E6.1), versus those that express both Zap-70 and Syk (so-called 'DNAX Jurkat'). However, no difference between the two clones was observed in dectin-1 signal inhibition by tetraspanin over-expression. This line of enquiry could be explored further by using Jurkat deficient in both Zap-70 and Syk. In addition, in Jurkat the dectin-1 signal is thought to be initiated by the Src family kinase Lck (Fuller et al., 2007), whereas Lyn and Fyn are the main players in DT40 and RBL-2H3 cells. The possibility of a specific tetraspanin effect on Lck has been suggested previously, since the hyperproliferative phenotype observed in CD37-deficient T-cells was found to be accompanied by elevated Lck kinase activity (van Spriel et al., 2004). It would be interesting to test whether dectin-1-induced Lck activity is impaired following tetraspanin over-expression in Jurkat. Moreover, it would be useful to generate stable Lck transfectants of Lyn-deficient DT40, to test whether tetraspanins could now inhibit dectin-1 signalling in this cell line.

If tetraspanin over-expression inhibits receptor-proximal signalling, a substantial decrease in tyrosine phosphorylation should in theory be observed. In an attempt to perform this experiment, Jurkat were transiently transfected with dectin-1, in the presence or absence of a tetraspanin, to study tyrosine phosphorylation over a time course following stimulation with curdlan. However, no inducible phosphorylation was observed, even in the absence of tetraspanin over-expression (data not shown). This may have been due to the relatively low transfection efficiencies achieved in Jurkat (approximately 5-6% dectin-1-positive cells), combined with the fact that dectin-1 and related receptors induce only relatively weak tyrosine phosphorylation in cell lines (J. Mori, personal communication). To attempt to address this issue in future, large-scale transfections could be followed by flow cytometry-based sorting of transfected cells. Alternatively, cells could be co-transfected with an epitope-tagged form of a signalling protein such as PLCγ1. Following cell stimulation, this 'reporter protein' could be immunoprecipitated with an anti-tag antibody and tyrosine phosphorylation detected by western blotting. This is an approach that has previously proved successful in Jurkat co-transfection experiments (Tomlinson et al., 2004).

In antigen-presenting cells from the CD37-deficient mouse, the observed increase in dectin-1 signalling might be due to an indirect effect caused by disruption of tetraspanin microdomains, rather than the proposed specific CD37-dectin-1 interaction (Meyer-Wentrup et al., 2007). To perform a somewhat analogous experiment in Jurkat, which express minimal CD37 but substantial levels of CD81, the latter was knocked down using RNAi. However, despite a substantial proportion of cells exhibiting almost complete CD81-knockdown, which presumably reflects those that were successfully transfected, no effect on dectin-1 signalling was

observed. There are a number of interpretations of this experiment. Firstly, it is possible that knockdown of CD81 does not disrupt tetraspanin microdomains in Jurkat to the same extent as over-expression of tetraspanins does. Secondly, it is possible that endogenous CD81 plays no role in dectin-1 signalling. These possibilities could be further investigated by knocking down other Jurkat tetraspanins, either alone or in combination.

Finally, co-immunoprecipitation studies in Jurkat suggest that dectin-1 is not a component of tetraspanin microdomains, as no interaction was observed between dectin-1 and the tetraspanins CD37 and CD151 in the relatively weak detergent Brij97, which is thought to maintain the integrity of tetraspanin microdomains by preserving tetraspanin-tetraspanin interactions (Hemler, 2005). Therefore, tetraspanin over-expression appears to somehow inhibit dectin-1 signalling without actually interacting with the receptor. It is possible that tetraspanin over-expression might indirectly affect dectin-1 signalling by disrupting tetraspanin microdomains, leading to the release of factors which inhibit dectin-1 signalling. This speculative idea will be expanded upon in Chapter Seven. However, the idea that tetraspanins can indirectly affect other proteins is not without precedent. Indeed, studies of the urokinase receptor, which is involved in plasminogen activation and proteolytic activity, indicate that its activity is reduced in the presence of CD82 over-expression, despite the authors finding no association between this receptor and tetraspanins at the cell surface and by co-immunoprecipitation studies (Bass et al., 2005). Despite the failure in this Chapter to detect dectin-1 co-immunoprecipitation with tetraspanins in Jurkat, studies were extended to determine whether a panel of 18 tetraspanins could interact with dectin-1 in HEK293T cells using the relatively stringent detergent Triton X-100. This particular cell line was utilised by the van Spriel group to show that dectin-1 and

CD37 interact (Meyer-Wentrup et al., 2007). However, data in this Chapter suggests that multiple tetraspanins may have the ability to interact with dectin-1 in HEK293T cells, which is difficult to envisage unless the receptor has a general tetraspanin-binding pocket. It is more likely that the interaction in HEK293T cells is an artefact of the very high expression levels that can be achieved in this cell line. Consistent with this, all tetraspanins were similarly able to co-immunoprecipitate an unrelated protein, integrin $\alpha_{IIb}\beta_3$, in these cells. This data suggests that co-immunoprecipitation studies in HEK293T cells may not be suitable to identify potential tetraspanin-partner protein interactions, and that they should be interpreted with some caution, as any interactions may be an over-expression artefact.

4.5 SUMMARY

In this Chapter a number of methods have been employed to investigate how tetraspanin over-expression inhibits dectin-1 signalling in Jurkat. These studies have demonstrated that tetraspanins inhibit both NFAT/AP-1 and AP-1 reporter activation by dectin-1. However, no such inhibition was observed in two other model cell lines, the DT40 B- and the RBL-2H3 mast-cell lines, suggesting that tetraspanins regulate a Jurkat-specific signalling protein. In addition, knockdown of CD81 did not affect dectin-1 signalling in Jurkat. Finally, co-immunoprecipitation experiments failed to definitively determine whether dectin-1 interacts with tetraspanins. No interaction was observed in transfected Jurkat using the detergent Brij97, but an interaction was detected between dectin-1 and many tested tetraspanins in transfected HEK293T cells using the detergent Triton X-100. The latter may be an artefact of the relatively high expression levels than can be achieved.

CHAPTER 5 –

TETRASPANIN OVER-EXPRESSION INHIBITS CLEC-2 SIGNALLING

5.1 INTRODUCTION

CLEC-2 signals via a single YXXL motif that undergoes tyrosine phosphorylation to induce platelet aggregation with a distinct lag phase when stimulated with its ligand, the snake venom toxin rhodocytin (Suzuki-Inoue et al., 2006). As well as studies in platelets from mutant mice, the NFAT/AP-1-luciferase reporter assay has been used in DT40 B- and Jurkat T-cell mutant lines to study the signalling pathway downstream of this receptor (Fuller et al., 2007). Together these studies have generated a model in which CLEC-2 signalling requires Src, Syk and Tec family tyrosine kinases and PLCγ, but is only partially dependent on the SLP-76/BLNK family of adaptor proteins (Fuller et al., 2007).

In the previous Chapters, tetraspanin over-expression was shown to inhibit signalling by dectin-1. Since CLEC-2 is a member of the dectin-1 family sharing the CTLD and cytoplasmic YXXL signalling motif, it is possible that tetraspanins also negatively regulate CLEC-2. At present, there has been no published literature regarding the ability of tetraspanins to regulate CLEC-2 signalling.

5.2 AIM

- To determine whether tetraspanin over-expression inhibits CLEC-2 signalling in a model cell line.

5.3 RESULTS

5.3.1 Rhodocytin induces NFAT/AP-1 activation in CLEC-2-transfected Jurkat

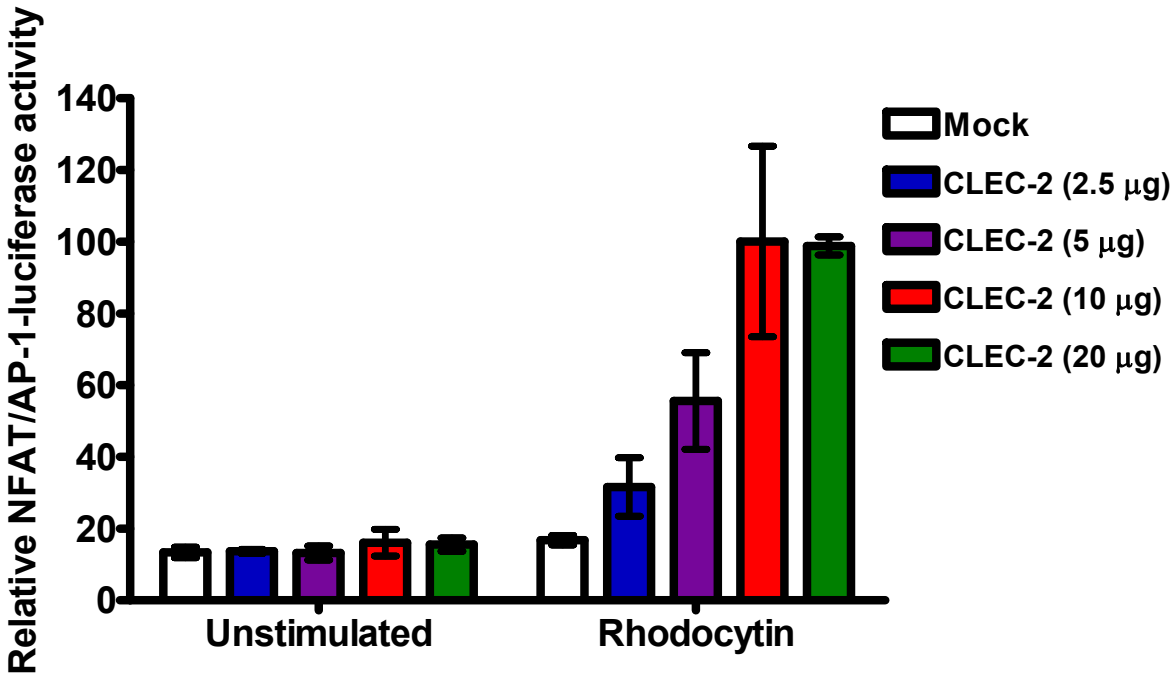
Fuller et al. have previously demonstrated that, in Jurkat cells, transfected CLEC-2 induces NFAT/AP-1 activation in response to its ligand rhodocytin (Fuller et al., 2007). To establish dose response data and to ultimately determine whether tetraspanins affect the signalling of CLEC-2, Jurkat cells were initially transfected with four different amounts of a CLEC-2 expression construct. The degree of NFAT/AP-1 activation was measured in response to 50 nM rhodocytin, a concentration used previously in this assay (Fuller et al., 2007). Rhodocytin-induced NFAT/AP-1 activation was maximal at 10 μg of the CLEC-2 expression construct (Figure 5.1A). As a positive control, all transfection conditions responded to PMA and ionomycin, although there is an unexplainable downwards trend in the degree of response (Figure 5.1B). Despite this, there was still a robust response even when 20 μg of CLEC-2 was stimulated with PMA and ionomycin. Consistent with the NFAT/AP-1 data, the percentage of cells expressing CLEC-2 at the surface increased with increasing amounts of transfected CLEC-2, to a maximal level with 10 μg of the expression construct (Figure 5.1C). As a result of the data gathered, 10 μg CLEC-2 was used in all subsequent experiments to investigate whether tetraspanins inhibit the signalling of this receptor.

5.3.2 Tetraspanins inhibit CLEC-2 signalling in Jurkat

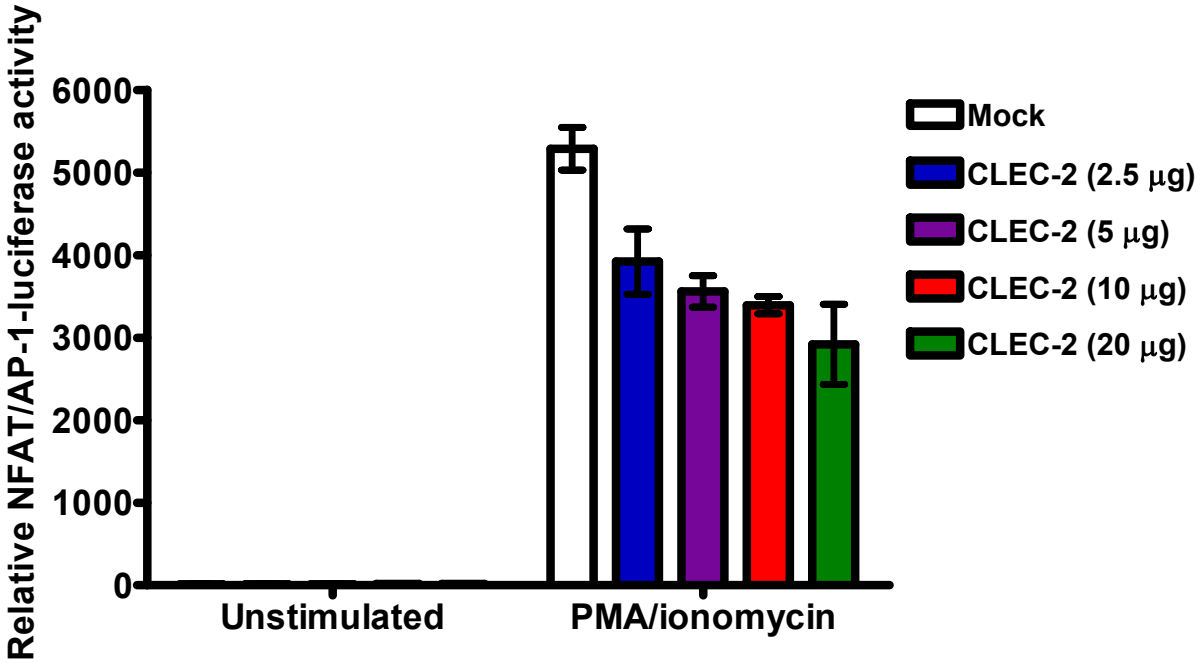
To determine whether CD37 and the platelet-expressed tetraspanins CD9, CD63, CD151 and Tspan32 could inhibit CLEC-2 signalling, Jurkat cells were co-transfected with the NFAT/AP-1-luciferase construct and CLEC-2 in the presence or absence of each tetraspanin. In each case, the tetraspanin abolished CLEC-2-induced NFAT/AP-

1 activation (Figure 5.2A). Moreover, all transfection conditions responded robustly to positive control stimulations, with some variability in the magnitude of the response (Figure 5.2B). The degree of PMA and ionomycin stimulation between mock, CLEC-2, CLEC-2-CD9 and CLEC-2-CD63 was robust and similar in response. However, the degree of PMA and ionomcyin response between CLEC-2-CD151, CLEC-2-Tspan32 and CLEC-2-CD37 was relatively weak in comparison. Despite this, the PMA and ionomycin data does not retract from the fact that over-expression of all tetraspanins tested inhibit CLEC-2 signalling following rhodocytin stimulation. In addition, CLEC-2 cell surface expression was not affected by tetraspanin expression (Figure 5.2C). This suggests that signal inhibition was not due to reduced cell surface expression of CLEC-2 when co-transfected with tetraspanins compared to receptor alone. This data shows that a number of tetraspanins inhibit the signalling of CLEC-2.

A.


Relative NFAT/AP-1-luciferase activity
140
120
100
80
60
40
20
0
Unstimulated
Rhodocytin
Mock
CLEC-2 (2.5 μg)
CLEC-2 (5 μg)
CLEC-2 (10 μg)
CLEC-2 (20 μg)

B.


Relative NFAT/AP-1-luciferase activity
6000
5000
4000
3000
2000
1000
0
Unstimulated
PMA/ionomycin
Mock
CLEC-2 (2.5 μg)
CLEC-2 (5 μg)
CLEC-2 (10 μg)
CLEC-2 (20 μg)

C.

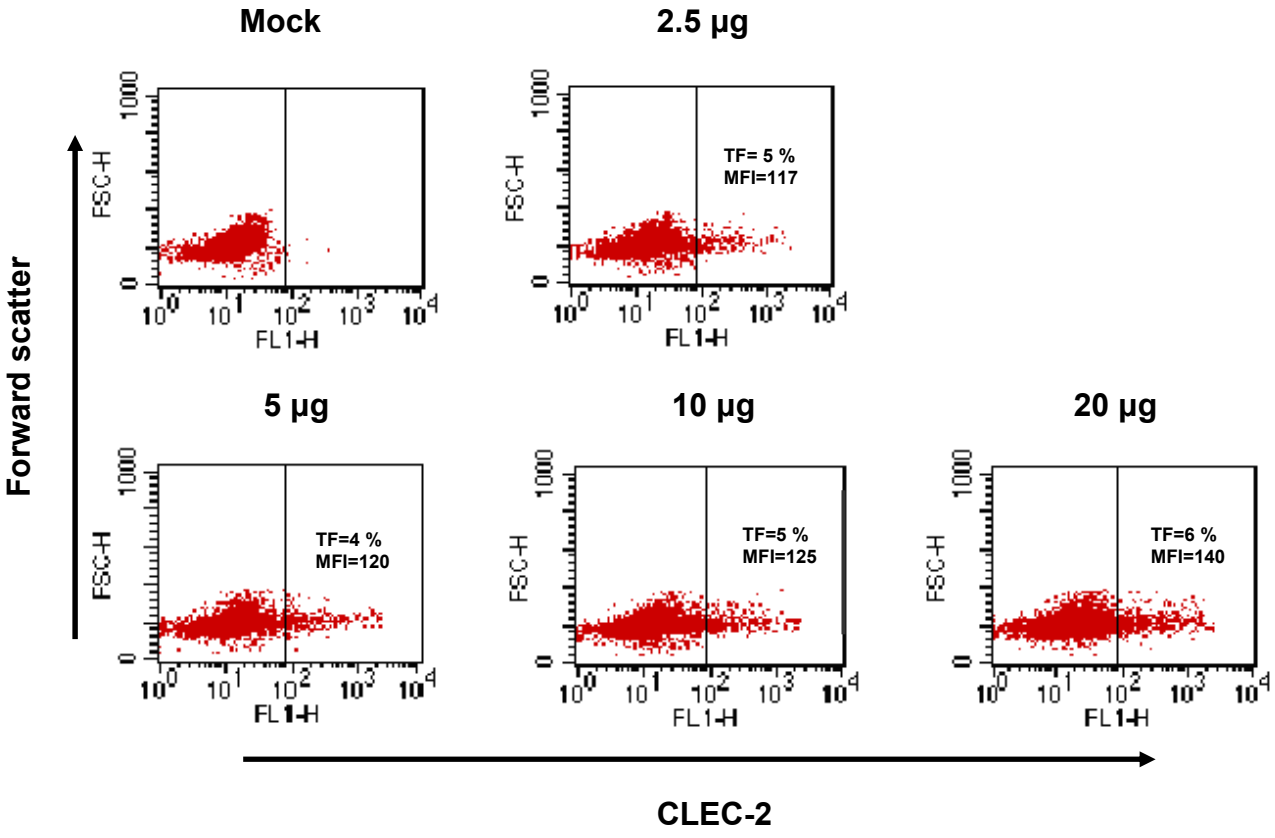
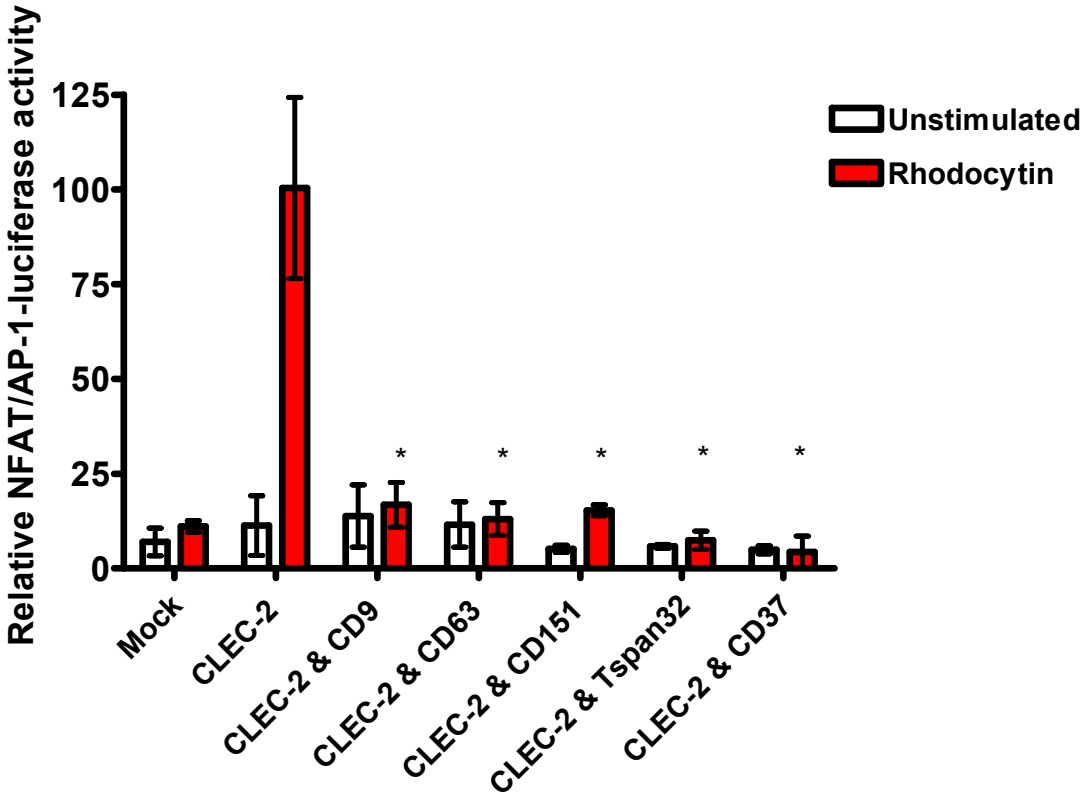


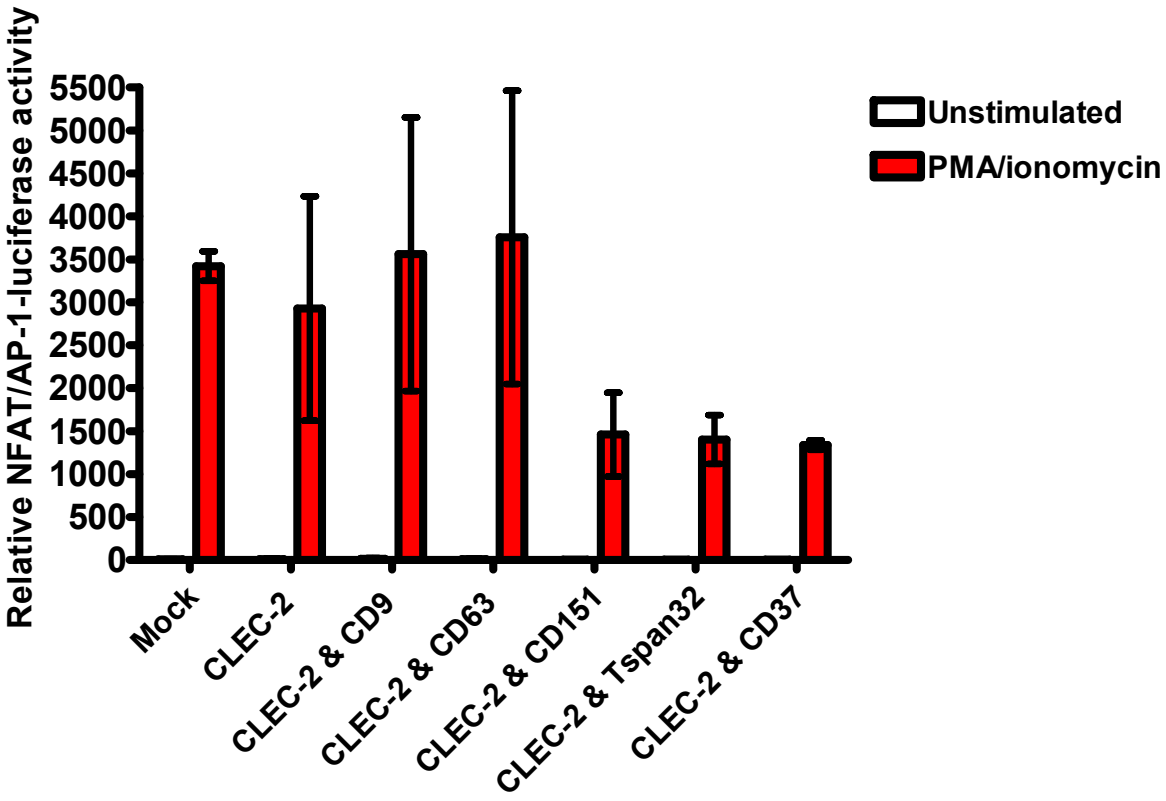
Figure 5.1 Rhodocytin induces NFAT/AP-1 activation in a CLEC-2 dose-dependent manner in Jurkat T-cells

Jurkat T-cells were transiently transfected with an NFAT/AP-1-luciferase reporter construct, β-galactosidase construct, pEF6-MYC-CLEC-2 or empty pEF6 vector (mock). Cells were stimulated with media alone (unstimulated), rhodocytin (50 nM) (A) and PMA/ionomycin (B) for six hours as indicated. Luciferase activity was measured following stimulation and transfection efficiency was corrected to β-galactosidase activity in individual experiments. The CLEC-2 (10 µg) with rhodocytin stimulation value was set to 100% and other conditions presented were calculated relative to this. Error bars represent standard error of the mean. Experiments were performed three times. (C) Cells were stained with MYC primary antibody and then anti-mouse FITC-conjugated secondary antibody. Dead cells were gated out using propidium iodide. Dot plots were analysed and values indicate the average transfection efficiency (TF) and mean fluorescence intensity (MFI) of protein on the cell surface. Data is representative of three independent experiments.

A.


Relative NFAT/AP-1-luciferase activity
125
100
75
50
25
0
Unstimulated
Rhodocytin
*
*
*
*
*
Mock
CLEC-2
CLEC-2 & CD9
CLEC-2 & CD63
CLEC-2 & CD151
CLEC-2 & Tspan32
CLEC-2 & CD37

B.


Relative NFAT/AP-1-luciferase activity
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Unstimulated
PMA/ionomycin
Mock
CLEC-2
CLEC-2 & CD9
CLEC-2 & CD63
CLEC-2 & CD151
CLEC-2 & Tspan32
CLEC-2 & CD37

C.

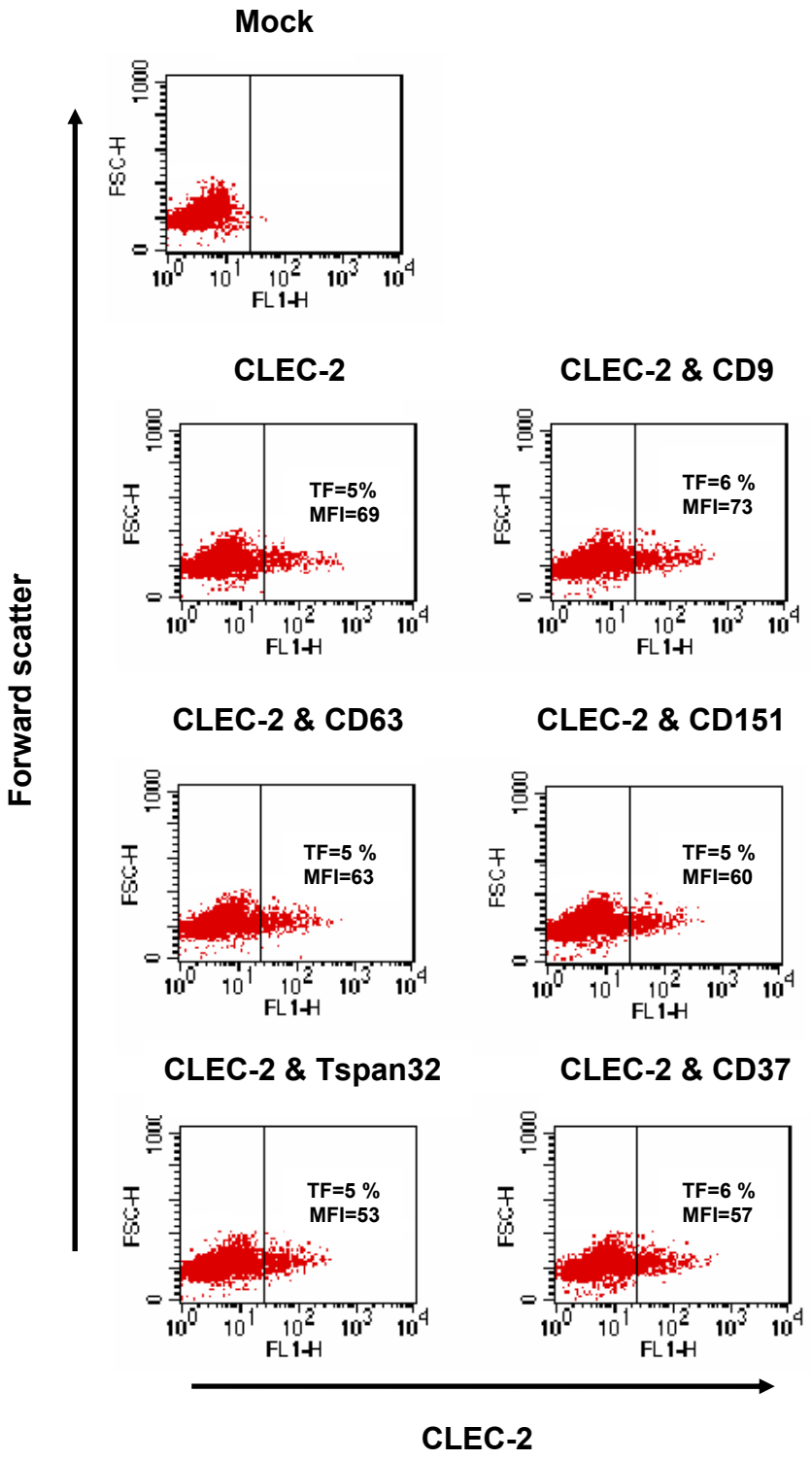

Mock
CLEC-2
CLEC-2 & CD9
TF=5%
MFI=69
TF=6 %
MFI=73
CLEC-2 & CD63
CLEC-2 & CD151
TF=5 %
MFI=63
TF=5 %
MFI=60
CLEC-2 & Tspan32
CLEC-2 & CD37
TF=5 %
MFI=53
TF=6 %
MFI=57
FSC-H
FL1-H
Forward scatter
CLEC-2

Figure 5.2 Tetraspanin over-expression inhibits CLEC-2 signalling in Jurkat T-cells

Jurkat T-cells were transiently transfected with an NFAT/AP-1-luciferase reporter construct, β-galactosidase construct, pEF6-MYC-CLEC-2 (10 μg) and pEF6-CD37, CD9, CD63, CD151, Tspan32 (20 μg) or empty pEF6 vector (mock). Cells were stimulated with media alone (unstimulated), rhodocytin (50 nM) (A) and PMA/ionomycin (B) for six hours as indicated. Luciferase activity was measured following stimulation and transfection efficiency was corrected to β-galactosidase activity in individual experiments. The CLEC-2 with rhodocytin stimulation value was set to 100% and other conditions presented were calculated relative to this. An unpaired Student's *t*-test indicated a statistically significant difference ($P<0.05$) between CLEC-2 transfected alone compared to CLEC-2 co-transfected with the tetraspanins as indicated *. Error bars represent standard error of the mean. Experiments were performed twice. (C) Cells were stained with MYC primary antibody and then anti-mouse FITC-conjugated secondary antibody and analysed by flow cytometry. Dead cells were gated out using propidium iodide. Dot plots were analysed and values indicate the average transfection efficiency (TF) and mean fluorescence intensity (MFI) of protein on the cell surface. Data is representative of two independent experiments.

5.4 DISCUSSION

In this Chapter, over-expression of CD37 or the platelet-expressed tetraspanins CD9, CD63, CD151 and Tspan32 was shown to potently inhibit CLEC-2-induced NFAT/AP-1 in the Jurkat T-cell line. Such data is remarkably similar to that of dectin-1 signal inhibition by tetraspanin over-expression in Jurkat cells, as described in Chapter Three.

The data in this Chapter suggests that tetraspanins may have the ability to regulate the signalling of CLEC-2 and dectin-1, compared to other receptors that signal via a similar tyrosine kinase-linked mechanism, for example, the platelet collagen receptor, GPVI. Unlike some of the data for dectin-1 and tetraspanins, the level of tetraspanin over-expression required to inhibit CLEC-2 signalling was not determined. However, the powerful inhibition of CLEC-2 signalling would suggest that the tetraspanins were expressed in this assay. It is tempting to speculate that tetraspanins may be the global regulators of this family as a result of the data gathered in respect of both dectin-1 and CLEC-2. The recently identified CLEC9A would be an ideal candidate to study to determine whether tetraspanins regulate this receptor, as it signals via a single YXXL motif contained within the cytoplasmic tail (Huysamen et al., 2008).

There is the possibility that tetraspanins may regulate CLEC-2 on platelets. Indeed, there are differences between CLEC-2 and GPVI signalling on the platelet, and an association with a tetraspanin may be a possible explanation. For example, CLEC-2 signals as a dimer via two single YXXL motifs whereas GPVI signals via an ITAM; upon CLEC-2 stimulation with its ligand rhodocytin there is a distinct lag phase before the onset of shape change and aggregation compared to GPVI stimulation; and there is a partial dependence on the SLP-76/BLNK family of adaptor proteins in

respect of CLEC-2 compared to GPVI (Fuller et al., 2007; Suzuki-Inoue et al., 2006; Watson et al., 2001). Therefore, data gathered in this Chapter paves the way for CLEC-2-induced platelet aggregation in response to rhodocytin stimulation to be investigated using mice deficient in a tetraspanin.

5.5 SUMMARY

In this Chapter it has been shown that tetraspanins completely inhibit CLEC-2 signalling. These data suggest that the dectin-1/CLEC-2/CLEC9A family might be regulated by tetraspanins and that CLEC-2 signalling might be abnormal in tetraspanin-deficient platelets.

CHAPTER 6 –

CLEC-2 SIGNALLING IS NORMAL IN CD9-DEFICIENT PLATELETS

6.1 INTRODUCTION

6.1.1 CLEC-2 and platelets

CLEC-2 is expressed on a number of human haematopoietic cells, including monocytes, granulocytes, dendritic cells and platelets (Colonna et al., 2000; Kerrigan et al., 2009; Suzuki-Inoue et al., 2006). CLEC-2 was identified on both human and mouse platelets as a receptor for the snake venom toxin rhodocytin by Suzuki-Inoue et al. (2006) using rhodocytin affinity chromatography and mass spectrometry. Indeed, it has recently been shown that treatment of mice with a CLEC-2 antibody leads to a specific loss of CLEC-2 in circulating platelets for several days resulting in defective aggregate formation *in vivo* and *in vitro*, and as a consequence increased bleeding times and impaired occlusive thrombus formation (May et al., 2009). In addition, the transmembrane glycoprotein podoplanin was found to be a ligand for CLEC-2 (Suzuki-Inoue et al., 2007). Podoplanin is expressed on tumour cells, lymphatic endothelial cells and renal cells (podocytes), and the physiological significance of the CLEC-2 interaction with podoplanin has yet to be determined (Christou et al., 2008; Ozaki et al., 2009; Wicki and Christofori, 2007).

6.1.2 The role of tetraspanins on platelets

The first indication that tetraspanins play a role in platelet function was the ability of both CD9 and CD151 antibodies to induce platelet activation (Roberts et al., 1995; Wu et al., 2000). Moreover, it was established that CD9 is expressed at approximately 49,000 copies per platelet, which has recently been confirmed (Higashihara et al., 1990; Protty et al., 2009). In addition, several groups have performed co-immunoprecipitation studies in relatively weak detergents suggesting an interaction between tetraspanins and integrin $\alpha_{IIb}\beta_3$ (Israels et al., 2001). However, our group

were unable to detect such an association suggesting the possibility of a relatively weak interaction (Protty et al., 2009). Nevertheless, studies suggested a role for tetraspanins on platelets. To date, at least five tetraspanins are known to be expressed on the platelet: CD9, CD63, CD151, Tspan32 and Tspan9 (Goschnick and Jackson, 2007; Protty et al., 2009; Tomlinson, 2009). In addition, recent proteomic studies suggest the additional expression of several other tetraspanins in human platelets (Lewandrowski et al., 2009). However, these cannot be confirmed to be platelet-expressed until antibodies are used due to the possibility of contamination from other blood cells (Tomlinson, 2009).

Mice deficient in CD151 or Tspan32 have a mild bleeding phenotype (Goschnick et al., 2006; Lau et al., 2004). In particular, the authors observe defective platelet spreading on fibrinogen, delayed clot retraction and a tendency to re-bleed in tail-bleeding assays, and attribute these observations to defective "outside-in" signalling of integrin $\alpha_{IIb}\beta_3$ (Goschnick and Jackson, 2007). In addition, an *in vivo* thrombus defect is observed in the Tspan32 knockout (Goschnick et al., 2006).

The role of CD9 on platelets had not been thoroughly investigated until relatively recently (Mangin et al., 2009). CD9 has a relatively high expression level of 49,000 copies per human platelet, second only to the major platelet integrin $\alpha_{IIb}\beta_3$ at 80,000 copies per cell (Higashihara et al., 1990; Protty et al., 2009; Wagner et al., 1996), and it was of great interest to determine its role on platelets. However, Mangin et al. found a minor phenotype in CD9-deficient platelets, including a shorter bleeding time in tail bleeding assays, a reduced overall blood loss, increased *in vivo* thrombosis and increased $\alpha_{IIb}\beta_3$ activation, as determined by fibrinogen binding, compared to wild-type controls (Mangin et al., 2009). This data was surprising given the relatively high

expression level of CD9, and is in contrast to CD151- and Tspan-32-deficient platelets, and suggests that in wild-type mice CD9 may prevent excessive thrombus growth by acting as a negative regulator of platelet recruitment (Mangin et al., 2009).

Recently, mice deficient in the tetraspanin CD63, which is localized to dense granules and lysosomes, have been reported with only very minor differences in platelet function (Schroder et al., 2009). The only difference between wild-type and CD63-deficient platelets are slightly stronger aggregation responses in the knockout *in vitro* (Schroder et al., 2009). In addition, CD63 has been shown to associate with integrin $\alpha_{IIb}\beta_{3,}$ with the authors hypothesising that it may regulate platelet spreading and signalling on fibrinogen, but any functional significance of this interaction has yet to be determined (Israels and McMillan-Ward, 2005).

Lastly, Tspan9, a previously uncharacterised tetraspanin, has recently been identified as a novel platelet tetraspanin (Protty et al., 2009). Expression of Tspan9 is relatively specific to platelets and megakaryocytes (Protty et al., 2009), suggesting an important role for this tetraspanin in common with other platelet/megakaryocyte-specific cell surface proteins such as integrin $\alpha_{IIb}\beta_3$, GPIb-IX-V and GPVI.

6.1.3 The role of tetraspanins in CLEC-2 signalling on platelets

In Chapter Five, tetraspanin over-expression potently inhibited CLEC-2-induced NFAT/AP-1 luciferase activity following stimulation with rhodocytin in the Jurkat T-cell line. Therefore, the basis of this Chapter was to investigate CLEC-2-induced platelet aggregation following stimulation with rhodocytin in CD9-deficient platelets. CD9 was selected due to its relatively high expression of 49,000 copies per cell (Protty et al., 2009).

6.2 AIMS

- To investigate CLEC-2-induced platelet aggregation following rhodocytin stimulation from mice deficient in the tetraspanin CD9.
- To determine whether tetraspanins co-immunoprecipitate with CLEC-2 in human platelets.

6.3 RESULTS

6.3.1 CLEC-2 cell surface expression is normal in the absence of CD9

CLEC-2 cell surface expression was determined on $CD9^{+/+}$ and $CD9^{-/-}$ platelets by incubating with a rabbit anti-mouse CLEC-2 antibody or with a relevant negative control. CLEC-2 cell surface expression was similar in $CD9^{+/+}$ and $CD9^{-/-}$ platelets (Figure 6.1A). A Student's *t*-test indicated that there was no significant difference between $CD9^{+/+}$ and $CD9^{-/-}$ when data was expressed as the geometric mean of CLEC-2 cell surface expression from three mice (Figure 6.1B).

6.3.2 CLEC-2-induced aggregation is normal in $CD9^{-/-}$ mouse platelets

It was pertinent to investigate CLEC-2 signalling in the platelet utilising mice deficient in CD9 to determine whether platelets would exhibit altered aggregation following stimulation of the receptor with rhodocytin. Platelets were stimulated with a high (100 nM), intermediate (15 nM) and a sub-threshold (5 nM) dose of rhodocytin and aggregation responses were measured. $CD9^{-/-}$ platelet aggregation was not altered compared to $CD9^{+/+}$ at 100 nM and 15 nM rhodocytin (Figure 6.2A-B). Control and CD9-deficient platelets stimulated with 5 nM rhodocytin did not respond (Figure 6.2C), even after 20 minutes (data not shown). As a control, aggregation responses were then measured to the platelet agonists ADP and collagen, which act upon the GPCRs $P2Y_1$ and $P2Y_{12,}$ and the collagen receptors GPVI and integrin $\alpha_2\beta_{1,}$ respectively. $CD9^{+/+}$ and $CD9^{-/-}$ platelets were similar in their aggregation response to 5 μg/ml collagen and 2 μM ADP (Figure 6.2D-E). Together these data demonstrate that CLEC-2-induced platelet aggregation is not dependent on CD9.

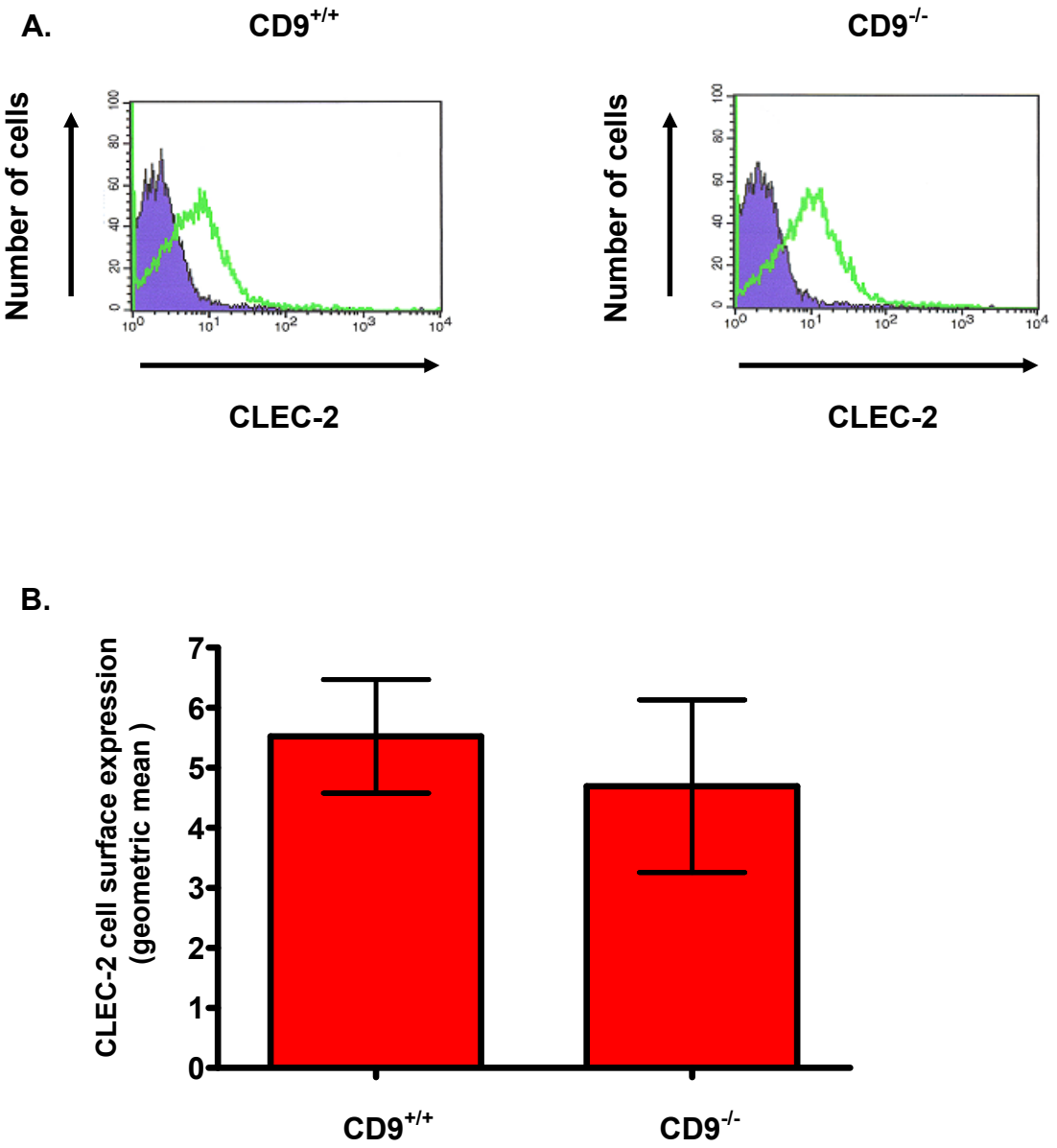


Figure 6.1 Normal CLEC-2 cell surface expression on $CD9^{+/+}$ and $CD9^{-/-}$ mouse platelets

(A) $CD9^{+/+}$ and $CD9^{-/-}$ washed mouse platelets were stained with either rabbit anti-mouse negative (filled histograms) or rabbit anti-mouse CLEC-2 polyclonal primary antibody (open histograms) and then anti-mouse FITC-conjugated secondary antibody. Antibody binding was visualized by flow cytometry. (B) Data is also represented as the geometric mean cell surface expression level from three independent experiments. An unpaired Student's *t*-test indicated no statistically significant difference between $CD9^{+/+}$ and $CD9^{-/-}$. Experiments were performed three times.

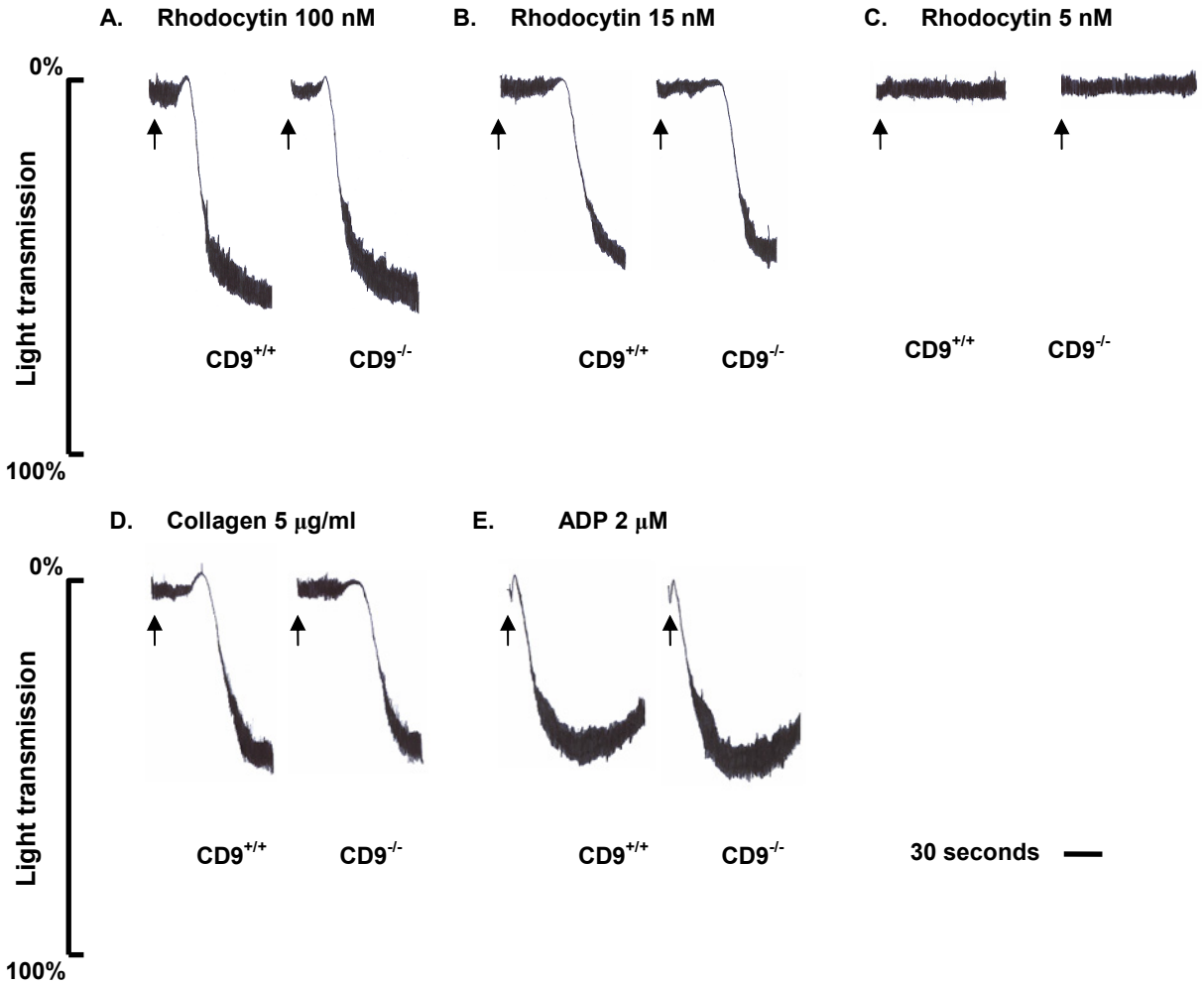


Figure 6.2 CD9$^{-/-}$ mouse platelets aggregate normally following stimulation of CLEC-2 with rhodocytin

Washed mouse platelets were pre-incubated with human fibrinogen (0.32 mg/ml) for 30 seconds and aggregation was measured for 3 minutes following stimulation with either 100 nM rhodocytin (A), 15 nM rhodocytin (B), 5 nM rhodocytin (C), 5 μg/ml collagen (D) or 2 μm ADP (E). The arrow indicates the addition of the agonist. Data are representative of 2-3 independent experiments.

6.3.3 CLEC-2-induced tyrosine phosphorylation is normal in the absence of CD9

Lysates in 1% NP-40 were prepared following aggregation to investigate the possibility of a phosphorylation defect in $CD9^{-/-}$ platelets. Samples were resolved on gradient 4-12% reducing SDS-PAGE and incubated with anti-phosphotyrosine (4G10) antibodies. There were no major differences in the phosphorylation profiles between $CD9^{+/+}$ and $CD9^{-/-}$ platelets following rhodocytin stimulation, although some phospho-proteins appeared to be slightly enhanced in the absence of CD9 (Figure 6.3A). As this experiment was performed once, it is worthy of further investigation, for example, repeating the experiment using a time course and blotting with phospho-specific antibodies to identify the up-regulated phospho-proteins. Indeed, if such small changes are reproducible, it is possible that aggregation is insufficiently sensitive to detect such small changes in reactivity, which nevertheless would be consistent with a role for tetraspanins in the negative regulation of CLEC-2 signalling. As a control, lysates were blotted with an anti-CD9 antibody (Figure 6.3B) to confirm the absence of CD9 in the $CD9^{-/-}$ platelets.

6.3.4 Other tetraspanins are not up-regulated in CD9-deficient megakaryocytes

One possibility for the lack of phenotype in $CD9^{-/-}$ mouse platelets is that other tetraspanins may be up-regulated thereby compensating for the lack of CD9. A phylogenetic analysis of mammalian tetraspanins (www.ebi.ac.uk/clustalw/) revealed that Tspan2 and CD81 are highly related to CD9 (48% and 45% respectively) according to amino-acid identity (Figure 1.1). Other candidates that might be up-regulated are the known platelet tetraspanins CD63, CD151, Tspan9 and Tspan32. However, antibodies are not available to most of these mouse tetraspanins, therefore mRNA levels were determined using semi-quantitative RT-PCR for megakaryocytes, the platelet progenitors. Megakaryocytes were used because the anulceate platelet

contains low levels of mRNA (Gnatenko et al., 2003) such that RT-PCR is technically difficult due to the potential for contamination with mRNA from other blood cells. Tetraspanins were amplified using cDNA from $CD9^{+/+}$ and $CD9^{-/-}$ megakaryocytes (Figure 6.4). A positive control RT-PCR was performed using primers against GAPDH. It was apparent that no tetraspanins were up-regulated in the $CD9^{-/-}$ megakaryocytes when compared to $CD9^{+/+}$. Slightly increased CD81 levels were apparent in the two lanes containing the highest concentration of target megakaryocyte cDNA (Figure 6.4). However, it is difficult to interpret this because these concentrations both appear to give a saturating signal. Indeed, the lack of any substantial CD81 upregulation was supported by a second, independent experiment, in which CD81 PCR bands were of identical intensity between the wild-type and knockout (M.G. Tomlinson, Appendix Figure I.I).

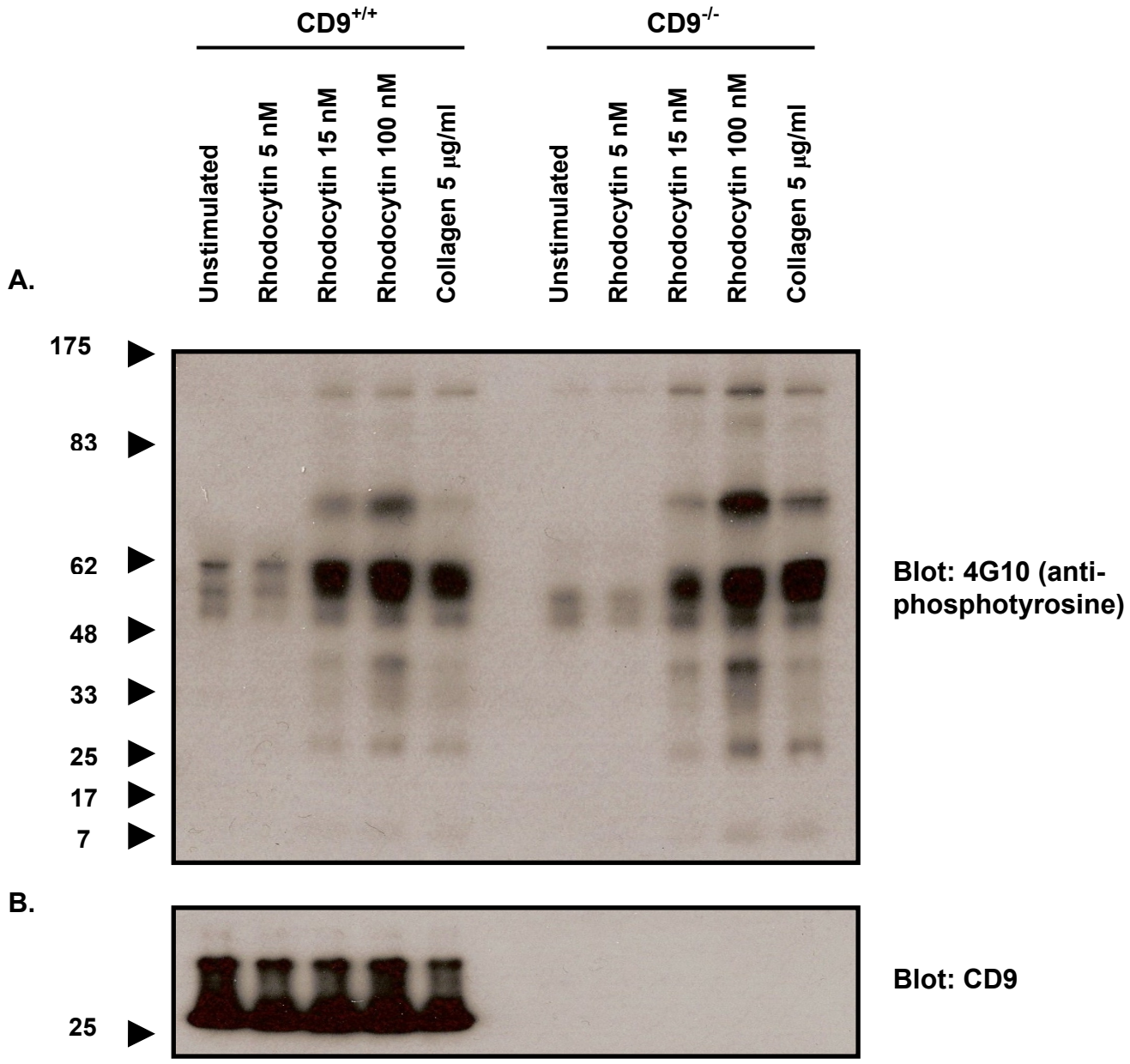


Figure 6.3 CD9$^{+/+}$ and CD9$^{-/-}$ mouse platelets phosphorylation profiles

Mouse platelets were lysed with 1% NP-40 following aggregation with the indicated agonists for 3 minutes. Proteins in whole cell lysates were visualised by SDS-PAGE and western blotting with indicated antibodies. This experiment was performed once.

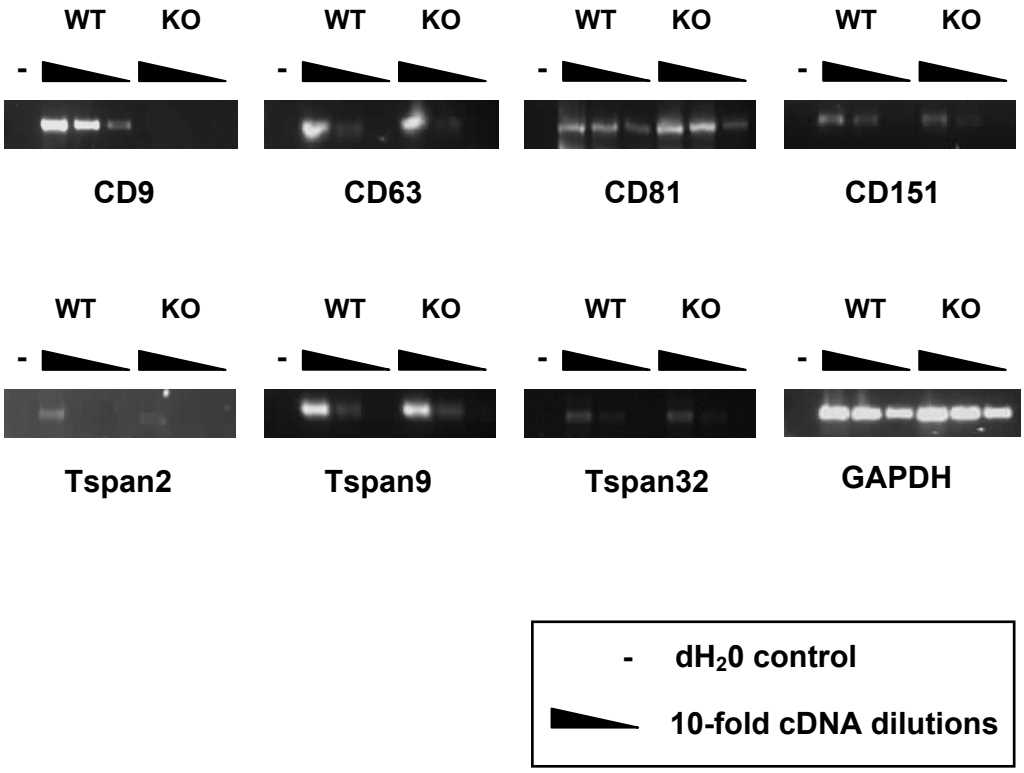


Figure 6.4 RT-PCR of CD9$^{-/-}$ megakaryocytes suggest that other tetraspanins are not up-regulated

RT-PCR was performed on CD9$^{+/+}$ (WT) and CD9$^{-/-}$ (KO) megakaryocytes using primers against particular tetraspanins as indicated, and cDNA templates at 10-fold dilutions. In addition, dH_20 and GAPDH were used as negative and positive controls respectively. Data is representative of two independent experiments.

6.3.5 Tetraspanins do not co-immunoprecipitate with CLEC-2 in rhodocytin-stimulated human platelets

To determine whether tetraspanins interact with CLEC-2 in platelets, a co-immunoprecipitation approach in rhodocytin-stimulated human platelets was performed. Washed human platelets were stimulated with either 300 nM rhodocytin or left unstimulated, and lysed in the relatively mild detergent Brij97, thought to maintain tetraspanin-tetraspanin interactions (Hemler, 2005). Aggregations were performed to ensure that the rhodocytin stimulation had worked (data not shown). Samples were immunoprecipitated for CLEC-2, CD9 or ADAM-10, the latter serving as a positive control as it is known to be a tetraspanin-associated protein (Arduise et al., 2008). Both CD9 and ADAM-10 did not co-immunoprecipitate with CLEC-2 in Brij97 lysis conditions in either unstimulated or rhodocytin-stimulated conditions (Figure 6.5A). However, CLEC-2 was detected in the whole cell lysate and CLEC-2 immunoprecipitations (Figure 6.5A). Moreover, CD9 co-immunoprecipitated with the ADAM-10 positive control (Figure 6.5B). Together, these data suggest that tetraspanins either do not interact with CLEC-2 or that the interaction is weak and not detectable by co-immunoprecipitation.

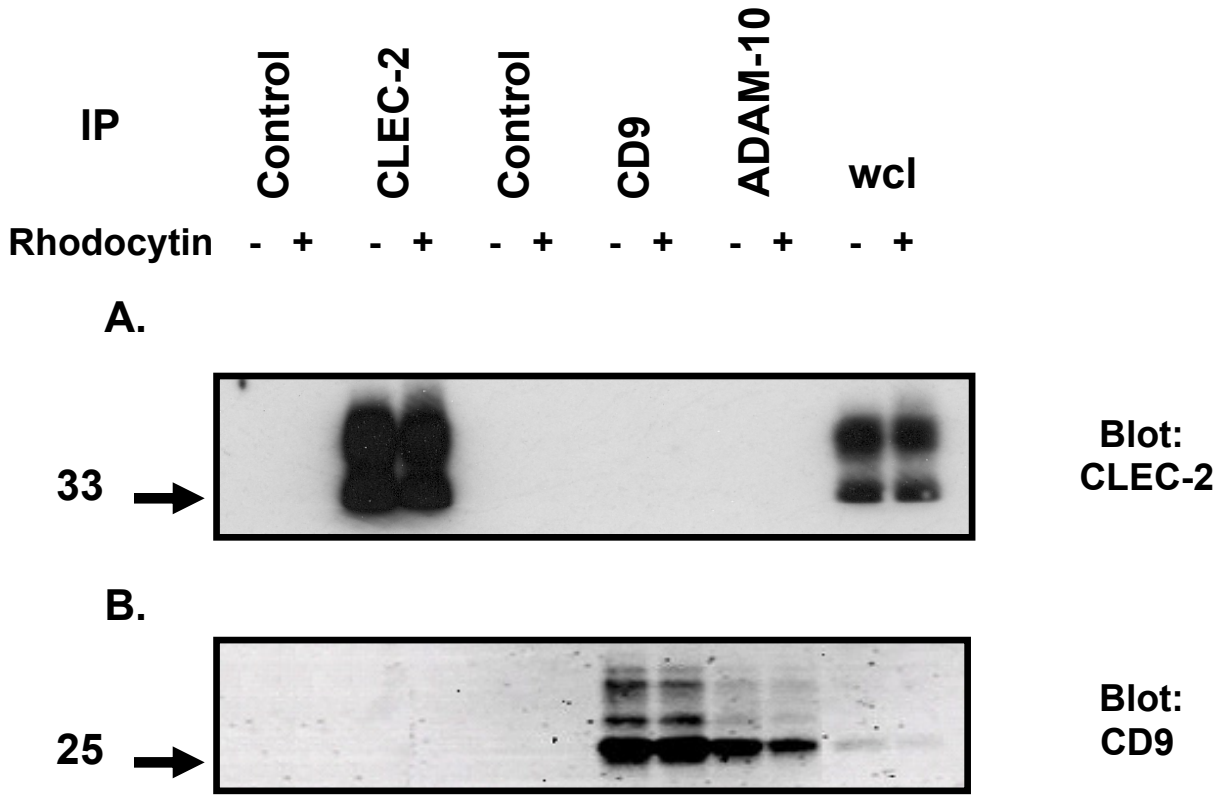


Figure 6.5 Tetraspanins do not co-immunoprecipitate with CLEC-2 in rhodocytin-stimulated human platelets

Washed human platelets were stimulated with 300 nM rhodocytin or left unstimulated, lysed with 1% Brij97 and immunoprecipitated for 1.5 hours with the indicated antibodies. Proteins were visualised by SDS-PAGE and western blotting with the indicated antibodies. Note that CD9 was only weakly detected in the whole cell lysate (wcl) at this level of exposure. Data was kindly provided by Dr. M.G. Tomlinson.

6.4 DISCUSSION

In this Chapter, it has been shown that mice deficient in the tetraspanin CD9 do not have a platelet aggregation or signalling phenotype following stimulation of CLEC-2 using high, intermediate or low doses of the snake venom toxin rhodocytin. In addition, no interaction was observed between CD9 and CLEC-2 in rhodocytin-stimulated human platelets using the relatively weak detergent Brij97, which is not thought to disrupt tetraspanin microdomains. This suggests that either CLEC-2 does not interact with tetraspanins or that if it does interact, but the interaction is not stable during co-immunoprecipitation in Brij97 lysis conditions.

One possibility for the lack of a platelet phenotype was the up-regulation of other tetraspanins to compensate for the lack of CD9. Functional redundancy has been suggested to account for the subtle phenotypes observed in mouse platelets deficient in either CD151 or Tspan32 (Goschnick et al., 2006; Lau et al., 2004). Other examples of compensation include the phenotype observed in $CD9^{-/-}$ oocytes where injection of CD81 mRNA restores the fertilization rate to approximately 50% of wild-type levels (Kaji et al., 2002). Therefore, a semi-quantitative RT-PCR approach was performed using the progenitor of the platelet, the megakaryocyte, to determine whether other tetraspanins are up-regulated in CD9-deficient megakaryocytes. RT-PCR is difficult to perform in platelets due to the potential for contamination as platelets express relatively low levels of mRNA (Gnatenko et al., 2003). No such mRNA upregulation was observed for the CD9-related tetraspanins Tspan2 and CD81, or the platelet-expressed tetraspanins CD63, CD151, Tspan9 and Tspan32. The RT-PCR approach used was not fully quantitative and therefore future experiments in the platelet would be necessary, as this does not prove that tetraspanins are not up-

regulated at the protein level. For example, a mouse CD81 antibody exists (Maecker et al., 2000), and so it is possible to determine whether CD81 is up-regulated in the platelet. However, this question will not be able to be addressed fully until antibodies have been generated for all tetraspanins.

Multiple tetraspanins may be expressed in platelets, in addition to CD9, CD63, CD151, Tspan32 and Tspan9 (Goschnick and Jackson, 2007; Protty et al., 2009; Tomlinson, 2009), and it is possible that CD9 is not the tetraspanin that regulates CLEC-2. Indeed, platelet phenotypes are observed in CD151 and Tspan32 knockouts, but mild phenotypes are only observed in CD9 and CD63 knockouts, thus highlighting the differences in tetraspanin-deficient mice (Goschnick et al., 2006; Lau et al., 2004; Mangin et al., 2009; Schroder et al., 2009). There is the possibility that CD151, Tspan32, CD63, Tspan9, or another platelet tetraspanin may specifically regulate the signalling of CLEC-2 on platelets, perhaps by binding directly to the receptor to regulate its clustering or downstream signalling. Further work to address these questions would include utilising mice deficient in CD151, Tspan32 and CD63, or indeed any tetraspanin-deficient mice that are generated in the future.

A final point is to question whether rhodocytin is the ideal agonist to activate CLEC-2. Podoplanin has been identified as the natural endogenous ligand for CLEC-2 (Suzuki-Inoue et al., 2007) and a potential second ligand has been proposed in plasma or on the surface of platelets (May et al., 2009). It is possible that a tetraspanin association with CLEC-2 is more important for activation by these endogenous agonists.

6.5 SUMMARY

In this Chapter it has been shown that mice deficient in the tetraspanin CD9 do not have a platelet aggregation or signalling phenotype following stimulation of CLEC-2 with rhodocytin. Moreover, CLEC-2 cell surface expression is similar between $CD9^{+/+}$ and $CD9^{-/-}$ platelets. In addition, it appears that other tetraspanins are not up-regulated in megakaryocytes. Finally, tetraspanins do not co-immunoprecipitate with CLEC-2 in rhodocytin-stimulated human platelets.

CHAPTER 7 –

GENERAL DISCUSSION

7.1 Summary of findings

The main objective of this thesis was to investigate the role tetraspanins and their ability to regulate the signalling of dectin-1 and CLEC-2 in model cell lines using a transcriptional reporter assay. A secondary aim was to investigate CLEC-2-induced platelet aggregation in mice deficient in the major platelet tetraspanin CD9. The work in this thesis was stimulated by a publication of the van Spriel group that suggests that the tetraspanin CD37 negatively regulates dectin-1-mediated signalling (Meyer-Wentrup et al., 2007). However, the role of other tetraspanins in both dectin-1 and CLEC-2 signal regulation had not been investigated until now. The work done in this thesis suggests that transient over-expression of multiple tetraspanins can negatively regulate dectin-1 and CLEC-2 signalling in a model cell line, suggesting that tetraspanins play a general role in regulating C-type lectin signalling. Specifically, this thesis demonstrates that: (a) over-expression of multiple tetraspanins can potently inhibit dectin-1 signalling; (b) multiple tetraspanins similarly regulate CLEC-2 signalling; and (c) CLEC-2-induced platelet aggregation is, however, normal in the absence of the major platelet tetraspanin CD9. The mechanism behind tetraspanin inhibition of dectin-1 and CLEC-2 signalling in the cell line is not clear. Nevertheless, we hypothesise that tetraspanins play an important role in the regulation of dectin-1 and CLEC-2 in the endogenous setting. This could be via a direct mechanism, by recruitment of the two receptors into tetraspanin microdomains. Alternatively the mechanism could be indirect, if a protein that regulates dectin-1 and CLEC-2 is itself regulated by tetraspanins.

7.2 Direct and indirect models for tetraspanin regulation of C-type lectin-like receptors

This thesis has attempted to establish a conclusive mechanism as to how tetraspanins can regulate both dectin-1 and CLEC-2 signalling, but a number of questions still remain. In particular, how can many different tetraspanins, in fact all that were tested, each almost completely inhibit dectin-1 and CLEC-2 signalling when over-expressed in Jurkat T-cells? It seems improbable that this could be achieved by a direct interaction between all these over-expressed tetraspanins and dectin-1 and CLEC-2. Therefore it would appear that transiently over-expressed tetraspanins perturb the cell in some way so as to largely prevent signalling by these receptors. Since the most well-characterised feature of tetraspanins is their capacity to interact with other tetraspanins, it is likely that tetraspanin over-expression perturbs the cell by associating with endogenous tetraspanins and disrupting the architecture and function of tetraspanin microdomains.

7.2.1 Direct models for tetraspanin regulation of C-type lectin-like receptors

There are a number of potential direct models for the negative regulation of C-type lectin-like receptor signalling by tetraspanins. Such models, which assume a direct interaction between one specific tetraspanin and the lectin-like receptor, and regulation of the receptor within tetraspanin microdomains, are based on the hypothesis that over-expression of any single tetraspanin can disrupt the normal make-up and function of tetraspanin microdomains. These microdomain disruption models include a loss of tetraspanin-associated signalling machinery and the impairment of lipid raft localization, both of which are discussed in more detail below.

7.2.1.1 Loss of tetraspanin-associated signalling machinery

This is a simple model by which tetraspanin over-expression might negatively regulate both dectin-1 and CLEC-2 (Figure 7.1). Recent studies have shown that the plasma membrane contains several hundred punctate tetraspanin microdomains, with an average size of 0.2 μm^2 (Espenel et al., 2008; Nydegger et al., 2006). In the endogenous setting, dectin-1 or CLEC-2 may be recruited into tetraspanin microdomains enabling the receptor to signal, perhaps because essential downstream signalling molecules are also recruited to these microdomains via interactions with specific tetraspanins. However, following transient tetraspanin over-expression, the normal microdomain organisation may become disrupted and dysfunctional due to domination of the microdomain by a single tetraspanin. Consequently, the lectin-like receptor may be unable to couple to the relevant signalling machinery resulting in signal inhibition. An argument against this model is the fact that CLEC-2 was not co-immunoprecipitated with tetraspanins in platelets, even in relatively low stringency lysis conditions (1% Brij97) that are thought to preserve microdomain integrity (Hemler, 2005). Moreover, there is no evidence that any of the signalling molecules utilised by lectin-like receptors are present in tetraspanin microdomains. Indeed, it has recently been shown that CLEC-2 translocates to lipid rafts upon ligand engagement (Pollitt et al., 2010).

7.2.1.2 Lipid raft localization

The lipid raft localization model is another possible direct mechanism of C-type lectin-like receptor regulation that is dependent on microdomain disruption by tetraspanin over-expression (Figure 7.2). In the endogenous setting, the lectin-like receptor may associate with an as yet unidentified tetraspanin. Following receptor cross-linking upon stimulation, it becomes activated and may localize to lipid rafts,

which are rich in C-type lectin-like-activating signalling molecules such as Src family tyrosine kinases. This hypothesis is supported by data which shows that CLEC-2 translocates to lipid rafts following stimulation with rhodocytin in platelets (Pollitt et al., 2010), and by very recent similar data for dectin-1 (Xu et al., 2009a). The putative C-type lectin-like receptor-associated tetraspanin might then become palmitoylated by a lipid raft-resident palmitate transferase, resulting in sustained localization to lipid rafts and sustained signalling. Such a mechanism would be analogous to the regulation of the B-cell receptor/CD19/CD21 complex by the tetraspanin CD81, where inducible palmitoylation of CD81 is required for sustained localization of the B-cell receptor-CD19/CD21/CD81 complex to lipid rafts, thus allowing productive signalling (Cherukuri et al., 2004a; Cherukuri et al., 2004b). If this is true, over-expression of any tetraspanin might out-compete the lectin-like receptor for binding to its specific tetraspanin partner, sequestering the tetraspanin away from the receptor. The latter would then fail to exhibit sustained raft localization and sustained signalling, resulting in a failure to activate NFAT/AP-1. This hypothesis could be easily tested by fractionation in a sucrose gradient of stimulated and unstimulated cells. In common with the previously described direct model, this model is not compatible with the failure to co-immunoprecipitate platelet CLEC-2 with tetraspanins. However, it is possible that a potential tetraspanin-CLEC-2/dectin-1 interaction might involve a tetraspanin that has yet to be tested, because of a current lack of antibodies to most tetraspanins.

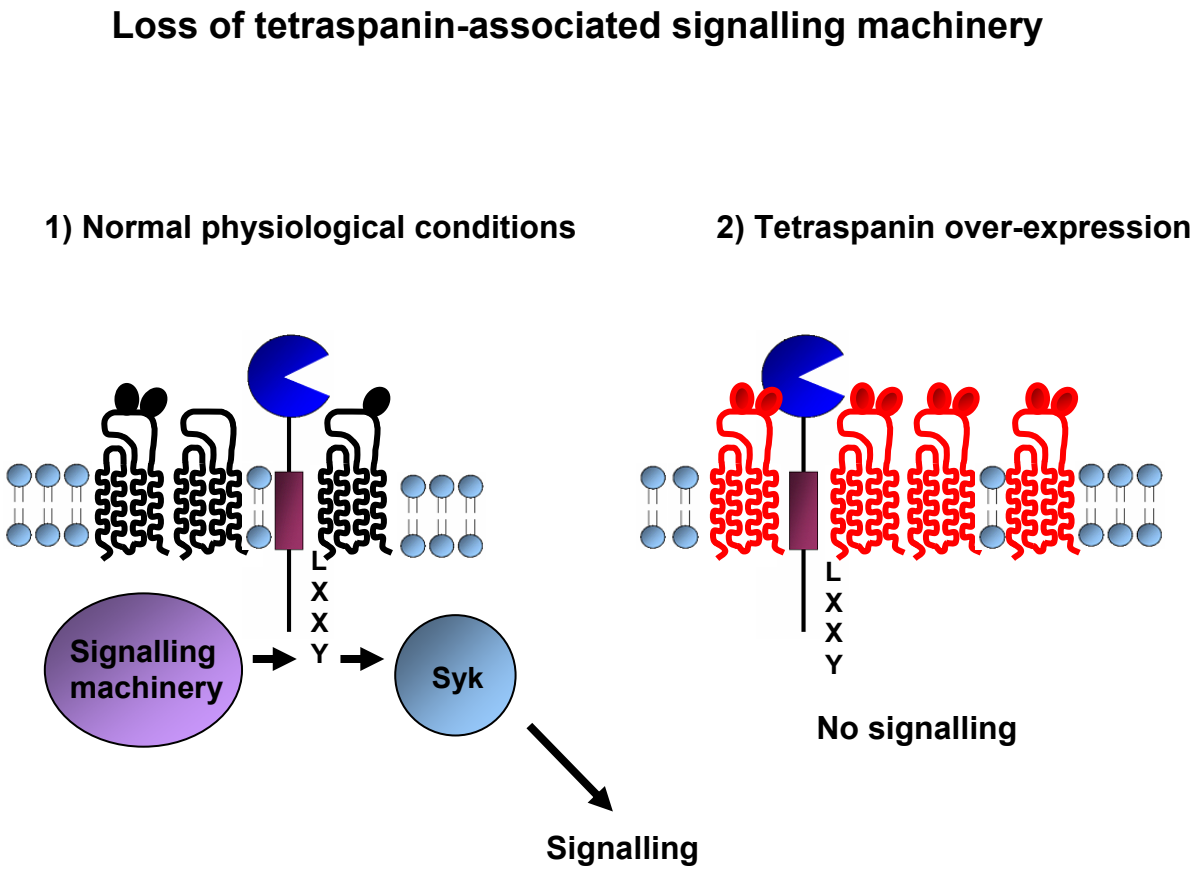


Figure 7.1 Loss of tetraspanin-associated signalling machinery

The loss of tetraspanin-associated signalling machinery is a simple model which assumes that the C-type lectin-like receptor and its signalling machinery is a component of tetraspanin microdomains (1). Tetraspanin over-expression (shown in red) could displace endogenous tetraspanins (three different tetraspanins shown in black) and associated signalling machinery, resulting in a failure of the receptor to signal (2).

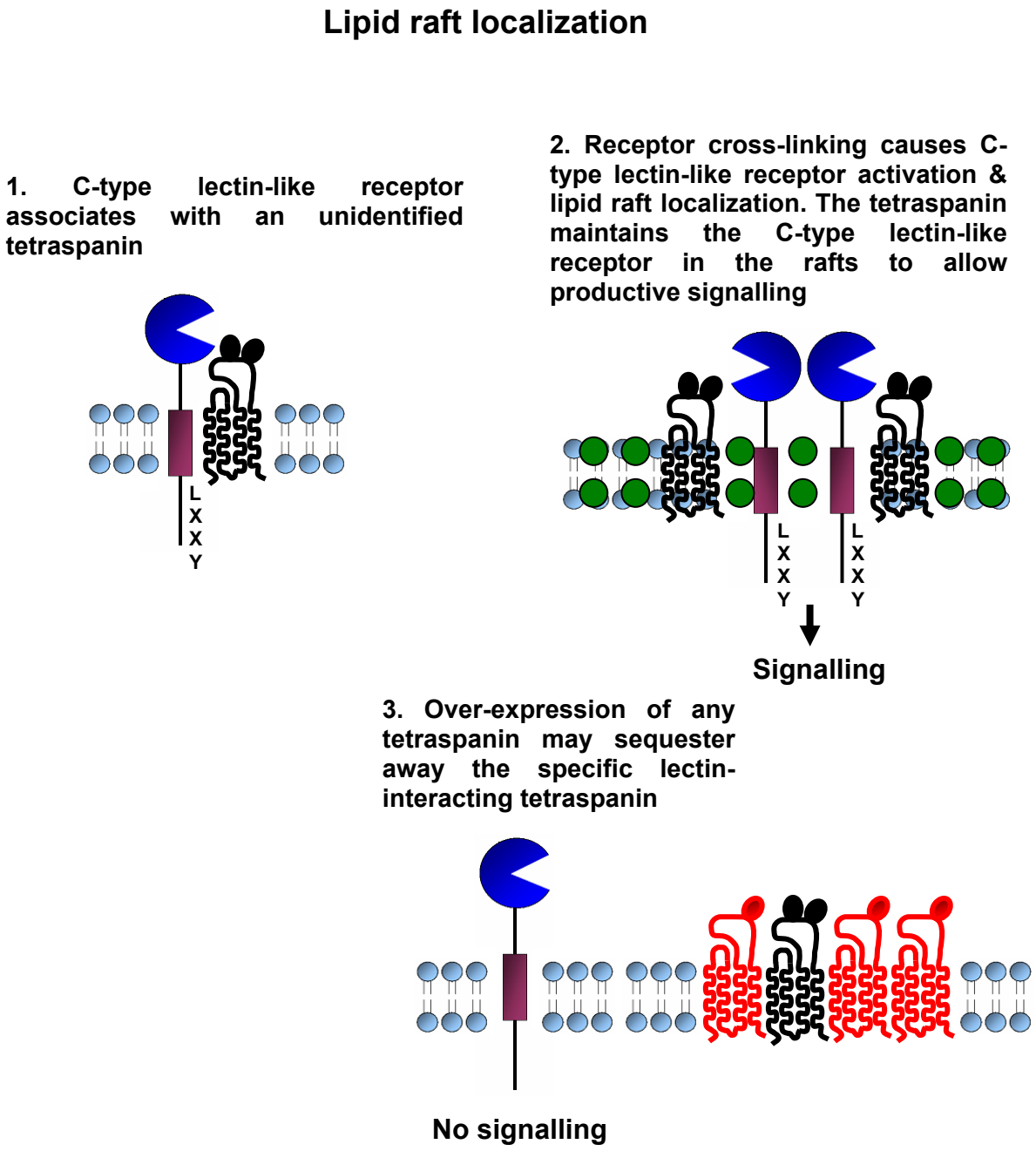


Figure 7.2 Lipid raft localization

The lipid raft localization model is one model by which tetraspanins may regulate C-type lectin-like receptor signalling. This model hypothesises that dectin-1/CLEC-2 associate with a specific tetraspanin, shown in black (1), which promotes lipid raft localisation upon receptor cross-linking (2) in a manner that is similar to that shown in regard to the B-cell receptor and the tetraspanin CD81 (Cherukuri et al., 2004a; Cherukuri et al., 2004b). Lipid raft-specific lipids are shown in green and the over-expressed tetraspanin in red. The latter could disrupt normal microdomain architecture, sequester away the specific dectin-1/CLEC-2-interacting tetraspanin, and so lead to a failure of dectin-1/CLEC-2 to be sustained in lipid rafts and consequently a failure to signal normally (3).

7.2.2 Indirect models for tetraspanin regulation of C-type lectin-like receptors

If dectin-1 and CLEC-2 do not interact with tetraspanins, which would be consistent with the failure to co-immunoprecipitate the latter with platelet tetraspanins, then an indirect mechanism must be responsible for the inhibitory effect of tetraspanin over-expression. Indeed, an unidentified protein may regulate dectin-1 or CLEC-2, which itself is regulated by tetraspanins and is dysregulated by tetraspanin over-expression. Putative tetraspanin-regulated candidates include transmembrane proteinases and signal-promoting molecules which are discussed in more detail below.

7.2.2.1 Dysfunction of a tetraspanin-associated transmembrane proteinase

Since the transmembrane proteinases ADAM-10 and MT1-MMP are thought to be regulated by tetraspanin microdomains (Arduise et al., 2008; Lafleur et al., 2009; Yanez-Mo et al., 2008), it is possible that over-expression of any tetraspanin might disrupt the microdomains leading to activation of the proteinase (Figure 7.3). The proteinase might cleave its targets, which could release soluble proteins that inhibit dectin-1/CLEC-2 signalling; dectin-1/CLEC-2 is unlikely to be the direct proteinase target because the experiments in this thesis found no evidence for dectin-1/CLEC-2 cleavage using flow cytometry and western blotting. If the proteinase or its dectin-1/CLEC-2-regulating target is present on Jurkat but not DT40 or RBL-2H3 cells, this could explain the failure to observe inhibition in the latter two lines. Such a model could readily be investigated by testing whether conditioned media from tetraspanin-transfected Jurkat cells could inhibit dectin-1 and CLEC-2 signalling.

7.2.2.2 Sequestration of an inhibitory protein within tetraspanin microdomains

Another possible indirect model could involve an inhibitory signalling protein that is normally tetraspanin-associated, and so sequestered away from the lectin-like receptor

(Figure 7.4). However, upon tetraspanin over-expression, the microdomain could become disrupted, so releasing the inhibitory protein that can subsequently inhibit dectin-1 and CLEC-2 signalling. The putative tetraspanin-associated inhibitory protein could be a phosphatase that dephosphorylates a molecule involved in C-type lectin-like signalling. Indeed, the van Spriel group allude to this model as a mechanism of dectin-1 signal regulation by the tetraspanin CD37 (Meyer-Wentrup et al., 2007). Moreover, the tetraspanin CD53 has been shown to associate with as yet, an unidentified phosphatase (Carmo and Wright, 1995).

Dysfunction of a tetraspanin-associated transmembrane proteinase

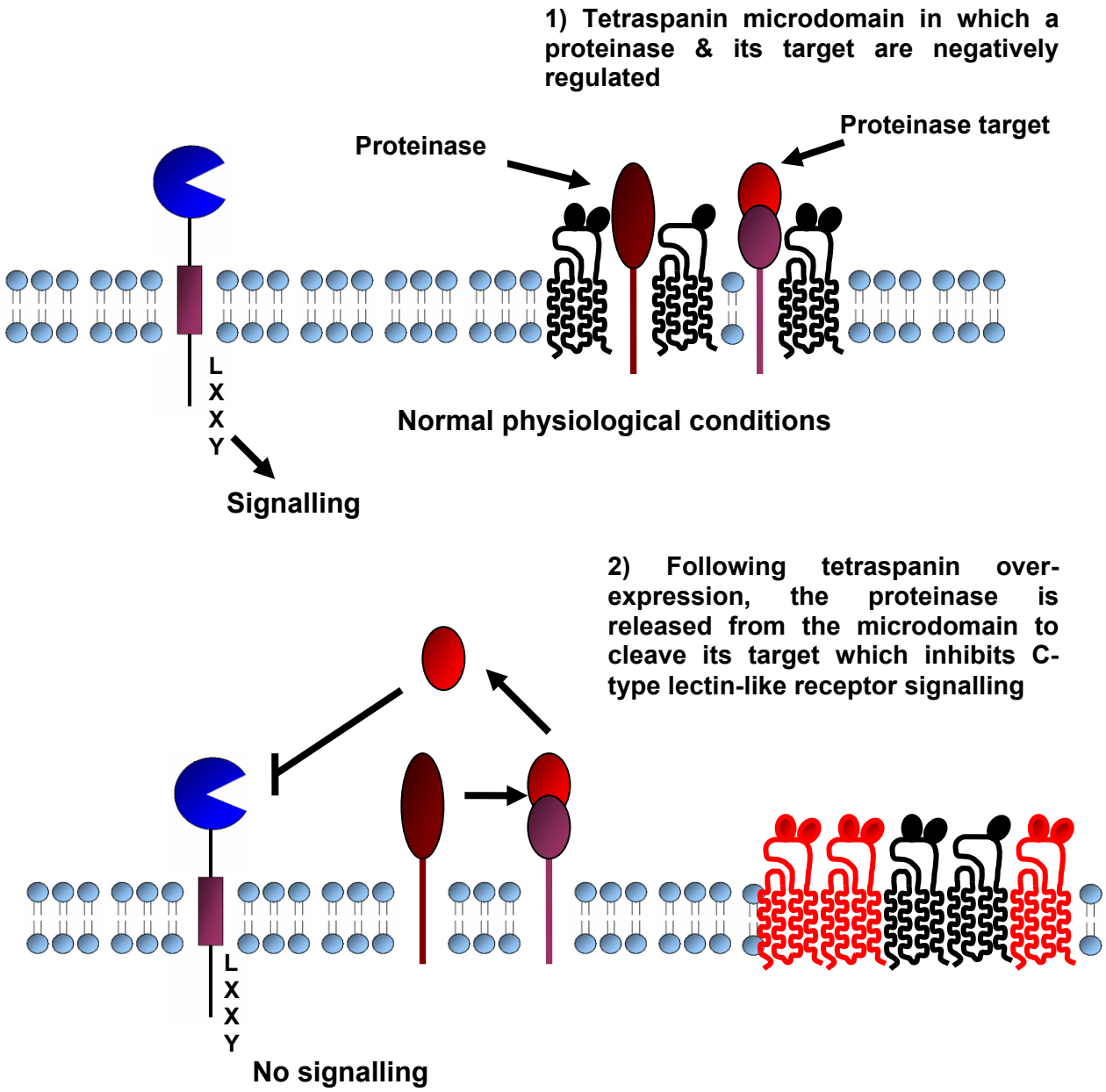


Figure 7.3 Dysfunction of a tetraspanin-associated transmembrane proteinase

A proteinase and its target are hypothesized to be negatively regulated within tetraspanin microdomains (1). Tetraspanin over-expression (shown in red) causes mis-localization and dysfunction of the proteinase, which can cleave its targets to release soluble proteins that inhibit C-type lectin-like receptor signalling (2).

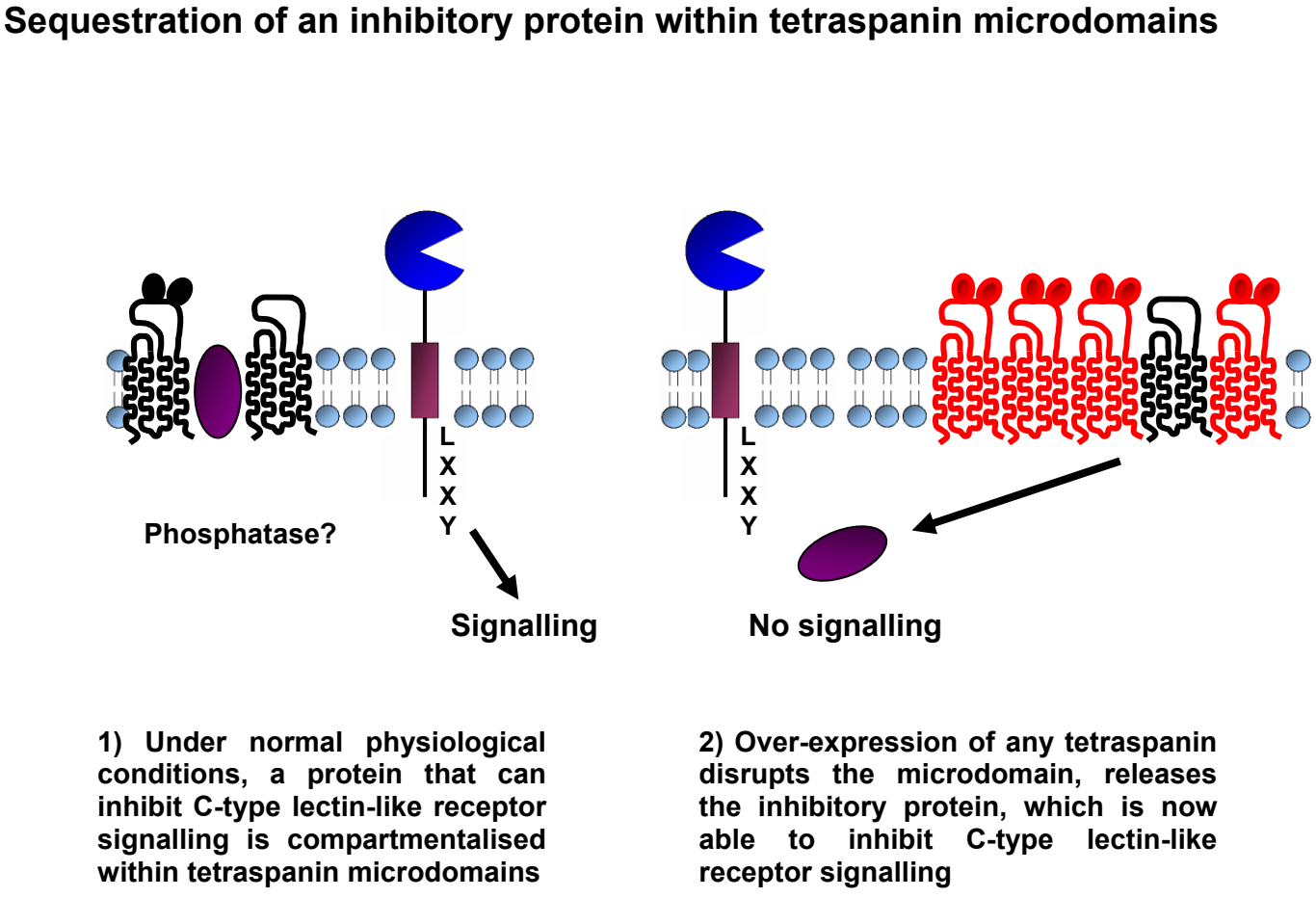


Figure 7.4 Sequestration of an inhibitory protein by tetraspanin microdomains

A membrane protein or an intracellular signalling protein, that can negatively regulate dectin-1 and CLEC-2 signalling, is normally compartmentalised into tetraspanin microdomains (1). However, upon tetraspanin over-expression (shown in red), the microdomain becomes disrupted releasing the protein, which is now able to inhibit dectin-1 and CLEC-2 signalling (2).

7.3 Future directions

This thesis has focussed on the regulation of the C-type lectin-like receptors dectin-1 and CLEC-2 by tetraspanins in model cell lines. However, the ultimate aim is to focus on the platelet-expressed CLEC-2 for the following reasons: the Nieswandt group have established an important role for CLEC-2 as a platelet activating receptor in thrombosis and haemostasis (May et al., 2009); there is no published data that suggests tetraspanins regulate CLEC-2 signalling; and our group's focus is on platelets.

7.3.1 Does tetraspanin over-expression disrupt tetraspanin microdomains?

As discussed previously, tetraspanin over-expression is likely to inhibit CLEC-2 signalling by interacting with endogenous tetraspanins and so disrupting the normal make-up and function of tetraspanin microdomains. There are a number of biochemical and microscopy-based experiments that could address the hypothesis that tetraspanin over-expression disrupts tetraspanin microdomains, as discussed below.

To biochemically test whether tetraspanin over-expression disrupts tetraspanin microdomains, one could use cell surface biotinylation followed by immunoprecipitation of an endogenous tetraspanin, under low stringency lysis conditions, and streptavidin blotting to detect interacting proteins. This could be done in Jurkat using the endogenous tetraspanin CD81 and transfected CD9, and the transfected cells would need to be first purified by flow cytometry sorting. Comparisons of banding patterns between CD9-transfected and control cells might reveal loss or reduced intensity of some bands following CD9 over-expression, indicating disruption of tetraspanin microdomains.

Another method to determine whether tetraspanin over-expression disrupts tetraspanin microdomains is to employ microscopy-based methods. One possibility is use confocal total internal reflection fluorescence (TIRF) microscopy to determine surface localization of endogenous tetraspanins, for example CD81, in the presence or absence of transiently over-expressed CD9. In addition, single molecule tracking of an endogenous tetraspanin, as used by others (Espenel et al., 2008), could be utilised to determine whether tetraspanin over-expression affects the mobility of endogenous tetraspanins in the plasma membrane.

7.3.2 Does CLEC-2 interact with tetraspanins?

Both direct and indirect models are proposed for tetraspanin regulation of C-type lectin-like receptors. Therefore, a further avenue to pursue is to perform more experiments to determine whether CLEC-2 interacts with tetraspanins or whether it is localised exclusively outside of tetraspanin microdomains.

Chemical cross-linking studies in cell lines have previously been used in the tetraspanin field to demonstrate direct interactions between tetraspanins and their partner proteins, for example, CD151 and integrin $\alpha_3\beta_1$, and CD81 and EWI-2 (Charrin et al., 2001; Yauch et al., 2000). Cells co-transfected with CLEC-2 and tetraspanins could be chemically cross-linked and co-immunoprecipitated with a view to identify interacting proteins. In addition, these experiments could be done in platelets to determine whether the endogenous proteins interact. An advantage of using chemical cross-linkers is their capacity to capture direct but relatively weak interactions that are lost during immunoprecipitation.

Any potential direct interaction can also be studied by bioluminescence resonance energy transfer (BRET), a method our group has used to study GPVI dimerisation

(Tomlinson et al., 2007). BRET involves first generating expression constructs encoding luciferase- and GFP-tagged forms of potentially interacting proteins. Upon transfection of these constructs into a cell line and addition of a luciferase substrate, a transfer of energy between the luminescent donor and fluorescent acceptor can be detected if the proteins are within 10 nm of each other (Pfleger and Eidne, 2006). A similar method, fluorescence resonance energy transfer (FRET), could also be employed and has previously been used to study tetraspanins and their associated partner proteins (Harris et al., 2008; Yanez-Mo et al., 2008).

7.3.3 CLEC-2 signalling in tetraspanin deficient mice

CLEC-2 signalling was investigated using mice deficient in the major platelet tetraspanin CD9. However, as no CLEC-2 phenotype was observed, it is possible that the wrong tetraspanin-deficient mouse was selected for study. Even though CD9 is relatively highly expressed at 49,000 copies per platelet (Higashihara et al., 1990; Protty et al., 2009), only a minor role has been reported, where tail bleeding assays reveal a shorter bleeding time and reduced blood loss compared to wild-type controls, along with increased $\alpha_{IIb}\beta_3$ activation (Mangin et al., 2009). In addition, increased thrombi are observed in CD9-deficient platelets, suggesting a model whereby CD9 negatively regulates platelet recruitment to prevent excessive thrombus growth (Mangin et al., 2009). Therefore, in light of this data, there are a number of other possible candidates for tetraspanin regulation of CLEC-2 signalling.

CD151 and Tspan32 are two such candidates for study to determine whether such tetraspanins regulate CLEC-2 signalling. Indeed, phenotypes are observed in the CD151- and Tspan32-knockout mice as both have a mild bleeding phenotype (Goschnick et al., 2006; Lau et al., 2004), and one may hypothesise that either CD151

or Tspan32 may regulate CLEC-2. The Jackson group observe defective platelet spreading on fibrinogen, delayed clot retraction and a tendency to re-bleed in tail-bleeding assays, and attribute these observations to defective "outside-in" signalling of $\alpha_{IIb}\beta_3$ (Goschnick and Jackson, 2007). Moreover, an *in vivo* thrombus defect is observed in the Tspan32 knockout (Goschnick et al., 2006). Further to the study of CLEC-2 signalling in these mice, there should be some consideration to generate mice deficient in both CD151 and Tspan32 to determine whether a more pronounced phenotype is observed. However, as both CD151 and Tspan32 are located on the same chromosome (Table 7.1), this is relatively difficult.

The dense granule- and lysosome-localised tetraspanin CD63 has been shown to play a minor role in platelet function, where slightly stronger aggregation responses are observed in the knockout (Schroder et al., 2009). Therefore, the possibility of identifying a CLEC-2 phenotype in CD63-deficient platelets is relatively slim compared to other candidates such as CD151 and Tspan32. Nevertheless, if such mice were readily available, preliminary platelet studies could be conducted to identify a CLEC-2 phenotype.

If the above tetraspanins are not the regulators of CLEC-2 as revealed by knockout mouse studies, there are a number of other potential candidates, for example the recently identified Tspan9 (Protty et al., 2009). In addition, recent proteomic studies suggest the additional expression of several other tetraspanins in human platelets (Lewandrowski et al., 2009) (Figure 7.1). However, as discussed previously, this cannot be confirmed until specific antibodies to these tetraspanins are generated. Of particular interest given that CLEC-2 is expressed on the megakaryocyte/platelet lineage but not on most other cell types, our group has shown that Tspan9 is relatively

platelet- and megakaryocyte-specific (Protty et al., 2009), and Tspan9-deficient mice have now been generated. Moreover, our group have recently acquired the Tspan33 knockout mouse from the Tsai group (Heikens et al., 2007). The study of CLEC-2 signalling in these mice might establish a role for tetraspanins in the regulation of CLEC-2 on platelets.

Table 7.1 - Potential platelet tetraspanins as revealed by proteomics (Lewandrowski et al., 2009)

Tetraspanin	Mouse chromosomal localization	Comments	Platelet phenotype in knockout mice
CD9	6qF3	-	Minor role in the negative regulation of $\alpha_{IIb}\beta_3$ activation & thrombus formation (Mangin et al., 2009).
Tspan9	6qF3	-	Knockout mouse not analysed (our group).
Tspan33	6qA3.3	-	Not reported (Heikens et al., 2007).
CD151	7qF5	-	Positive role in the regulation of $\alpha_{IIb}\beta_3$ signalling, thrombus formation & stability (Lau et al., 2004).
CD63	10qD3	-	Minor role in the negative regulation of platelet aggregation *in vitro*, but no effect on thrombus formation *in vivo* (Schroder et al., 2009).
CD82	2qE1	Potential contamination from other blood cells as unable to detect on platelets by flow cytometry.	No knockout mouse.
Tspan14	14qB	-	No knockout mouse.
Tspan32	7qF5.7	-	Positive role in the regulation of $\alpha_{IIb}\beta_3$ signalling, thrombus formation & stability (Goschnick et al., 2006).
Tspan15	10qB4	-	No knockout mouse.
Tspan4	7qF5	Potential contamination from other blood cells as unable to detect on platelets by flow cytometry.	No knockout mouse.
CD81	7qF5	Potential contamination from other blood cells as unable to detect on platelets by flow cytometry.	Not reported (Cherukuri et al., 2004b) .
CD37	7qB4	Potential contamination from other blood cells as unable to detect on platelets by flow cytometry.	Not reported (Knobeloch et al., 2000).
Tspan2	3qF3	-	No knockout mouse.
Tspan18	2qE1	-	No knockout mouse.

7.3.4 Does tetraspanin over-expression affect CLEC-2 localization or dimerisation?

A further avenue to pursue is to determine whether tetraspanin over-expression affects CLEC-2 localization or dimerisation. In spread platelets, CLEC-2 has recently been shown to exhibit a punctuate localisation pattern, as detected using confocal microscopy (Pollitt, A.L. & Watson S.P., unpublished data). Such imaging experiments could be extended to Jurkat cells transfected with CLEC-2, which could be stained for this protein in the presence or absence of a co-transfected tetraspanins. Additional recent experiments have shown that CLEC-2 can exist as a dimer at the platelet surface, as detected using chemical cross-linking followed by western blotting (Hughes et al., 2010; Watson et al., 2009). Indeed, Hughes et al. have shown that dimerisation allows the tandem SH2 domains of Syk to effectively engage the two YXXL motifs of a CLEC-2 dimer. The dimerisation methodology could also be extended to transfected Jurkat cells, to determine whether tetraspanin over-expression affects CLEC-2 dimerisation.

7.3.5 The recently identified C-type lectin-like receptor CLEC9A

The novel C-type lectin-like receptor CLEC9A was recently identified, and signals in a similar way to dectin-1 and CLEC-2 via a YXXL motif (Huysamen et al., 2008). Therefore, the next logical step was to determine whether tetraspanins regulate this receptor. Preliminary studies regarding the role of tetraspanins in CLEC9A signal regulation were conducted as part of this thesis project (data not shown). An NFAT/AP-1 reporter assay in Jurkat cells was performed following co-transfection of HA epitope-tagged CLEC9A and tetraspanins. However, CLEC9A was not activated when cross-linked with the HA antibody, and thus the role of tetraspanins upon signalling was not determined (data not shown). Moreover, flow cytometry studies in

human platelets suggested that CLEC9A is not expressed, even following activation of the platelet (data not shown). Nevertheless, the future study of the role of tetraspanins in CLEC9A signalling on antigen-presenting cells is an exciting prospect.

7.4 The role of CLEC-2 on platelets

The role of CLEC-2 on platelets and the literature regarding this activation receptor has grown rapidly since its discovery (Suzuki-Inoue et al., 2006). Indeed, its ability to signal in a novel manner via a single YXXL motif through interaction with the Syk kinase has fuelled the study of this receptor (Fuller et al., 2007; Ozaki et al., 2009). The Nieswandt group have recently shown that CLEC-2 is essential in haemostasis and thrombosis (May et al., 2009). For these experiments, CLEC-2-deficient platelets were generated by treatment of mice with a CLEC-2 antibody, which lead to the loss of CLEC-2 at the surface of platelets. The reason for this loss of CLEC-2 is not clear, but the authors propose that it may involve ectodomain shedding by a metalloprotease, or receptor internalisation and degradation, similar to what they have observed for GPVI (Rabie et al., 2007). *In vitro* studies showed that CLEC-2-deficient platelets failed to respond to rhodocytin. Under flow conditions, the CLEC-2-deficient platelets adhered normally to collagen, but then failed to form stable aggregates. Similarly, *in vivo*, CLEC-2-deficient platelets adhered to the site of ferric chloride-injury in arterioles, but were unable to form thrombi that block the vessel. In tail bleed assays in these mice, about two thirds of CLEC-2-deficient mice bled for twice as long, while one third failed to stop bleeding during the 20 minute experiment. Together, these data suggest that CLEC-2 is necessary for normal thrombus growth and stability, and lead the authors to hypothesize that CLEC-2 has a novel ligand on platelets (May et al., 2009). However, one argument against this idea is that tyrosine phosphorylation of CLEC-2 is not detected when platelets are aggregated using

agonists such as thrombin, which should in theory result in CLEC-2 activation by engagement with its putative ligand on other platelets (Pollitt et al., 2010). The generation of CLEC-2-deficient mice is now essential to firmly establish a role for CLEC-2 on the platelet and to determine whether this receptor has any other functions in the adult mouse or during development. In light of this exciting data from the Nieswandt group, it is tempting to speculate that CLEC-2 might be an ideal drug target for the treatment of thrombotic disease. It is also possible that tetraspanins could regulate thrombosis through regulation of CLEC-2 function, particularly since CD151 and Tspan32, are like CLEC-2, essential for normal thrombus formation and stability (Tomlinson, 2009).

A role for CLEC-2 in cancer cell metastasis has also been proposed, since the identification of a tumour cell ligand, podoplanin, for this receptor (Ozaki et al., 2009; Suzuki-Inoue et al., 2007). Podoplanin is a protein that has been implicated in both tumour cell-induced platelet aggregation and tumour metastasis (Wicki and Christofori, 2007). Indeed, it is tempting to speculate that tumour cells that have disengaged from the solid tumour mass and entered the blood stream, might bind to circulating platelets via a podoplanin/CLEC-2 interaction. This might benefit the tumour in several ways. For example, a tumour cell that is coated with platelets might more easily avoid detection by immune cells, and might more easily become lodged at a site in the microvasculature where it can develop into a metastic tumour. In addition, since platelets contain several growth factors, these may promote tumour growth once the tumour has come to rest. Tetraspanins have recently been implicated in the regulation of this podoplanin/CLEC-2 interaction. Indeed, the tetraspanin CD9, when over-expressed on tumour cells, appears to interact with podoplanin and to inhibit podoplanin-induced platelet aggregation and pulmonary metastasis (Nakazawa et al.,

2008). Although one important aspect of the study that the Fujita group did not address, was whether CD9 affected podoplanin expression (Nakazawa et al., 2008). Nevertheless, the data suggests that CD9 may act as a metastasis suppressor, a proposal that has been suggested by other groups previously (Takeda et al., 2007a), and indicates how tetraspanins might regulate CLEC-2 function at the level of its ligand, podoplanin. Moreover, CLEC-2 might be a promising drug target in the treatment of cancer.

Finally, CLEC-2 may have a role in facilitating HIV infection, as platelets can engulf a significant amount of the virus. In both CLEC-2-transfected cells and platelets, CLEC-2 has the ability to capture HIV-1, irrespective of the viral envelope protein (Chaipan et al., 2006). Moreover, treatment with CLEC-2 antibodies potently reduced the association of HIV-1 with platelets, implicating CLEC-2 in HIV-1 spread in the bloodstream of infected individuals. However, the related lectin DC-SIGN also facilitated HIV-1 binding to platelets, suggesting that CLEC-2 is not indispensable in this process (Chaipan et al., 2006). Despite this, CLEC-2 may be a drug target in the treatment of HIV-1.

7.5 The role of tetraspanins

Tetraspanins are an intriguing family of proteins that have been shown to associate with, and in some cases, regulate a plethora of partner proteins within tetraspanin-enriched microdomains. These microdomains are thought to regulate fundamental cellular functions such as adhesion, signalling, intracellular trafficking and cell-cell fusion (Charrin et al., 2009; Hemler, 2008).

In contrast to early suggestions that tetraspanin microdomains might form a ‘web’ across the entire plasma membrane (Maecker et al., 1997), recent evidence suggests

that tetraspanin microdomains form several hundred distinct clusters across the membrane, with an individual size of 0.2 μm^2 (Nydegger et al., 2006). Further evidence using single molecule tracking suggests that tetraspanin microdomains are highly dynamic and can readily move across the plasma membrane (Espenel et al., 2008). It has also become apparent that most cell surface proteins do not appear to localize to tetraspanin microdomains (Charrin et al., 2009; Hemler, 2008). The full complement of tetraspanin microdomains on different cell types awaits proteomic investigation.

Despite much research on this family of proteins, few definitive functions have been assigned to individual family members. A good example is the proposed role for CD37 in the negative regulation of dectin-1 signalling (Meyer-Wentrup et al., 2007). In this study, it was not proven that CD37 and dectin-1 form a direct interaction. Moreover, other tetraspanins were not tested for an ability to regulate dectin-1 (Meyer-Wentrup et al., 2007). Indeed, the data in this thesis does not support the theory that CD37 specifically regulates dectin-1: multiple tetraspanins could co-immunoprecipitate with dectin-1 following over-expression in HEK293T cells, and multiple tetraspanins could inhibit dectin-1 signalling when over-expressed in Jurkat T-cells.

One problem with assigning functions to tetraspanins is that 33 members have been identified in mammals. This allows considerable scope for functional redundancy between family members. For example, CD9 and CD81 share approximately 45% protein sequence identity, which is consistent with some compensatory roles, as CD81 over-expression in CD9-deficient oocytes partially restores sperm-egg fusion (Higginbottom et al., 2003). A second problem arises from the fact that tetraspanins

cluster together in tetraspanin-enriched microdomains. This would suggest that deletion of different tetraspanins in the same microdomain might similarly disrupt microdomain function and yield similar phenotypes. This is certainly possible in CD151- and Tspan32-deficient platelets, which are defective in spreading and clot retraction leading to a mild bleeding phenotype (Goschnick et al., 2006; Lau et al., 2004), and in CD37-, CD81-, CD151- and Tspan32-deficient T-cells, which are hyperproliferative in response to T-cell receptor triggering (Knobeloch et al., 2000; Miyazaki et al., 1997; Tarrant et al., 2002; van Spriel et al., 2004; Wright et al., 2004). Whilst such studies have revealed some interesting tetraspanin-regulated functions, the precise mechanisms behind the observations have yet to be elucidated, and in particular the role of individual tetraspanins is not clear. A final problem is that the tetraspanin field is dominated by a select few tetraspanins. These were discovered at the beginning of the field due to the relative ease by which monoclonal antibodies could be generated to these proteins. This has left some of the more recently identified members unstudied and has left a void in tetraspanin research. In particular it is dangerous to draw general conclusions from the functional analyses of a small subset of the tetraspanin family. Our ideas on tetraspanin function may change considerably when all members have been studied.

As discussed previously, one of the best studied examples of a strong, highly stoichiometric, direct interaction is in regard to CD151 and the laminin-binding integrins, such as $\alpha_3\beta_1$. Indeed, it has been shown that CD151 recruits these integrins to tetraspanin microdomains and in the absence of CD151, association with microdomains is lost and integrin adhesion strength, signalling, trafficking and glycosylation is impaired (Serru et al., 1999; Winterwood et al., 2006; Yang et al., 2008; Yauch and Hemler, 2000). Consistent with these findings in cell lines, knockout

mouse studies have shown decreased pathologic angiogenesis both *in vivo* and *in vitro* by impaired integrin signalling and failure to associate with tetraspanin-enriched microdomains (Takeda et al., 2007b). In addition, mice deficient in CD151 exhibit kidney failure (Baleato et al., 2008; Sachs et al., 2006). However, there are few similar examples of such a direct interaction being assigned a specific function. This prompts the question as to whether CD151-$\alpha_3\beta_1$ functional interaction is the norm for other tetraspanins. Do all tetraspanins have one or more specific partners that they recruit into tetraspanin microdomains? Or do some only bind other tetraspanins and play an anchoring role or organising role at the core of the microdomain? Or do some tetraspanins fail to interact with other tetraspanins, perhaps regulating a partner protein in a non-microdomain environment? These questions will only be answered when good antibody reagents exist to all tetraspanins, allowing an integrated study using biochemical and imaging studies in wild-type and tetraspanin-knockdown cells.

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APPENDIX

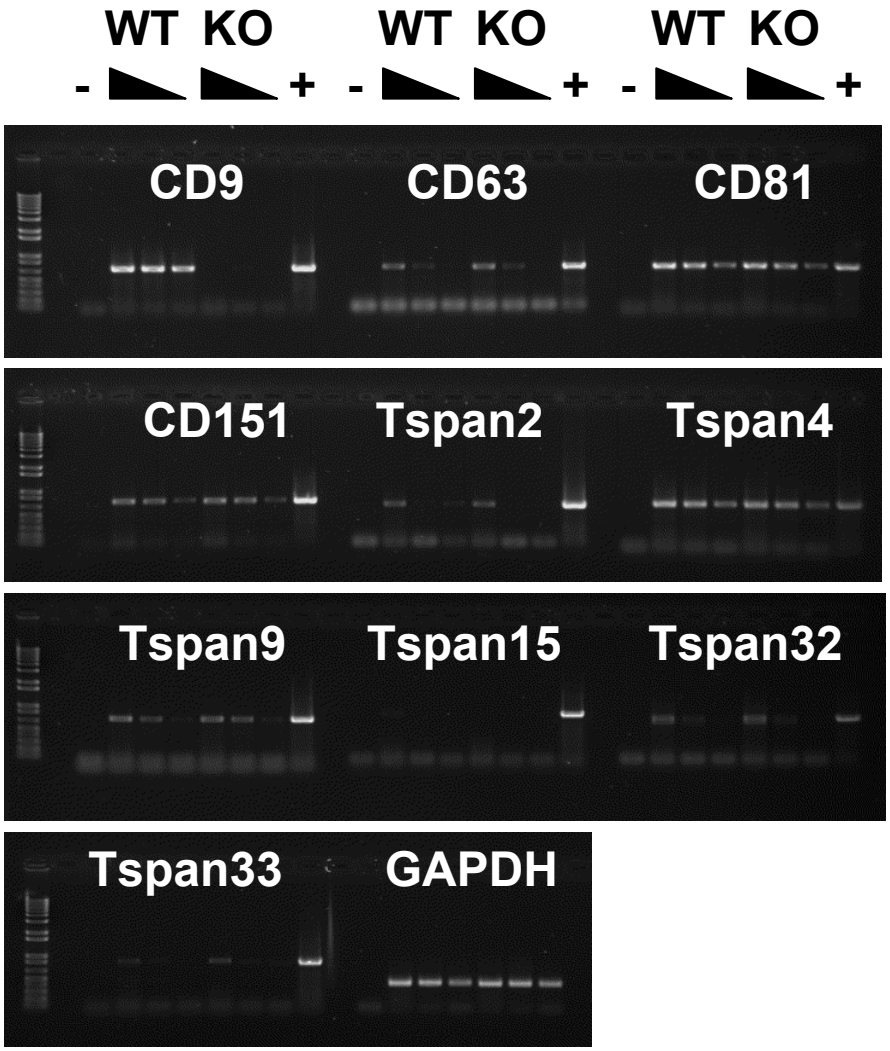

Figure I.I RT-PCR of CD9$^{-/-}$ megakaryocytes suggest that other tetraspanins are not up-regulated

RT-PCR was performed on CD9$^{+/+}$ (WT) and CD9$^{-/-}$ (KO) megakaryocytes using primers against particular tetraspanins as indicated, and cDNA templates at 10-fold dilutions. Data was kindly provided by Dr. M.G. Tomlinson.